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Effects of Nifedipine, Metronidazole, and Nitric Oxide Donors on Spore Germination and Cell Culture Infection of the Microsporidia *Encephalitozoon hellem* and *Encephalitozoon intestinalis*

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Two species of microsporidia, *Encephalitozoon hellem* and *Encephalitozoon intestinalis*, were isolated from AIDS patients and cultured in green monkey kidney cells. A spore germination assay and a cultured-cell infection assay were used to test the efficacy of candidate antiparasitic agents. The calcium channel blocker nifedipine, metronidazole, and two nitric oxide (NO) donors, *S*-nitroso-*N*-acetylpenicillamine and sodium nitroprusside, were tested in the two assays. Nifedipine (10^{-8} M) significantly inhibited *E. hellem* spore germination in three of four germination media. Metronidazole (10^{-5} M) inhibited germination weakly and significantly inhibited *E. intestinalis* germination in a single germination medium. The inhibitory effect of nifedipine and metronidazole used together was greater than the sum of the effects of the drugs used alone in all *E. hellem* germination assays. The NO donors also inhibited spore germination. The inhibitory effect of nifedipine and metronidazole could be reversed by washing the spores, while that of the NO donors was not reversible. In early cultured-cell infections, both nifedipine (10^{-8} M) and metronidazole (10^{-5} M) significantly reduced the number of cells being infected. As the infection spread, these agents were less effective. Some inhibition of the spread of the infection was also demonstrated with the NO donors at a concentration (10^{-5} M) not obviously toxic to the cultured cells. These data suggest that combination drug therapy targeting spore germination and intracellular parasite development is promising.

Microsporidia are obligate intracellular protozoan parasites that are widely distributed in nature. While they have been recognized as pathogens in animals for decades, they were not considered significant pathogens of humans until the AIDS epidemic (6, 9, 22, 27). To date, several hundred cases of human microsporidiosis have been reported worldwide for human immunodeficiency virus-infected patients (25). It appears that at least six species of microsporidia infect immunodeficient individuals (3, 6, 9). The sites of these infections include the cornea, kidneys, lungs, liver, peritoneal cavity, and, most commonly, the gastrointestinal tract (3, 7, 11, 21, 22). Recently, the microsporidian species responsible for most cases of intestinal microsporidiosis, *Enterocytozoon bieneusi* (11), was detected in a human immunodeficiency virus-negative individual with traveler's diarrhea (26, 34), suggesting that microsporidiosis may be more than an opportunistic infection of immunodeficient or immunosuppressed hosts.

Successful treatment of microsporidiosis in immunodeficient patients is limited (22). Treatment with various antibiotics or antiprotozoal drugs has been attempted. With the possible exceptions of some eye infections and a few cases of disseminated microsporidiosis, chemotherapy has failed to eradicate the parasites (6, 10, 14, 21, 25).

Microsporidia are characterized by environmentally resistant spores with an internally coiled polar filament, a membranous polaroplast, and a sporoplasm with one or two nuclei and no mitochondria (6, 9, 22, 27). Once the spores have been ingested and stimulated by the appropriate host environment, the coiled filament everts and elongates (germination) to form a long tube that impales any nearby host cell. Osmotic forces within the spore may power this process (29, 30). The cytoplasm and nucleus of the spore (sporoplasm) pass through the tube into the injected cell, and the cycle of proliferation and infection is maintained. Germination is a major step in the infection process of this organism and a key site for interdiction of the spread of the infection (20).

Four microsporidian genera (*Plesitophora*, *Encephalitozoon*, *Nosema*, and *Enterocytozoon*) have been reported to cause disease in humans (6, 7, 9, 11, 21, 22). *E. bieneusi* is the most common cause of human intestinal microsporidiosis (11, 25, 34). *Encephalitozoon intestinalis*, formerly called *Septata intestinalis* (16), also infects enterocytes, but unlike *E. bieneusi*, it may disseminate (7, 32). While *E. bieneusi* has not been grown in long-term culture, *E. intestinalis* and two other *Encephalitozoon* species, *Encephalitozoon hellem* and *Encephalitozoon cuniculi*, have been cultured (10, 32, 33). We have chosen to study candidate antiparasitic agents with *E. intestinalis* and *E. hellem* because these are the two microsporidia that can be grown in long-term culture which are most commonly encountered in AIDS patients. This investigation was undertaken to determine whether and to what extent nitric oxide (NO) donors, nifedipine, and metronidazole could suppress the germination of isolated spores or the infection of cultured cells. The choice of agents was based on prior observations that calcium channel blockers inhibited microsporidian spore germination (20, 24), that metronidazole and related benzimidazoles exhibited some clinical efficacy in AIDS-related microsporidiosis (10, 14, 15, 34), and that nitric oxide had antiparasitic activity against several intracellular parasites (19, 31), including microsporidia (13).

