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Semisynthetic Echinocandins Affect Cell Wall Deposition of *Pneumocystis carinii* In Vitro and In Vivo

MARILYN S. BARTLETT,[1]* WILLIAM L. CURRENT,[2] MICHAEL P. GOHEEN,[1] CAROLE J. BOYLAN,[2] CHAO H. LEE,[1] MARGARET M. SHAW,[1] SHERRY F. QUEENER,[1] AND JAMES W. SMITH[1]

Departments of Pathology and Pharmacology and Toxicology, Indiana University School of Medicine,[1] and Eli Lilly & Co.,[2] Indianapolis, Indiana

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Cyclic lipodepsipeptide compounds of the echinocandin class exhibit broad-spectrum antifungal activity and have been shown to be effective in the treatment of *Pneumocystis carinii* pneumonia in laboratory animal models. Previous studies have led investigators to propose that these compounds, active against fungal cell walls, are selectively active against the cyst forms of *P. carinii*. We demonstrate that a semisynthetic, water-soluble echinocandin analog, LY307853, is effective in reducing the numbers of all life cycle forms of *P. carinii* and is more effective in mice immunosuppressed with monoclonal antibody to L3T4$^+$ cells than in mice immunosuppressed with dexamethasone. Treatment of *P. carinii* isolates with LY307853 in a short-term in vitro culture model resulted in cytoarchitectural alterations suggesting that this echinocandin may interfere with the export of surface glycoprotein and the formation of the tubular elements normally found on the surfaces of trophic forms. The cytoarchitectural changes in trophic forms treated in vitro with LY307853 were also observed in trophic forms in the lung tissue of rats treated with a closely related echinocandin analog, LY303366.

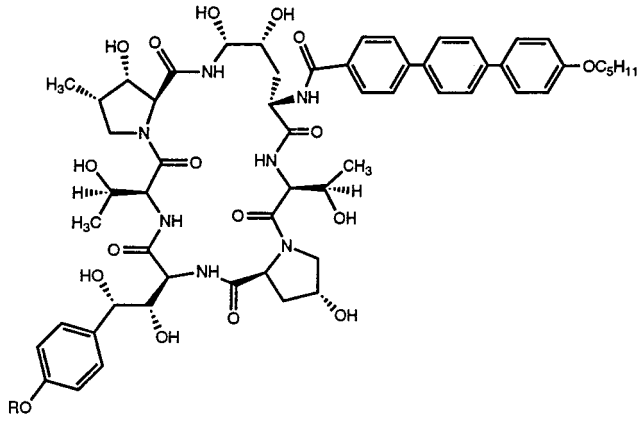
Pneumocystis carinii is an important pathogen that continues to be a significant cause of pneumonia in individuals with AIDS (21) and is an important opportunistic pathogen of immunocompromised patients receiving immunosuppressive drugs to prevent transplant rejection or for the treatment of malignancies (19). Although the combination of trimethoprim and sulfamethoxazole has been used for the prophylaxis and treatment of *P. carinii* pneumonia for 20 years (15), adverse reactions to the drug combination are common and there is concern that *P. carinii* strains resistant to these drugs may be developing. Current regimens for *P. carinii* prophylaxis have given poor responses, and more effective agents are needed (22).

Cyclic lipodepsipeptides of the echinocandin class are known inhibitors of fungal (1,3)-β-D-glucan synthase, and their fungicidal activities have been associated with the disruption of cell wall biosynthesis (9). Semisynthetic analogs of echinocandin B and pneumocandins (also members of the echinocandin class) exhibit broad-spectrum fungicidal activity in vitro and are very effective in animal models of fungal infection (9, 23–25). These compounds have also been shown to be active against *P. carinii* isolates both in vitro and in vivo (1, 8, 23–26). Because it has been reported that the cyst walls of *P. carinii* contain (1,3)-β-D-glucan and because cysts are the first life cycle form to disappear from the lungs of laboratory animals treated with echinocandins (9, 25), it has been suggested that these compounds work by preventing cyst wall synthesis and, thus, affect only cysts (25).

