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Use of a Fluorescent Probe To Assess the Activities of Candidate Agents against Intracellular Forms of *Encephalitozoon* Microsporidia

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Microsporidia are obligate intracellular protozoan parasites. Three species of the genus *Encephalitozoon* are among the microsporidia that infect immunodeficient humans. These species, *Encephalitozoon cuniculi*, *Encephalitozoon hellem*, and *Encephalitozoon intestinalis*, all develop in a parasitophorous vacuole within a host cell. The present study describes a method that uses the fluorescent probe calcein and confocal microscopy to detect drug-induced effects in *Encephalitozoon*-infected green monkey kidney cells. The effects were as follows: (i) changes in parasite organization within the parasitophorous vacuole; (ii) swelling and gross morphological changes of parasite developing stages in situ; (iii) killing of developing parasite stages in situ, detected by their uptake of the fluorescent probe; and (iv) reduction in the viability of the host cell population, assessed by the loss of the probe. Verapamil and itraconazole were used to increase the vital dye loading by both uninfected and infected cells. Agents with known antimicrosporidial activity, albendazole and fumagillin, caused all three types of parasite changes at concentrations that had no detectable effect on host cell viability. The effective doses of albendazole and fumagillin that caused swelling and disorganization of parasite developing stages were 5×10^{-7} and 10^{-6} M respectively. Killing of developing stages was detected at 10-fold-higher concentrations for these agents and at 10^{-5} M for metronidazole. This method can be used to screen candidate antimicrosporidial agents in infected cultured cells.

Microsporidia are obligate intracellular protozoan parasites (4) that have been considered significant human pathogens only since the onset of the AIDS epidemic (3, 7–9, 23, 27, 33). The most frequently encountered species, *Enterocytozoon bieneusi*, is almost always restricted to the intestinal epithelium (8), while three species of the genus *Encephalitozoon* readily disseminate (5, 33). *E. bieneusi* has not yet been successfully maintained in long-term culture (31), but the three *Encephalitozoon* species *Encephalitozoon intestinalis* (formerly *Septata intestinalis* [3, 15]), *Encephalitozoon hellem*, and *Encephalitozoon cuniculi* can be readily cultured (7, 9, 29, 30). These *Encephalitozoon* microsporidia also differ from *E. bieneusi* in that they proliferate and differentiate within a parasitophorous vacuole (2, 4).

A number of model systems have been developed to test candidate antimicrosporidial agents. The unique spore polar filament germination is a hallmark of this class of parasite (4). During germination a coiled tube within the spore is everted and extrudes to impale a nearby target cell. The infectious sporoplasm is then injected from spore to target cell through this tube (polar filament). In this manner the parasite infection spreads. Spore germination seems to be a promising target for possible antiparasitic chemotherapy (19, 21). Therefore, a spore germination assay has been employed in evaluating potential antimicrosporidial agents (16). A second assay system involves assessing an agent's efficacy in reducing or preventing the spread of infection through a monolayer of cultured cells (1, 16). A third type of assay system involves the use of whole-animal models (10).

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The present study describes a method that has been developed to test the parasiticidal activity of agents against various viable stages of *Encephalitozoon* microsporidia in situ. The purpose of establishing such a test system is to screen candidate agents and determine their effects on the gross morphology and viability of the various parasite stages within the parasitophorous vacuole while also assessing the viability of the host cells. This method utilizes confocal microscopy to visualize the fluorescent probe calcein within infected cells. The hydrophobic acetoxymethyl ester (AM) derivative of calcein readily passes the host cell plasma membrane and is converted to the fluorescent free-acid form by intracellular esterases. Nonviable cells do not fluoresce either because they lack esterases or because their loss of membrane integrity results in the loss of the free-acid form of the probe from the cell. These properties have made calcein a popular probe for measuring viability in eukaryotic cells (e.g., LIVE/DEAD viability/cytotoxicity kit; Molecular Probes Inc., Eugene, Oreg. [25]). In *Encephalitozoon*-infected cells the parasitophorous vacuole membrane, but not the membranes of viable parasite stages, is permeable by the free-acid form of calcein, so that parasite stages can be readily distinguished within viable host cells (20). Thus, the shape of the parasitophorous vacuole, its organization, and the viability of the various parasite stages can be studied with this probe. The present work details this method and illustrates how it can be applied to assess parasiticidal activity of test agents.

MATERIALS AND METHODS

Parasite cultures. Green monkey kidney (E6) cells were infected with *E. hellem*, *E. intestinalis*, and *E. cuniculi* isolates (7, 29, 30). All infected and uninfected cell cultures were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and 50 μg of gentamicin/ml.

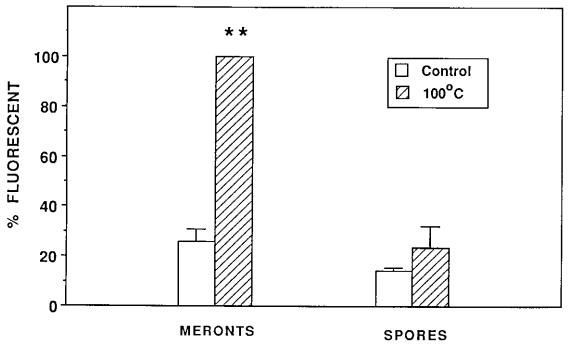

FIG. 1. Effects of prior immersion of a sample at 100°C for 60 s on the percentage of isolated *E. hellem* meronts and mature spores loaded with the free-acid form of calcein (means ± standard errors of the means). The difference between the heated sample and the control was significant at $P < 0.01$ (**) ($n = 5$).