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MATERIALS AND METHODS

E. hellem and *E. intestinalis* spores were isolated from infected AIDS patients and cultured in a green monkey kidney cell line (E6) (32, 33). All cell cultures were grown in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal bovine serum, 100 μg of piperacillin per ml, 50 μg of gentamicin per ml, and 2 μg of amphotericin B (Fungizone) per ml. Heavily infected cells were periodically harvested and stored at 4°C to provide the spores for these experiments. Spores were purified from pools of infected cells by the method of Didier et al. (12, 20).

Spore germination. For germination (spore polar filament extrusion) experiments, spores were stimulated to germinate by incubation at 37°C for 45 min in calcium-free or calcium-containing media, with or without 5% H_2O_2, and with or without test agents as described previously (20). Samples of stimulated spores were divided into two sets. One set was fixed immediately after incubation by the addition of an equal volume of 0.1 M cacodylate-buffered 2.5% glutaraldehyde (pH 7.4) and was then kept overnight at 4°C for light microscopic assessment of the effect of the drug on spore germination. To assess the reversibility of any drug effect, a second set of spores was treated as described above and washed three times with deionized water at 400 × *g* for 10 min and stored overnight in water at 4°C. The next day, these preparations were centrifuged for 15 min at 400 × *g* and then either fixed (time zero) or reincubated at 37°C for 30 min in calcium-containing and calcium-free incubation solutions without H_2O_2 and free of any test agent. Fixation was again accomplished with cacodylate-buffered 2.5% glutaraldehyde.

The percentage of spores that germinated under each set of conditions was assessed by observing fixed spores by phase-contrast microscopy. A minimum of 200 spores was counted, with those which had a protruding polar filament scored as germinated and the white, refractile spores scored as ungerminated (20).

Cultured-cell infection assay. Control (uninfected) and infected green monkey kidney cells (E6) were mixed in a ratio of 3 to 1, respectively, and placed in 24-well plates in which sterile circular glass coverslips had previously been placed. The mixed cells were grown in Dulbecco's modified Eagle's medium–5% serum and incubated at 37°C in 5% CO_2–air. Twenty-four hours later, the old medium was removed and fresh medium containing test agents was added. This procedure was repeated daily. Thus, infected cells were exposed to fresh agent daily for the duration of the experiment. The spent medium was examined for evidence of removed spores. Fewer than four spores were observed per high-power field, indicating that the majority of the extracellular spores in a well were adsorbed to, or otherwise attached to, the cultured cells, in many cases by their extruded polar filaments. Coverslips were removed from the wells on days 5 through 9, fixed in 10% formalin for 30 min, and washed twice with saline, and the samples were stained with Giemsa stain. The percentage of infected cells was assessed by counting 500 cells in five randomly chosen fields from each coverslip. The infected cells were easily distinguished from uninfected cells, as mature spores appeared green and spores in developing stages appeared light blue within the infected cells.

Nifedipine, metronidazole, and sodium nitroprusside (NP) were obtained from Sigma Chemical Company, St. Louis, Mo., and *S*-nitroso-*N*-acetylpenicillamine (SNAP) was obtained from Biomol Research Laboratories, Inc., Plymouth Meeting, Pa. For nifedipine and the two NO donors, the drug concentrations used in both germination and infected-cell assays approximated the 50% inhibitory concentration observed in spore germination assays alone (20). As metronidazole significantly inhibited spore germination only at concentrations well above therapeutic values, a concentration of this agent that was clinically relevant was chosen.

Statistical evaluation. The data are presented as means ± standard errors of the means of five or more separate experiments. Statistical analyses were performed by using one-way analysis of variance of repeated measures followed by Tukey's protected *t* tests. Differences between mean values were considered statistically significant at the level of $P < 0.05$.