In the present studies, a short-term in vitro culture and spinner flask cultures for transmission electron microscopy were used to study the effects of echinocandins on trophic forms. Two immunosuppressed mouse models of *P. carinii* pneumonia were used to test the water-soluble echinocandin B analog LY307853 against all life cycle forms of *P. carinii*. Brefeldin A was included in the studies because it has been shown to inhibit protein secretion and to break down the Golgi complex (16, 17). If the transport of glycoproteins in *P. carinii* is via a Golgi complex, the inclusion of brefeldin A might cause the same ultrastructural changes seen in echinocandin-treated *P. carinii* cells. Forskolin (colforsin) blocks the activity of brefeldin A and was included to further evaluate brefeldin A activity. Electron and immune electron microscopy were used to demonstrate damage to *P. carinii* cells treated with echinocandins both in culture and in rats.

MATERIALS AND METHODS

Compounds used to treat cultures or animals. The semisynthetic echinocandin B analogs LY303366 and LY307853 (Fig. 1) were prepared at the Lilly Research Laboratories (Eli Lilly & Company, Indianapolis, Ind.). LY307853, a water-soluble, phosphate analog of LY303366, was used in the short-term, in vitro culture model and in the two mouse models of *P. carinii* pneumonia



R = H LY303366
R = PO_3H Na LY307853

FIG. 1. Semisynthetic echinocandin B analogs LY303366 and LY306853.

* Corresponding author. Mailing address: Department of Pathology and Laboratory Medicine, MS A-128, Indiana University School of Medicine, 635 Barnhill Dr., Indianapolis, IN 46202-5120. Phone: (317) 274-5767. Fax: (317) 278-2018. Electronic mail address: mbartlet@indyvax.iupui.edu.

described below. The more water-insoluble parent compound, LY303366, was used in the rat model of *P. carinii* pneumonia. When LY307853 is administered intravenously or intraperitoneally to rats and mice, virtually all of it is rapidly converted, presumably by tissue phosphatases, to the parent LY303366. One hour after intravenous administration of LY307855 to rats, only parent LY303366 was detected in plasma (unpublished data). Brefeldin A and colforsin were purchased from Sigma Chemical Co.

Short-term culture. Short-term cultures were performed by using tissue cultures of human embryonic lung fibroblasts (HEL cells) inoculated with 7×10^5 viable trophozoites per ml (6). *P. carinii* isolates were prepared from infected rat lung (3). Plates of cell monolayers were incubated at 35°C in an atmosphere of 5% O_2, 10% CO_2, and the balance N_2. Cultures were treated with 0 (nontreated control), 1.0, and 0.1 μg of LY307853 per ml and sampled on days 1, 3, 5 and 7, and the numbers of *P. carinii* organisms were determined by microscopic evaluation of 10-μl samples transferred to 1-cm^2 areas on glass slides and stained with Giemsa stain.

Spinner flask cultures. Spinner flask cultures of *P. carinii* (11, 18) were used to determine the effects of LY303366 on the ultrastructure of trophic forms of *P. carinii*. Spinner cultures of human embryonic lung fibroblasts were inoculated with *P. carinii* isolates obtained from infected rat lung (3), and after 24 h, LY307853 was added at concentrations of 10.0 or 1.0 μg/ml. Spinner flask cultures were also treated with brefeldin A at concentrations of 100 and 10 μM, forskolin at concentrations of 100 and 10 μM, and the combination of forskolin and brefeldin A. A *P. carinii*-inoculated spinner flask without drug served as a control. Fifteen-milliliter samples of culture medium containing *P. carinii* isolates were removed from spinner flask cultures at 0, 1, 2, 3, 5, and 7 days. Tissue culture fluids were centrifuged to obtain pellets. The supernatants were decanted and replaced with Karnovsky's fixative, and the pellets were fixed for 2 h at 20°C and processed for transmission electron microscopy.