Visualization of the probe in infected cells. Infected and uninfected cells were plated on 35-mm-diameter culture dishes that were modified by replacing the plastic bottom with a no. 1 glass coverslip. The test cells were treated with a candidate agent for 24 and 48 h, and control cells were treated with the same volume of drug vehicle (dimethyl sulfoxide [DMSO]). Calcein AM (Molecular Probes Inc.) was added to the culture dishes 30 min prior to examination by confocal microscopy. Immediately prior to microscopy, 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 $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.5 mM glucose, and 1 mg of bovine serum albumin/ml. All steps, including imaging, were performed at 37°C. The cells were imaged with an inverted confocal laser scanning microscope (Multiprobe 2001; Molecular Dynamics, Sunnyvale, Calif.) with software running on a Silicon Graphics Indigo workstation. Calcein was excited at 488 nm, and the emission was collected at 510 nm (20).

Methods for optimizing dye loading and imaging. Calcein AM readily penetrated the plasma membranes of both infected and uninfected cells when added to the medium in the concentration range of 0.5 to 50 μM. In infected cells there was evidence of fluorescence in parasite stages when higher concentrations of calcein AM were used, suggesting that host cell esterase activity had not converted all of the probe to the free-acid form before it entered the parasitophorous vacuole. Electron microscopy indicated that in some infected cells the parasitophorous vacuole was very near the cell surface, so that the host cell plasma membrane and the vacuole membrane were almost adjacent to one another, favoring the direct passage of the AM form of the probe into the vacuole. This was most evident in cells in which large parasitophorous vacuoles formed a dome that ballooned out from the dish surface. Such cells were readily detected by vertical plane (Z-scan) imaging. To ensure that all the calcein within the host cell was in the free-acid form and that there was no buildup of calcein AM, the rate-limiting step in calcein production had to be the ability of the AM form of the probe to permeate the plasma membrane and not the host cell esterase activity. To accomplish this, the concentration of calcein AM used to load cells was kept at or below 1 μM and large, domed parasitophorous vacuoles were avoided.

The distribution of calcein in both uninfected and infected cells was not uniform either within or between cells. There was evidence of intracellular sequestering of the probe that increased with time. For this reason, calcein AM loading was limited to 30 min and imaging was limited to 45 min. The probe loading was made more uniform between cells by adding verapamil to the medium. In the experiments reported here, the verapamil was added to a final concentration of 10^{-5} M 90 min prior to the addition of the calcein AM. Itraconazole was tested as an antimicrosporidial agent in this system. Like verapamil, itraconazole increased dye loading, so that in those experiments where itraconazole was used, verapamil was omitted. Calcein AM and test agents were added in DMSO.

In order to detect probe uptake by intracellular parasite stages, the fluorescent image had to be compared with the transmitted light image taken at the same focal plane. The apparent fluorescence (gray scale) of a parasite varied as the parasite stages came into and went out of focus. This made it necessary to optically section the entire parasitophorous vacuole to allow comparison of the fluorescent image with the transmitted light image taken at the same focal plane, thereby ensuring that a fluorescing parasite stage was in focus. Sections were made at 0.5 μm. In thick cells, the large number of scans resulted in bleaching of the probe. In such cases this photooxidation was reduced by the addition of 1 mg of ascorbic acid/ml to the medium.

Visualizing the probe in isolated parasite stages. To ensure that calcein uptake by parasite stages actually represented a loss of viability, infected E6 cells were harvested, washed in HEPES buffer free of glucose and albumin, and twice forced through a 26-gauge needle. This resulted in the release of many of the parasite stages into the medium. The samples were exposed to the free-acid form of calcein at 100 μM in the HEPES buffer for 15 min at 37°C, sedimented in a microcentrifuge tube, and washed until the dye was removed from the medium. Parasite stages were then allowed to settle on polylysine-coated dishes and were imaged as described above. This method readily distinguished meronts, which rounded when isolated, from the refractile mature spores. Occasionally chains of sporogonial stages were seen intact. To test whether stage viability could be determined with calcein, parasites were killed by immersing the microcentrifuge tube in boiling water for 60 s or exposing them to 70% ethanol for 15 min prior to the addition of the probe. To test the effect of spore germination on dye loading, hydrogen peroxide was added to the samples, which were then incubated for 15 min at 37°C and washed free of H_2O_2 prior to loading with calcein. Hydrogen peroxide is known to cause germination of mature spores in a dose-dependent manner (19).

Administration of test agents. Because this paper describes the development of a method to assess microsporidial stage morphology and viability while the parasite is within a parasitophorous vacuole, agents that are known to have antimicrosporidial activity in in vitro or in vivo systems were employed as positive controls. These agents were albendazole (2, 11, 12), fumagillin (14, 26), itraconazole (19, 22), and metronidazole (13, 16). Each was added in DMSO (final drug concentration, 10^{-4} to 10^{-7} M) to the culture medium 24 or 48 h prior to imaging.