RESULTS

Figure 1 summarizes the effects of nifedipine and metronidazole, alone and in combination, on *E. hellem* and *E. intestinalis* spore germination in each of the four incubation media. Nifedipine (10^{-8} M) significantly inhibited *E. hellem* spore germination in three of the four incubation media and *E. intestinalis* spore germination in two of the four media. Metronidazole (10^{-5} M) caused a statistically significant inhibition of *E. intestinalis* spore germination in only one of the test media. When the two agents were used together at these concentrations, their inhibitory effect was greater than the sum of the effects of the agents used alone on *E. hellem* germination in all four incubation media. No such apparent potentiation of the effect of one agent by the other was observed in the case of *E. intestinalis* spore germination.

Figure 2 shows the percentages of cultured epithelial cells that were infected with microsporidia on days 5 and 8 (*E. hellem*) or days 6 and 9 (*E. intestinalis*) after the initiation of treatment. The results were dependent on the extent to which the infection had spread through the culture. Nifedipine (10^{-8} M) and metronidazole (10^{-5} M), alone and in combination, caused a peak inhibition of infection on day 5 for *E. hellem* and day 6 for *E. intestinalis*. As the infections were allowed to spread, these agents were less effective in reducing the number of infected cells. In contrast to the results of the germination experiments, metronidazole was at least as effective as nifedipine in the cell infection experiments. A true potentiation of the inhibitory effect of both agents was observed on day 9 of the experiment in which *E. intestinalis*-infected cells were used.

Nitric oxide donors SNAP and NP inhibited germination of *E. hellem* and *E. intestinalis* spores. Table 1 shows the 50% inhibitory concentrations of these NO donors in calcium-containing and calcium-free media. Hydrogen peroxide was not added to the media in these experiments because of its reaction with NO. SNAP appeared to be more effective than NP. Both NO donors appeared more effective in the calcium-containing incubation medium than in the calcium-free medium, and *E. hellem* appeared more sensitive to both NO donors than *E. intestinalis*. However, none of these differences were statistically significant. Figure 3 shows that NO donors reduced the spread of the microsporidian infection through monolayers of cultured cells. The observed inhibition peaked on day 7 of the experiment. SNAP (10^{-5} M) had a significant effect on only the *E. intestinalis* infection on this day, while NP (10^{-5} M) significantly decreased the percentage of cells infected by either parasite species. Unlike the germination experiments, which were conducted for a short period with closed tubes, the cell culture experiments were conducted with multiwelled plates with loosely fitting lids that allowed loss of NO to the atmosphere. NO donors were replaced daily with new medium, and the indicated concentration of NO in the cultured cell experiments was therefore the peak daily value. Higher concentrations of either NO donor in the medium caused a reduction in the viability of the cultured cells, as evidenced by cell rounding, blebbing, formation of vacuoles, and cell detachment.

Figure 4 illustrates the reversibility of the inhibitory effects of agents on the germination of *E. hellem* spores in the calcium-free medium and the calcium-containing medium. Hydrogen peroxide germination media were excluded from this group of experiments to avoid the production of irreversible damage by this reactive oxygen compound. Control spores showed significant differences between the percentages of spores germinating during the initial incubation period and during the postwash incubation. In the case of NP-treated *E. hellem* spores germinating in the calcium-free medium but not the calcium-containing medium, there was a slight but statistically significant distinction between the initial incubation and postwash incubation values. Washing failed to restore germination in SNAP-treated microsporidian spores that germinated in either medium. In contrast, the germination inhibition caused by nifedipine and, to a lesser extent, metronidazole and by the two agents in combination could be partially or totally reversed by washing the treated spores.