Transmission electron microscopy. Karnosky's fixative-fixed pellets from spinner flasks and fixed rat lung tissues were rinsed in cacodylate buffer and then immersed in a mixture of 1.5% OsO_4 and 2.5% K_4 Fe(CN) · $6.3H_2O$ for 2 h. The specimens were rinsed in buffer, dehydrated, and embedded in polybed 812 resin. Thin sections were stained with uranyl acetate and lead citrate and were examined with a Philips CM10 transmission electron microscope at an accelerating voltage of 80 kV. Infected rat lung samples were fixed (14), rinsed in buffer, dehydrated, embedded, sectioned, stained, and examined as described for the culture samples.

For immune-specific staining, pellets from the spinner flasks were fixed with a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde for 24 h at 4°C, rinsed with 0.1 M sodium cacodylate, dehydrated with up to 95% ethanol, and embedded in LR White resin (13). Sections were picked up on Formvar- and carbon-coated grids and were subjected to the following immunostaining procedure. Sections were floated on drops of phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) for 5 min and were placed on drops of PBS containing 1% fish gelatin for 10 min and then PBS containing 0.02 M glycine for 3 min. Subsequently, the sections were incubated for 16 h at 4°C in a 1/200 dilution of polyclonal antibody to a *P. carinii* surface glycoprotein complex. The antibody was prepared by injecting two New Zealand rabbits with the 120-kDa band eluted from polyacrylamide electrophoresis gels along with incomplete adjuvent, boosting twice, and collecting blood by marginal ear vein puncture. The antibody was checked for reactivity by Western blotting (immunoblotting). The sections were rinsed in PBS-BSA for 5 min, placed on drops of rabbit immunoglobulin G Au30 for 1 h, rinsed in PBS, and placed on drops of 1% glutaraldehyde in PBS for 3 min. Control samples were incubated without the primary antibody. The sections were rinsed in distilled water, stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy.

Mouse model for efficacy testing of LY307853. BALB/c mice were immunosuppressed either with dexamethasone, 1.2 mg/kg of body weight per day (5), or with antibody (0.2 mg intraperitoneally twice a week) from hybridoma clone GK1.5 to deplete $L3T4^+$ cells (1, 10). We tested with each immunosuppressive regimen because in previous studies demonstrated improved activity in immunologically immunosuppressed mice. Albendazole and the echinocandin LY30246 were more inhibitory to *P. carinii* in mice immunosuppressed with antibody than in mice immunosuppressed with corticosteroids (1, 2). After 4 days of dexamethasone immunosuppression or 14 days of antibody immunosuppression, the mice were inoculated intratracheally with *P. carinii* isolates from mice. Infections were allowed to develop for 3 weeks, after which treatment was initiated. A group of 10 *P. carinii*-infected mice immunosuppressed with dexamethasone and a group of 10 $T4^+$-cell-depleted mice were used for each drug dose. LY307853 was administered intraperitoneally once daily at 2.5 mg/kg/day. Corresponding groups of 10 immunosuppressed untreated mice served as nonmedicated controls. Trimethoprim-sulfamethoxazole (50/250 mg/kg/day) was used as a positive treatment control. After 3 weeks of treatment, the mice were anesthetized and exsanguinated by cardiac puncture, and their lungs were removed. Infection levels were determined by examining impression smears of lung tissue stained both with Giemsa stain for trophozoite and cyst forms and with methenamine-silver nitrate stain for cyst forms. The slides were examined as unknowns by two skilled microscopists. An infection score for each slide was determined by using the following roughly logarithmic scale: 5+ was more than 100 organisms per ×1,000 field, 4+ was 11 to 100 organisms per ×1,000 field, 3+ was 1 to 10 organisms per ×1,000 field, 2+ was 2 to 9 organisms in 10 ×1,000 fields, 1+ was <1 organism in >10 ×1,000 fields, and 0 was no organisms in 50 ×1,000 fields. An infection score was the mean of two examiners' scores for 10 mice.