In addition to drug-induced dye uptake by developing parasite stages, swelling of developing stages (particularly meronts) and disorganization of the parasitophorous vacuole content were also observed. Meronts were considered swollen when more than 25% of them measured 5 μm or more on their longest axis. In untreated cells less than 10% of these stages exceeded this length. The assessment of disorganization of parasitophorous vacuole content was subjective. Drug-induced disorganization was considered to have occurred when more than 25% of infected cells were scored as having disorganized parasitophorous vacuoles. Fewer than 5% of control infected cells were scored as having disorganized vacuoles.

Statistical analyses. In experiments in which mean values of several groups were being compared, the data were first analyzed by a one-way analysis of variance. This was followed by a post hoc Tukey's protected *t* test to determine the significance of differences between individual mean values. Student *t* tests were used to determine the significance of differences between two means.

RESULTS

Experiments involving calcein loading of isolated parasite stages suggested that dye loading could be used to assess viability in those stages that did not have a spore coat (i.e., stages other than sporoblasts and mature spores). Figure 1 shows the effect of heat killing on the subsequent uptake of the free-acid form of the probe by meronts and mature spores. The parasite stages were released from intact cells by passing infected cells through a 26-gauge needle. Only the obviously intact meronts and mature spores were counted. The preparations differed depending on the relative numbers of the various parasite stages detected, the percentage of mature spores that had already germinated, and the amount of mechanical damage to

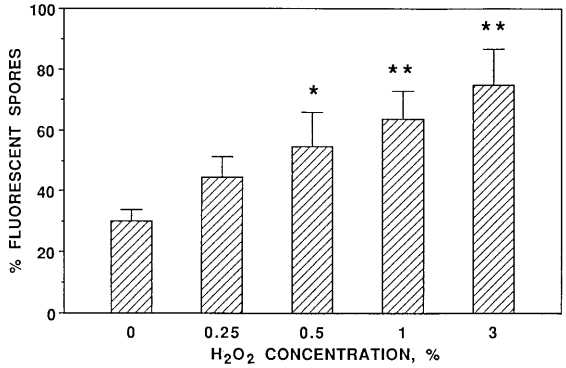

FIG. 2. Effects of H_2O_2 exposure for 15 min on subsequent loading of *E. hellem* spores with the free-acid form of calcein (means ± standard errors of the means). The differences between the H_2O_2-treated and the untreated group were significant at $P < 0.5$ (*) and $P < 0.01$ (**) ($n = 5$).