DISCUSSION

Spore germination undoubtedly plays an important role in the spread of a microsporidian infection between and within hosts (9, 22, 33). The present study was designed to assess the effects of three types of agents on the germination of spores of

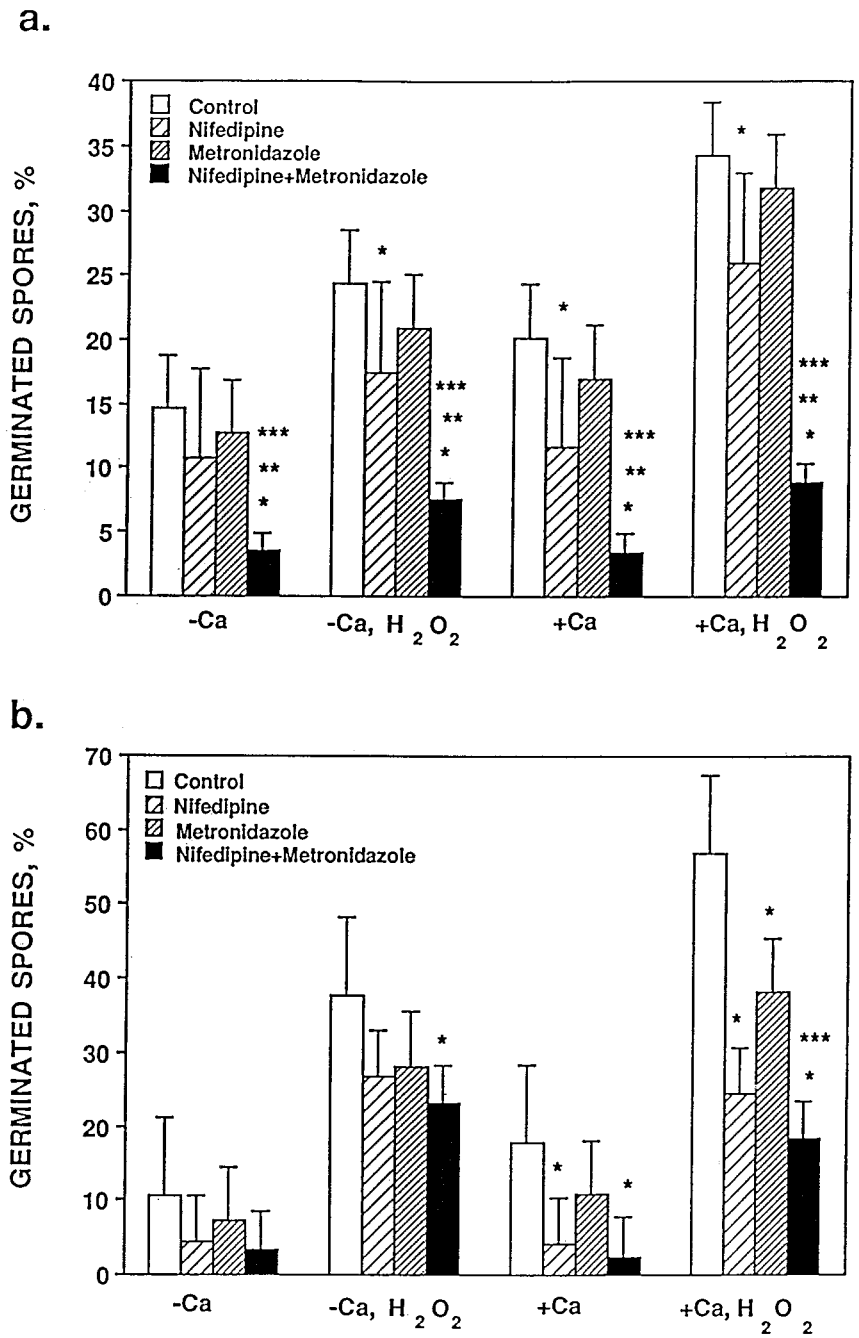

FIG. 1. Effects of nifedipine (10^{-8} M) and metronidazole (10^{-5} M), alone and in combination, on *E. hellem* (a) and *E. intestinalis* (b) spore germination in a calcium-free medium (−Ca) and a 1 mM calcium medium (+Ca) with and without 5% hydrogen peroxide. *, significantly different from control; **, significantly different from nifedipine-alone group; ***, significantly different from metronidazole-alone group.

two microsporidian species known to infect humans and to assess their effects on the spread of the infection through a monolayer of cultured cells. In the latter case, the rate of parasite proliferation, development, and the germination of both extracellular and intracellular spores all contribute to the spread of the infection.