TABLE 1. *P. carinii* infection scores for treated and untreated mice[a]

Treatment	Dexamethasone immunosuppression				$L3T4^+$ immunosuppression			
	Trophozoite (Giemsa) score		Cyst (silver) score		Trophozoite (Giemsa) score		Cyst (silver) score	
	1	2	1	2	1	2	1	2
Echinocandin	4.0	4.0	2.0	2.0	3.0	2.0	1.0	0.0
	4.0	3.5	1.5	1.5	2.5	2.5	1.5	1.0
	3.0	3.0	2.0	2.0	0.0	0.0	0.0	0.5
	3.0	3.0	2.0	2.0	0.0	0.5	1.0	1.0
	4.0	4.0	4.0	4.0	0.0	1.0	0.5	1.0
	2.0	2.0	1.0	0.0	3.0	3.0	0.5	1.0
	4.5	4.0	1.5	1.5	2.0	2.0	0.0	0.0
	4.0	4.5	2.0	2.0	1.0	0.0	0.5	0.0
	4.0	4.0	1.0	1.0	0.0	1.0	1.0	0.0
					0.0	1.0	0.0	0.0
None	5.0	5.0	4	4	5.0	5.0	3.0	3.0
	5.0	5.0	4	4	5.0	5.0	3.5	3.0
	5.0	5.0	4	4	4.0	4.0	3.5	2.5
	5.0	5.0	4	3	4.5	5.0	1.5	2.0
	5.0	5.0	4	4	5.0	5.0	3.0	3.0
	5.0	5.0	4	4	5.0	4.5	3.0	3.5
	5.0	5.0	4	3	5.0	5.0	3.0	3.5
	5.0	5.0	4	4	4.0	4.0	3.0	3.0
	5.0	5.0	4	4	4.0	4.5	3.0	3.0
					4.0	5.0	3.0	2.0

[a] 1 and 2 represent the scores of independent evaluators who microscopically examined slides as unknowns. Treated mice had significantly reduced infections ($P < 0.0001$ for both groups by Mann-Whitney nonparametric test). Average trophozoite and cyst scores for echinocandin-treated, dexamethasone-immunosuppressed mice were 3.6 ± 0.2 and 1.6 ± 0.3, respectively; those for echinocandin-treated, $L3T4^+$-immunosuppressed mice were 1.3 ± 0.4 and 0.5 ± 0.1, respectively; those for untreated, dexamethasone-immunosuppressed mice were 5.0 ± 0.0 and 3.9 ± 0.1, respectively; and those for untreated, $L3T4^+$-immunosuppressed mice were 4.6 ± 0.1 and 2.5 ± 0.2, respectively.

Rat model for ultrastructural studies. A dexamethasone-immunosuppressed rat model (7) was used to determine if LY303366 affected the ultrastructure of trophic forms of *P. carinii* in the lungs of rats. After 1 week of immunosuppression, approximately 10^6 *P. carinii* organisms were inoculated intratracheally, and infections were allowed to develop for 3 weeks. Eight heavily infected rats were then treated intravenously once daily with 2 mg of LY303366 per kg in 33% (vol/vol) aqueous polyethylene glycol. Eight heavily infected rats were not treated and served as controls. Two rats from the LY303366-treated group and two from the control group were killed by carbon dioxide asphyxiation on days 1, 3, 5, and 7, and their lungs were perfused with citrated saline to remove blood and then fixed rapidly by simultaneous intravascular and intraluminal perfusion with Karnovsky's fixative. Fixed lung tissue was then processed for transmission electron microscopy.

RESULTS

In short-term culture, LY307853 at 0.1 μg/ml inhibited the proliferation of the *P. carinii* isolates so that at 7 days the average number of *P. carinii* organisms per ×1,000 field was 4.3 ± 0.75, whereas the number in the untreated control culture was 9.1 ± 0.81 *P. carinii* organisms per ×1,000 field. Thus, LY307853 decreased the numbers of organisms by >50%. LY307853 appears to be active against trophic forms of *P. carinii* since these life cycle stages predominate in short-term culture (4).

In the mouse models, LY307853 at 2.5 mg/kg/day depleted the numbers of *P. carinii* organisms so that treated mice had significantly fewer organisms than untreated mice ($P < 0.0001$).