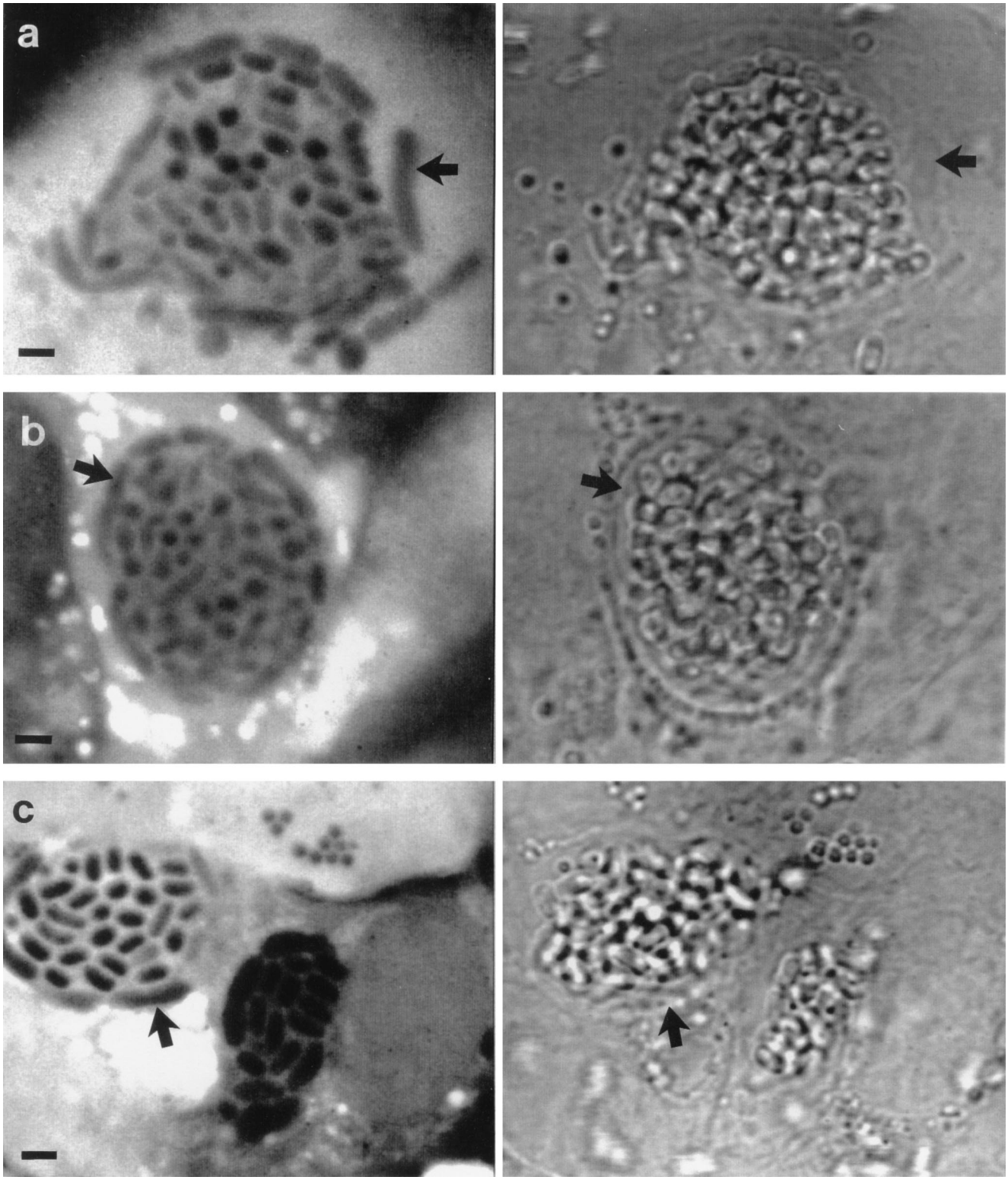

FIG. 3. Calcein fluorescence (left) of infected green monkey kidney cells and transmitted light images (right) of the same cells. (a) *E. hellem*-infected cell; (b) *E. cuniculi*-infected cell; (c) *E. intestinalis*-infected cells. Nonrefractile developing stages (arrows) are concentrated around the walls of the parasitophorous vacuoles, while refractile mature spores are concentrated in the centers of the vacuoles. Bar = 2 μm.

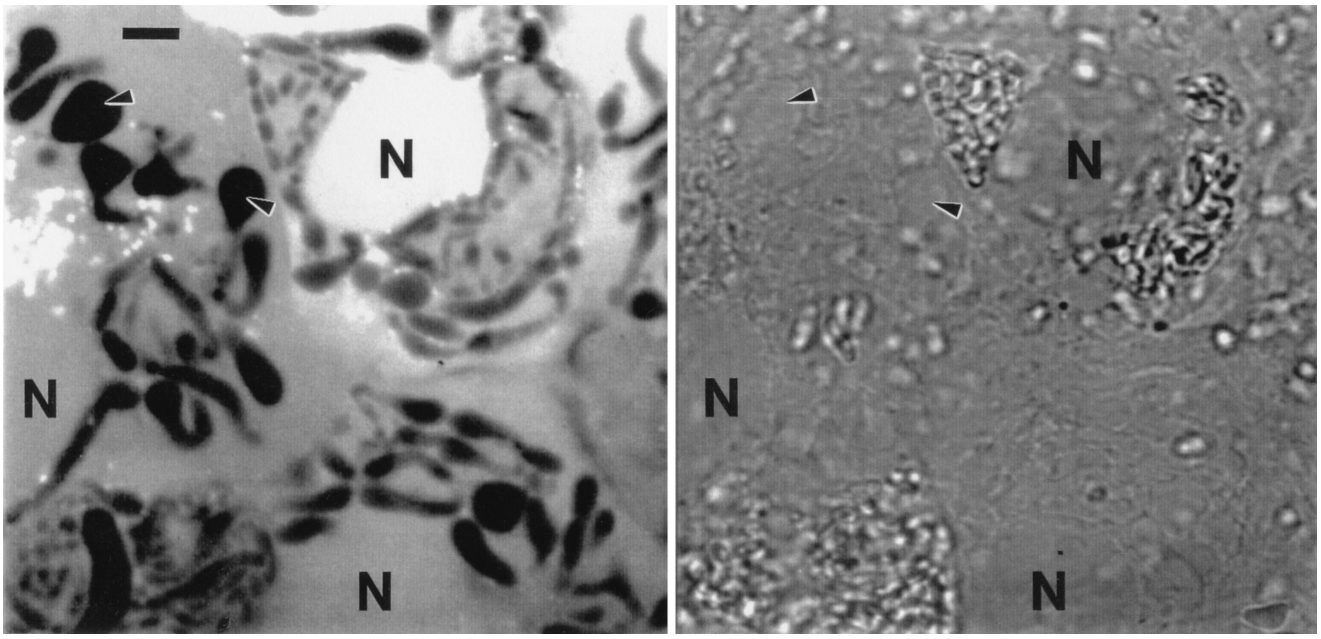

FIG. 4. *E. hellem*-infected cells (left, calcein fluorescence; right, transmitted light) treated for 24 h with 10^{-6} M albendazole and for 90 min with 10^{-5} M verapamil prior to loading with calcein AM. Arrowheads indicate the locations of swollen developing parasite stages, and "N" indicates a host cell nucleus. Bar = 5 μm.

developing stages that had occurred. Figure 1 illustrates that while heat killing resulted in uptake of the probe by all grossly intact meronts, it did not cause a significant increase in the number of spores that became permeable by the dye. Exposure to 70% ethanol for 15 min, which is known to kill microsporidia (5), resulted in the loading of all meronts and spores with the free-acid form of the probe.