Calcium plays some undefined role(s) in spore germination. While removal of calcium from the incubation medium inhibits germination in some microsporidian species (20), increasing the medium calcium concentration has been shown to inhibit germination in other species (17). Calcium mobilization appears to be associated with spore germination, whether from the membranous polaroplast (35) or as an influx from the area of the spore coat (24). The observation that the calcium channel blocker nifedipine in nanomolar concentrations inhibits germination by *E. hellem* (20) and *E. intestinalis* spores suggests that voltage-gated calcium channels play a key role in the extrusion of the polar filament. Presumably, the site of action of nifedipine in this system is at the cell membrane, as is the case in mammalian tissue (28). The nifedipine effect was re-

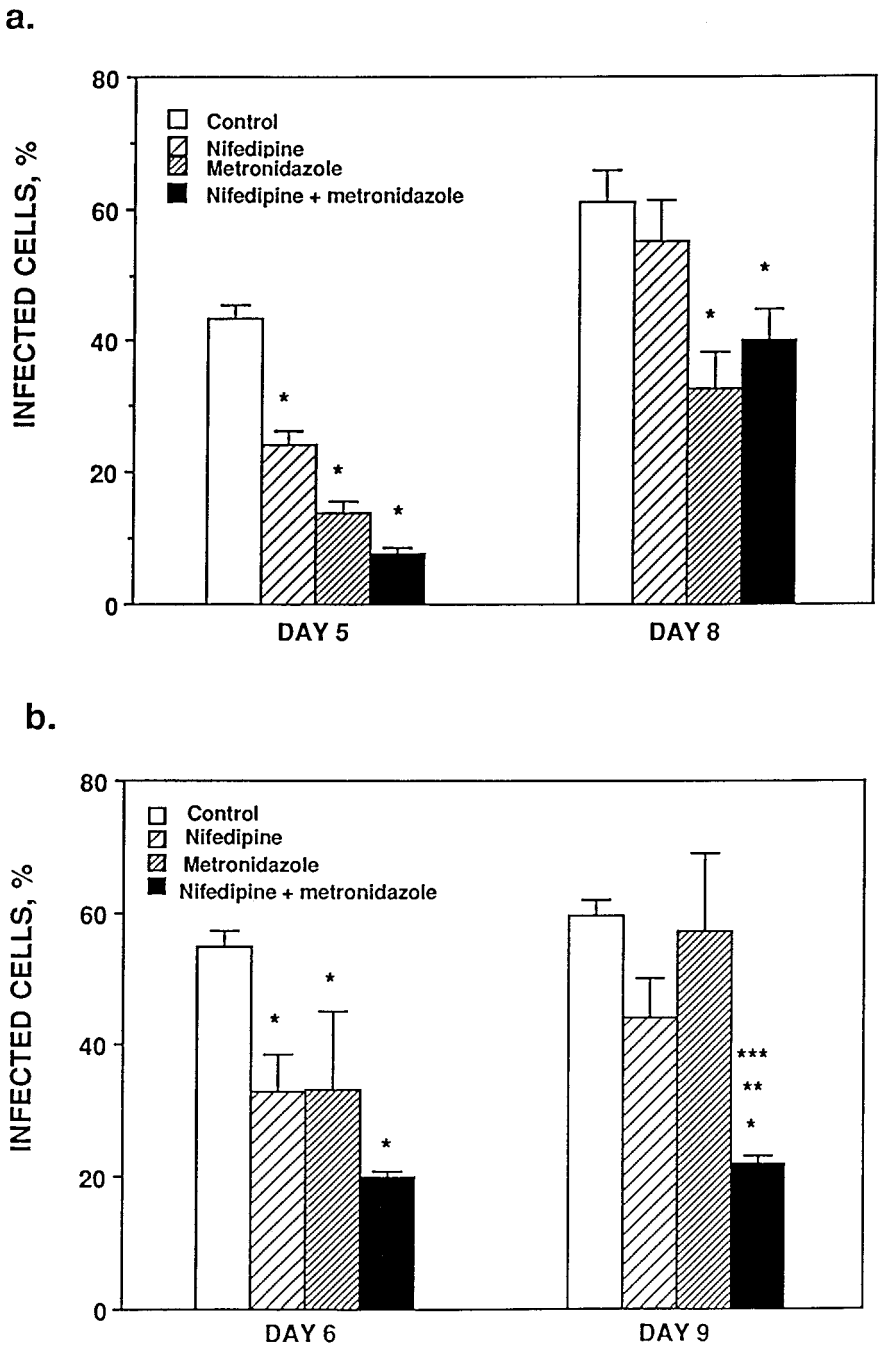

FIG. 2. Effects of nifedipine (10^{-8} M) and metronidazole (10^{-5} M), alone and in combination, on the percentage of cultured green monkey kidney cells infected with *E. hellem* (a) and *E. intestinalis* (b). *, significantly different from control; **, significantly different from nifedipine alone; ***, significantly different from metronidazole alone.