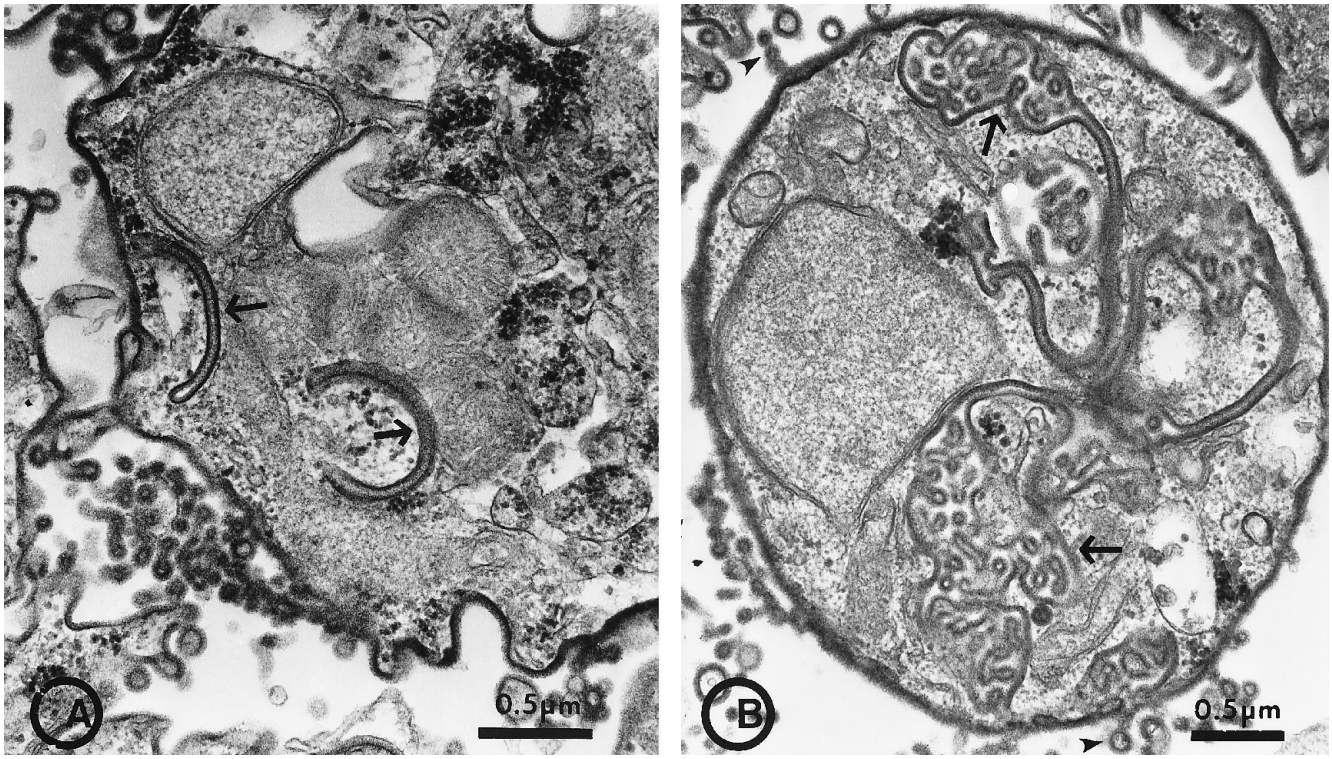


FIG. 2. (A) After 1 day of exposure to the compound LY307853, cell wall-like material (arrows) is present in a trophozoite of *P. carinii* from culture. (B) After 7 days of exposure to the compound in culture, numerous profiles of cell wall-like material are observed. Much of this internalized material (arrows) resembles the tubular projections (arrowheads) normally seen projecting from the surface of *P. carinii* isolates.