The uptake of calcein-free acid by mature spores might also be influenced by spore germination. During the germination process a filamentous polar tube is rapidly everted and extruded from within the spore. This tube is the conduit through which infectious material is passed from the spore to the impaled target cell. In order to determine if dye loading was a function of germination, germination was induced by the addition of H_2O_2 to the medium prior to exposure to calcein. Figure 2 shows that the percentage of mature spores that fluoresced increased as the H_2O_2 concentration increased, suggesting that germination did indeed provide a site for entry of the probe through the spore coat. Since most of the germinated polar filaments were torn off during the post-H_2O_2 centrifugation and washing steps that occurred prior to calcein exposure, the free-acid form of the probe may have gained entry into the spore through the opening of the extruded polar filament. However, there was no difference in dye loading between spores exposed to the calcein-free acid at the same time that germination was stimulated by H_2O_2 and spores exposed to the dye after H_2O_2 had been used to induce germination, suggesting that the dye entered through the spore coat elsewhere than at the opening made by the extruded polar filament. Taken together, these observations indicate that while calcein-free-acid uptake can be used to assess the viability of developing parasite stages, fluorescence of mature spores should be interpreted with caution since such dye uptake may signify either spore killing or germination.

Figure 3 shows examples of parasitophorous vacuoles from calcein-loaded cells infected with the three *Encephalitozoon* species discussed herein. The method clearly can be used with all three species, and this figure shows typical overall arrangements of parasite stages within the vacuoles and the morphology of the individual stages in living cells. Mature spores were found to be refractile when viewed by transmitted light, while the larger developing stages that lined the walls of the parasitophorous vacuoles were not refractile.

Albendazole is the antimicrosporidial agent that is most widely used clinically (2, 11, 12, 33). Figures 4 and 5 show the effects of this agent on *E. hellem*-infected cells. At the lower magnification (Fig. 4) the marked swelling of developing parasite stages was readily apparent in two cells, while the parasite content of a third cell appeared to be relatively unaffected. The typical organization of the contents of parasitophorous vacuoles seen in Fig. 3 was also disrupted in the more affected cells. A third effect of albendazole was the apparent killing of developing stages. Figure 5a illustrates calcein uptake by a developing stage of *E. hellem* in an albendazole-treated cell that had also been treated with itraconazole. The incidence of dye uptake by developing stages was variable but greatly exceeded that seen in untreated infected cells. Figure 5b shows disarray and swelling in a number of stages of *E. intestinalis* in an albendazole-treated preparation in which dye loading was optimized with verapamil. Figure 6 demonstrates that metronidazole caused dye uptake by developing stages of both *E. hellem* and *E. intestinalis*. It is unlikely that the effects observed in these experiments were a direct result of itraconazole or verapamil, as these agents had little effect on parasite viability or morphology at the concentrations used, whereas metronidazole, and particularly albendazole, showed these effects when used alone.

Figure 7 shows fumagillin-induced swelling of developing stages resembling that seen with albendazole and metronidazole. The degree of dye uptake and the magnitude of swelling in developing stages were lower with fumagillin; however, infected cells in fumagillin-treated preparations were more fragile than in albendazole-treated preparations as evidenced by a marked increase in the number of free-floating mature spores