versible, as washing the treated spores overnight restored their germinating ability.

Metronidazole was first introduced to treat trichomoniasis. Its therapeutic uses were subsequently expanded to include amoebiasis, giardiasis, and anaerobic infections. Metronidazole is administered in an inactive form and enters cells by passive diffusion (5). The drug is activated in cells via the transfer of electrons from ferredoxin to its nitro group (18), and a resulting toxic intermediate is believed responsible for the chemotherapeutic effect. In the present study, when used alone at a concentration of 10 μM, metronidazole could be

TABLE 1. Fifty percent inhibitory concentrations of NO donors NP and SNAP for microsporidian spore germination

Species	IC_{50} (μM)[a]			
	NP		SNAP	
	Without Ca	With Ca	Without Ca	With Ca
E. hellem	39.4 ± 1.1	14.1 ± 0.4	7.6 ± 6.8	3.9 ± 3.0
E. intestinalis	95.6 ± 48.6	28.2 ± 12.2	77.7 ± 56.2	26.0 ± 10.2

[a] IC_{50}, 50% inhibitory concentration. Values are means ± standard errors of the means. The germination medium contained or lacked calcium as indicated.

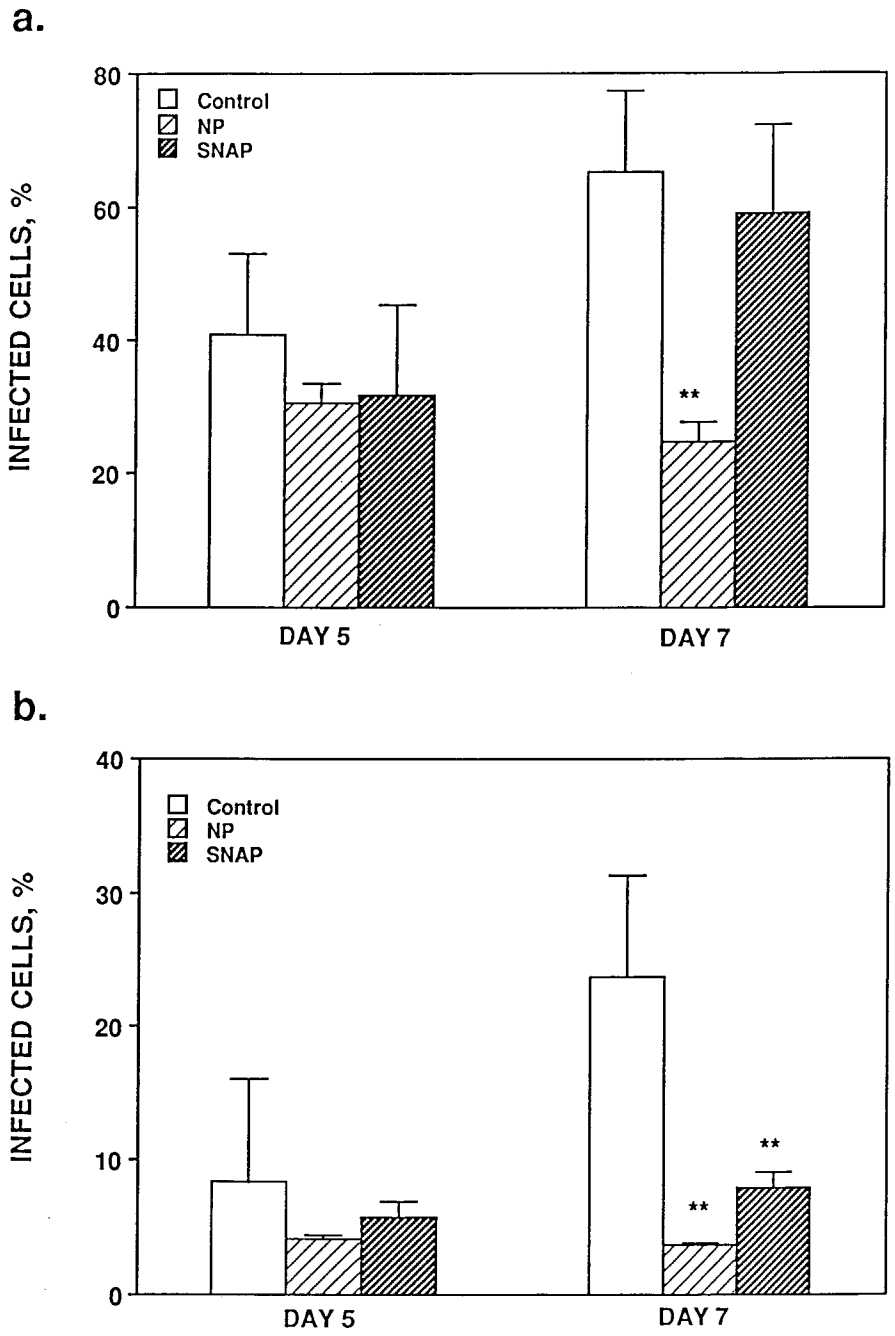

FIG. 3. Effects of nitric oxide donors NP and SNAP (10^{-5} M) on the percentage of cultured green monkey kidney cells infected with *E. hellem* (a) and *E. intestinalis* (b). **, significantly different from either of the other two groups.

shown to significantly inhibit only *E. intestinalis* spore germination in the calcium-containing H_2O_2 medium. This agent significantly inhibited spore germination in both species when used at a concentration of 100 μM (data not shown). When used in conjunction with nifedipine, 10 μM metronidazole potentiated the inhibitory effect of the former drug on *E. hellem* spore germination but not on *E. intestinalis* spore germination. While the mechanism of action of metronidazole in microsporidia is not known, it is likely that its site of action is intracellular (18). Initial attempts at treating human microsporidiosis with metronidazole showed transient improvement in some patients (15).

Both nifedipine and metronidazole were able to reduce the number of cultured cells infected with either *E. hellem* or *E. intestinalis*, and potentiation could be demonstrated by appropriately choosing the day of infection when both agents were used together. In these experiments, it is presumed that nifedipine's mode of action was to inhibit germination of spores located outside host cells, while metronidazole presumably inhibited both intracellular and extracellular spore germination. Metronidazole may have also slowed or inhibited intracellular parasite proliferation and differentiation, accounting for the fact that it appeared to be more effective in the cultured-cell assay than in the in vitro germination assay, particularly for *E. hellem* infection.