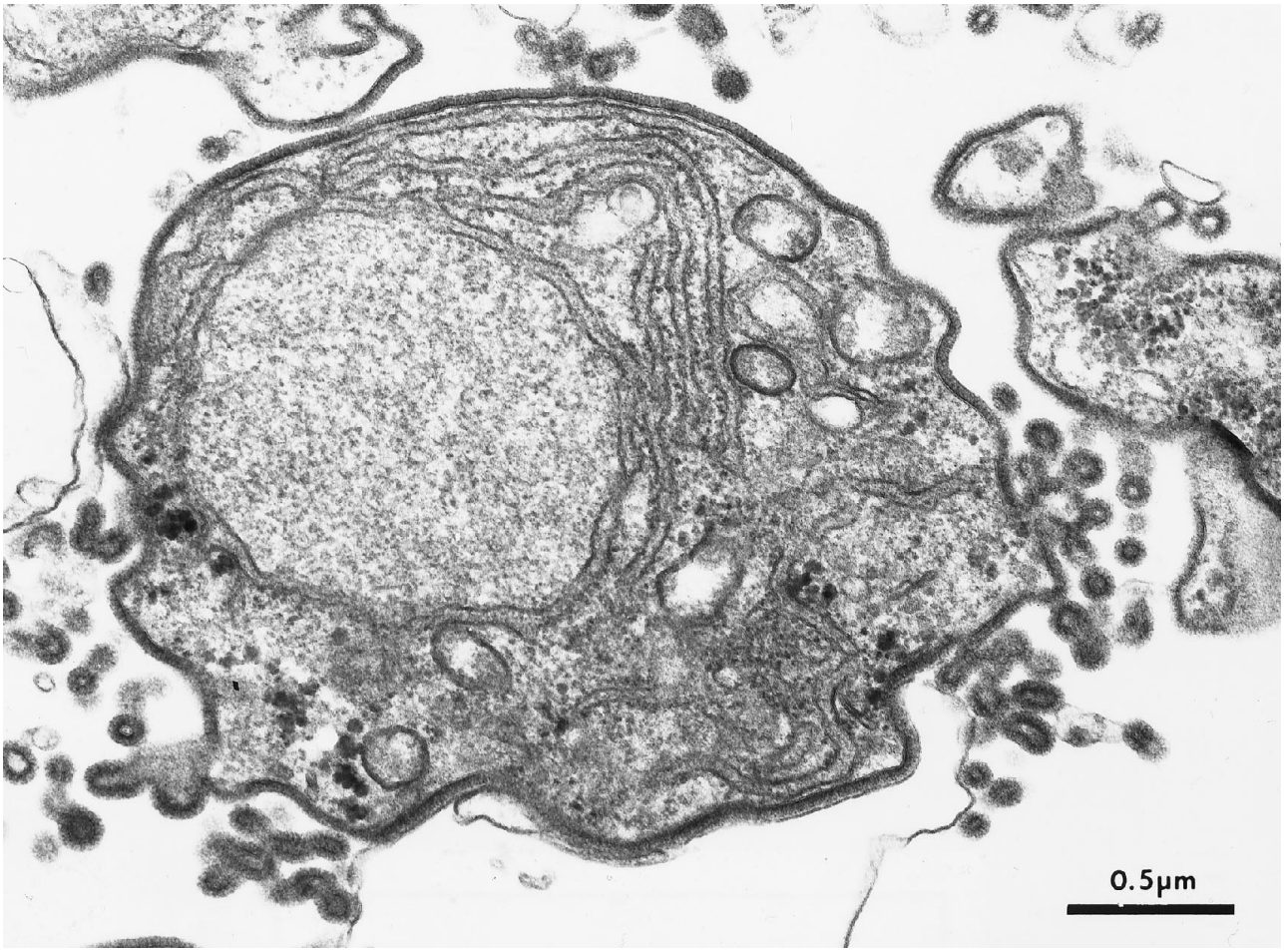


FIG. 3. Trophozoite of *P. carinii* from a 7-day control culture demonstrating the normal cellular morphology associated with this form of the organism.