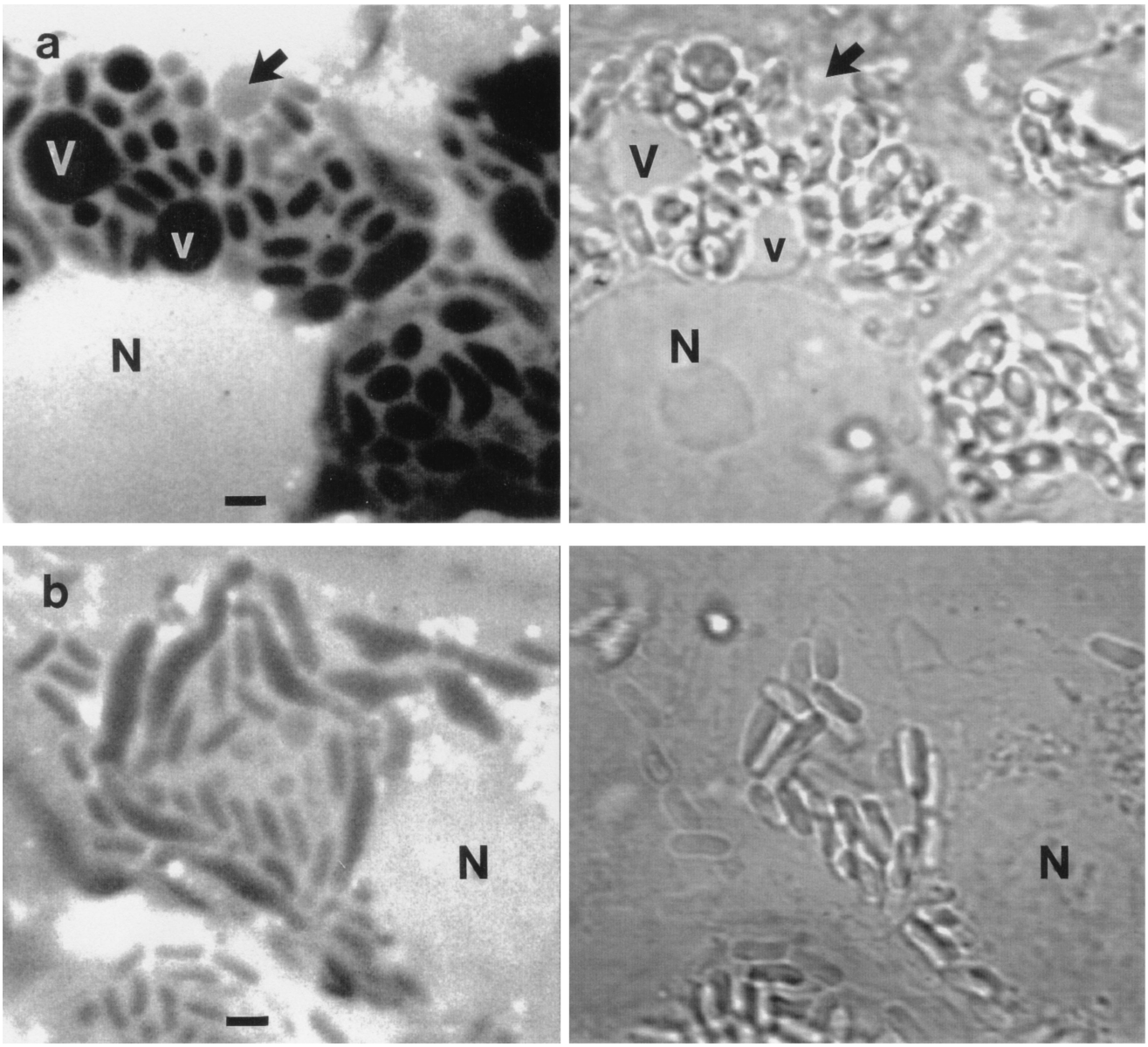

FIG. 5. Microsporidian-infected cells (left, calcein fluorescence; right, transmitted light) treated with 10^{-6} M albendazole for 24 h. (a) *E. hellem*-infected cell also treated with 10^{-6} M itraconazole for 24 h prior to loading with calcein AM showing dye uptake by a developing stage (arrow) and two aqueous vacuoles (V) within the parasitophorous vacuole. (b) *E. intestinalis*-infected cell also treated with 10^{-5} M verapamil for 90 min prior to dye loading showing swollen developing stages (left) and elongated refractile spores (right). "N" indicates a host cell nucleus. Bar = 2 μm.

in the fumagillin-treated preparations (data not shown). With all three of these antiparasitic agents there was a high degree of cell-to-cell variability in the observed morphological effects. In general, the parasitophorous vacuoles that contained the fewest mature spores and the largest number of developing stages exhibited the greatest evidence of drug-induced effects.

In one experiment in which infected cells were treated with 10^{-6} M itraconazole and an antiparasitic agent for 24 h it was possible to determine the effective concentrations of the agent that caused swelling of developing parasite stages and disorganization of the parasitophorous vacuole content. The doses effective at inducing both swelling and disorganization for albendazole and fumagillin were 5×10^{-7} and 10^{-6} M, respectively. While metronidazole caused some swelling of parasite stages, this was not a consistent phenomenon and did not meet the criterion for swelling described in Materials and Methods.

DISCUSSION

The present work describes the development of a method to evaluate the effects of candidate antimicrosporidial agents on parasite stages in situ. Uninfected and infected calcein-loaded cells were used, and three drug-induced parasite changes were observed. These changes were disorganization of the parasitophorous vacuole content, swelling of developing stages, and parasite dye uptake. The last was taken to indicate killing when it occurred in developing stages. Dye uptake in mature spores depends upon the way in which the spores were killed, or it