Nitric oxide is known to be cytotoxic to viruses, some bac-

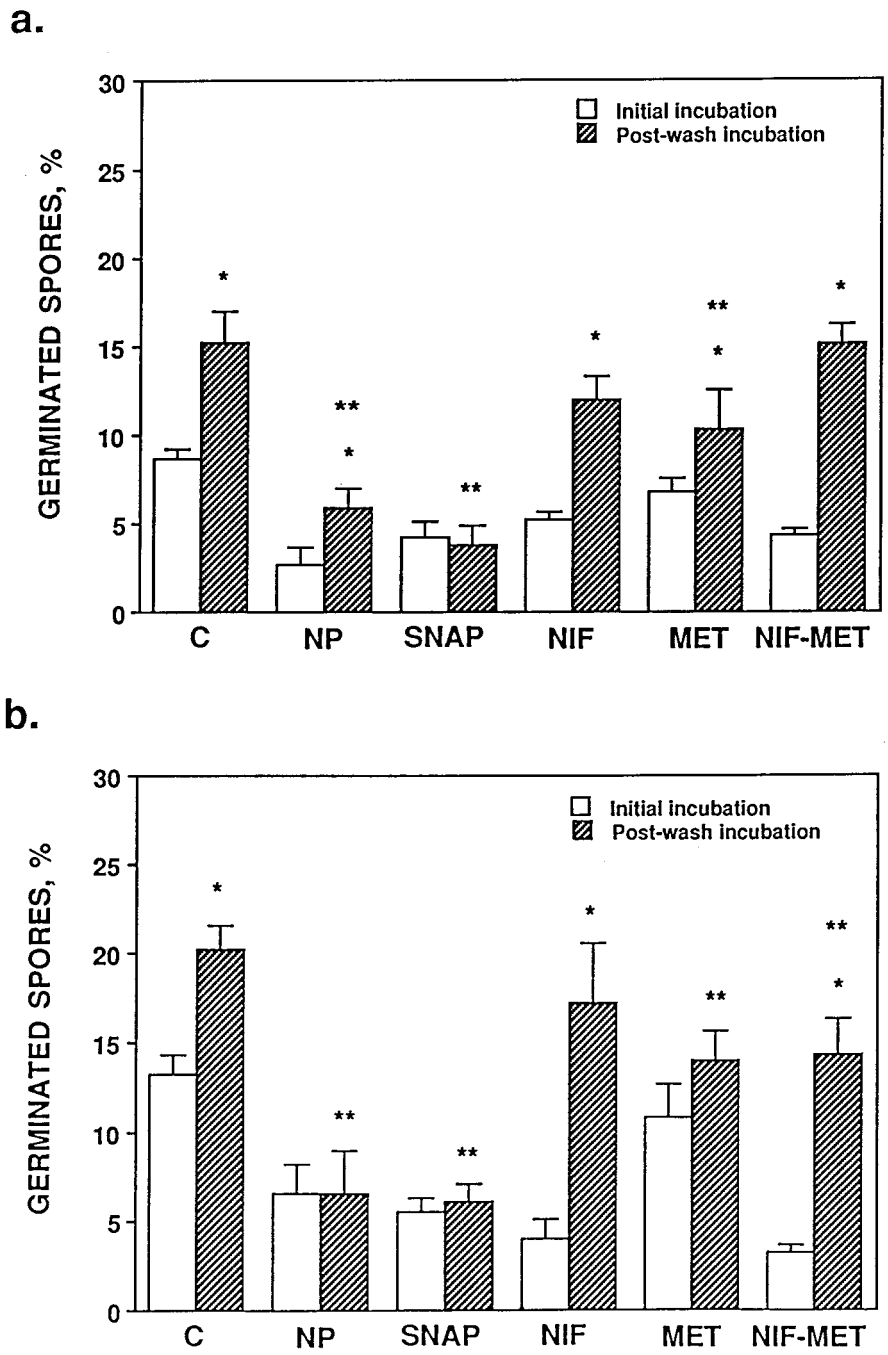

FIG. 4. Effect of washing *E. hellem* spores in water overnight after they were treated with nitric oxide donors NP and SNAP (10^{-5} M) or with nifedipine (NIF) (10^{-8} M) and metronidazole (MET) (10^{-5} M), alone or in combination, on the inhibition of germination in calcium-free medium (a) and calcium-containing medium (b). *, significantly different from the initial incubation group with the same treatment; **, significantly different from the postwash incubation control group (C).

teria, and some fungi (2, 4, 8). It has also been implicated in the killing of both intracellular and extracellular protozoan parasites (19, 31). The present data suggest that NO donors irreversibly inhibit microsporidian spore germination and reduce the infection of cultured-cell monolayers at concentrations that are not overtly toxic to the host cells. NO may be more effective in vivo in cells such as macrophages, in which its parasitocidal activity may result from its interaction with reactive oxygen molecules to produce highly toxic products such as peroxynitrite (36). Microsporidian-infected macrophages can be activated by lipopolysaccharide and gamma interferon to kill the parasites, and this killing appears to involve reactive nitrogen intermediates (13). To date, nitric oxide donors have not been very effective as antiparasitic agents in vivo (19), probably because pharmacologic tolerance to many of them develops rapidly (23), and in high doses they cause generalized vasodilation and hypotension (1).

The present study employed two in vitro assays to evaluate the efficacy of candidate antiparasitic agents. Nifedipine, metronidazole, and nitric oxide donors all exhibited antimicrospo-

ridial activity in one or both of these assays. The former two agents may have clinical application value when used in combination in infected individuals who have normal cardiovascular function, while NO may be an important element in the host's natural defense against microsporidian infection.

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