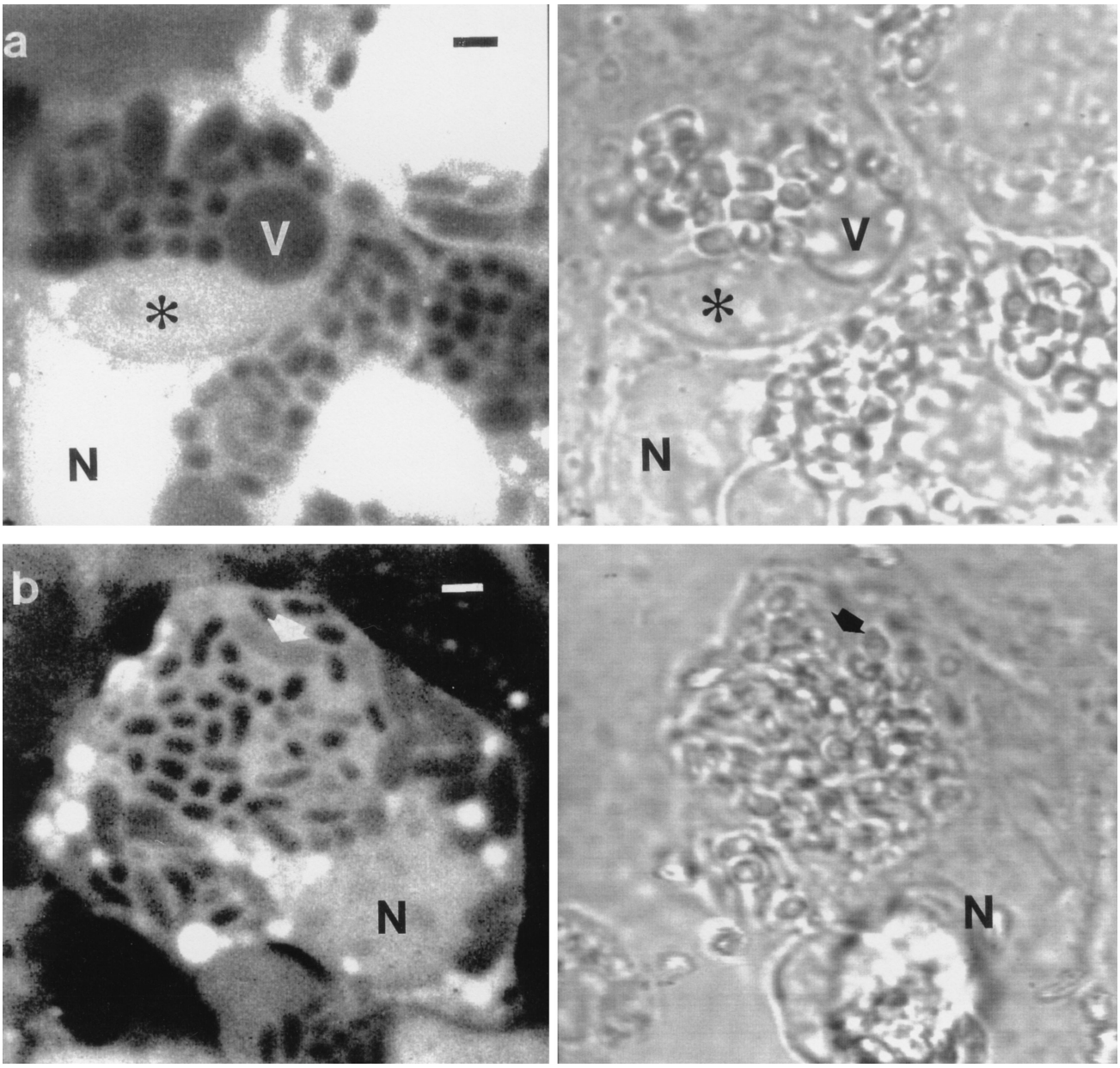

FIG. 6. Microsporidian-infected cells (left, calcein fluorescence; right, transmitted light) treated with 10^{-5} M metronidazole for 24 h. (a) *E. hellem*-infected cells also treated with 10^{-6} M itraconazole for 24 h prior to loading with calcein AM. One parasitophorous vacuole contains swollen developing stages, one of which has taken up calcein (asterisk), and an aqueous vacuole (V). (b) *E. intestinalis*-infected cell also treated for 90 min with 10^{-5} M verapamil. The arrow indicates one of several developing stages that took up calcein. "N" indicates a host cell nucleus. Bar = 2 μm.

may accompany spore emptying following germination. Large merogonial stages were found around the walls of the parasitophorous vacuole, while sporogonial stages were usually found extending as chains into the vacuole from the vacuole wall. The smaller mature spores appeared to be loose and were mainly concentrated in the middle of the vacuole (4, 9, 30). To the degree that it is possible to identify parasite developing stages by this method, the stages that appeared most susceptible to killing by the agents used were merogonial. This method also allows the simultaneous determination of host cell viability: in contrast to in situ parasite death, which is inferred from probe uptake, host cell death is inferred from the failure to take up and/or retain the probe.

One limitation of applying this method to studying intracellular parasites is the variation seen in dye loading between different host cell types or between different clonal expansions of the same cell type. In studies involving intracellular parasites the choice of host cell type is often dictated by the ease with which the parasite infection occurs rather than by the way the experimental host cell handles drugs or fluorescent probes. Green monkey kidney cells have proven to be an effective host for in vitro cultivation of *Encephalitozoon* microsporidia, but individual cells show a great deal of variability in the degree to which they take up or retain calcein. Verapamil and itraconazole, but not probenecid, increased fluorescence of both uninfected and infected E6 cells, suggesting that dye uptake

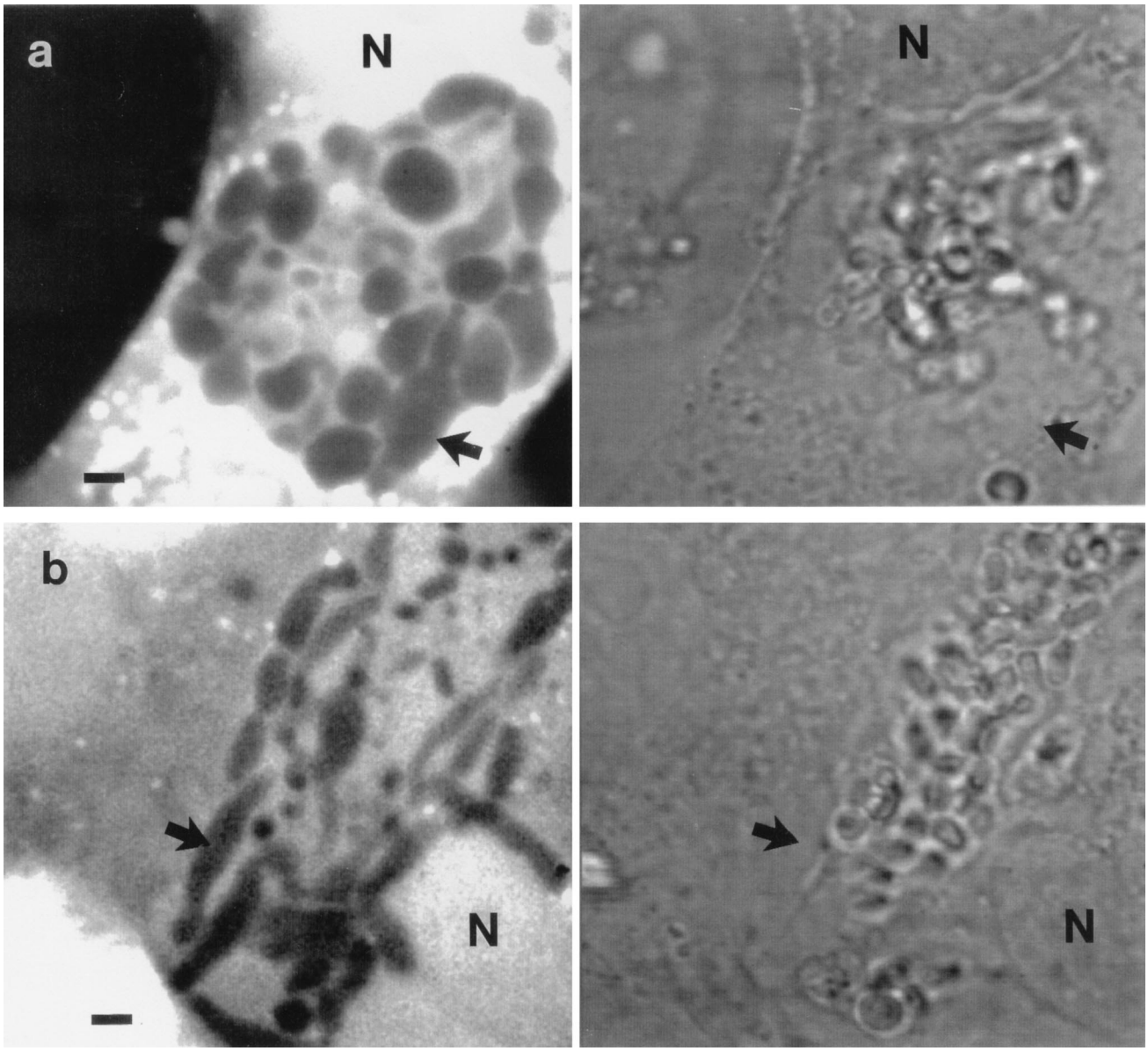

FIG. 7. Microsporidian-infected cells (left, calcein fluorescence; right, transmitted light) treated with 10^{-6} M fumagillin for 24 h and 10^{-5} M verapamil for 90 min prior to dye loading. (a) *E. hellem*-infected cell demonstrating disorganization of the parasitophorous vacuole content and swelling of developing stages (arrows). (b) *E. intestinalis*-infected cell demonstrating some swelling of developing stages (arrows). "N" indicates a host cell nucleus. Bar = 2 μm.

and/or retention of calcein or calcein AM is influenced by a P-glycoprotein-like transport system (17, 18, 32) but not by a probenecid-inhibitable anion transporter (28).

Itraconazole was not detectably effective at affecting parasite or host cell viability when used alone in this assay. We assumed that when used in conjunction with agents like metronidazole and albendazole its only action was to increase calcein loading. This may not be the case, however, because itraconazole has been shown to have some effects on spore morphology in at least one microsporidian species (22), and it rapidly inhibits spore germination in *E. hellem* (19), more rapidly than would be expected if its effects had been caused by alterations in parasite cytochrome P-450-mediated sterol biosynthesis (6). Similarly, verapamil was not detectably antimicrosporidial in this assay when used alone. However, other calcium channel blockers have been shown to inhibit *E. hellem* spore germination (19, 24) and to potentiate the metronidazole-induced inhibition of the spread of *E. hellem* and *E. intestinalis* infections through monolayers of cultured cells (16).

To ensure that host cell cytoplasmic calcein AM levels did not build up, allowing probe uptake by viable parasite stages, the calcein AM concentration of the medium was kept at or below 1 μM. This necessitated pretreating the cells with verapamil or itraconazole before dye loading in order to obtain detectable and uniform fluorescence. Under such conditions any in situ drug-induced gross morphological changes were readily detected. Detecting dye uptake by the various parasite stages within the parasitophorous vacuole was more difficult because of the dependence of the gray level on the plane of focus. To ensure that a given parasite stage had actually taken up calcein, 0.5-μm serial sections of the entire vacuole were made. This allowed bracketing of the parasite stage under

study, ensuring that it was in the plane of focus in at least one section. The necessity of serial sectioning large numbers of cells reduces the usefulness of this method for the rapid screening of in situ parasiticidal activity of a large number of agents. However, if an agent causes widespread killing of parasite stages without an effect on host cell viability, this method could be used to detect such an effect. With the criteria established here to assess drug-induced disorganization of parasitophorous vacuole contents and swelling of developing stages, this method can be used to rapidly screen candidate antiparasitic agents. Since it can be simultaneously used to study the viability of intracellular parasites and host cells, this method also has obvious advantages over more time-consuming and costly ultrastructural methods.

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