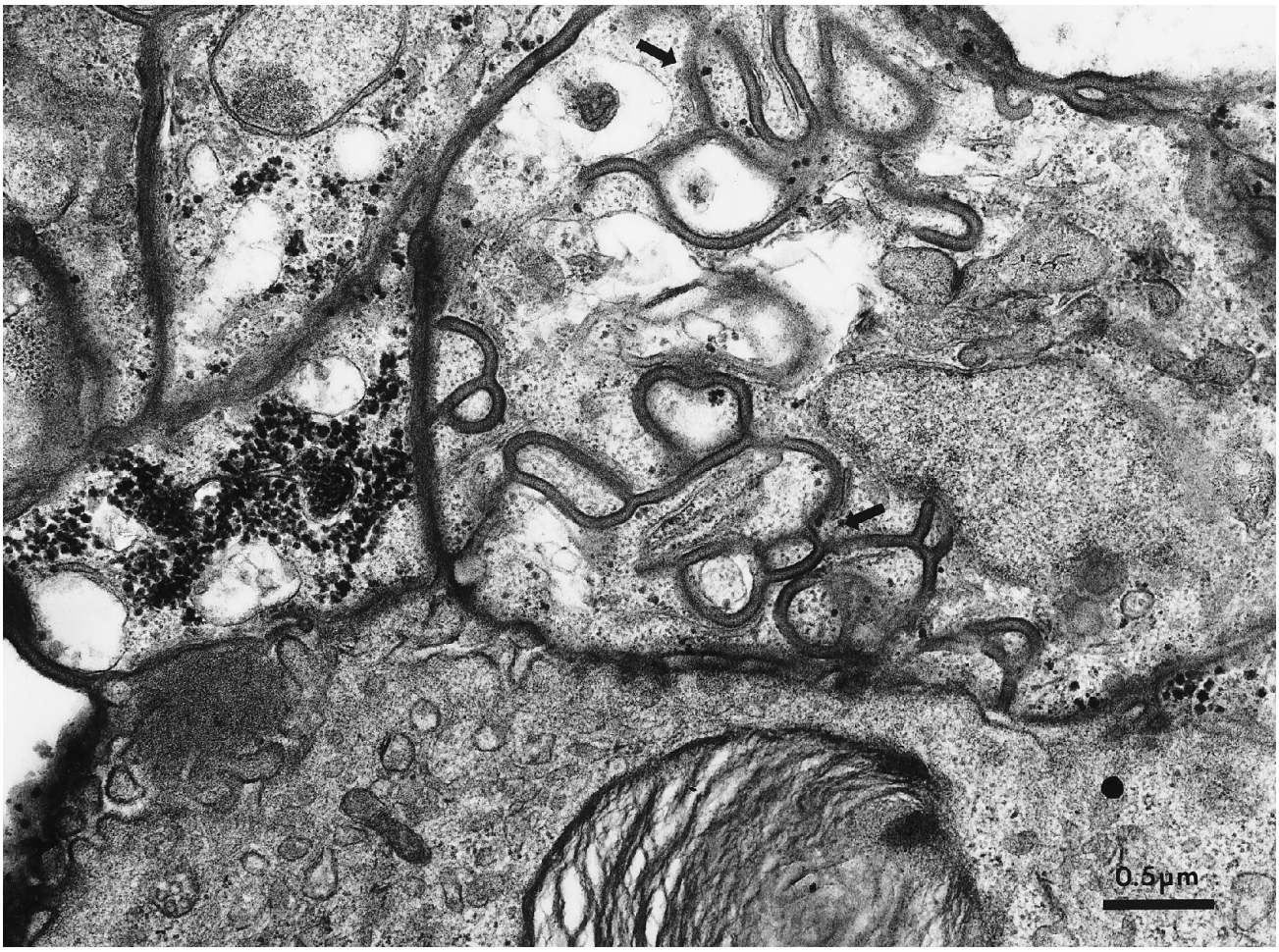


FIG. 4. Trophozoite of *P. carinii* in lung tissue of a rat exposed to the compound LY303366 for 4 days found adjacent to a type II pneumocyte in rat lung. The same type of abnormality seen in culture after exposure to LY30783 is demonstrated by the numerous internalized profiles of cell wall-like material (arrows).

Mice immunosuppressed with dexamethasone and treated with LY307853 had a mean Giemsa stain infection score of 3.6 ± 0.2, whereas untreated dexamethasone-immunosuppressed mice had a mean Giemsa stain infection score of 5.0 ± 0. Since the scoring system is roughly logarithmic, with a score of 5 representing >100 organisms per ×1,000 field and a score of 3 representing 1 to 10 organisms per ×1,000 field, drug treatment reduced infection by >90%. This reduction was of both trophozoites and cysts. The infection scores for each mouse are provided in Table 1.

In L3T4$^+$ antibody-immunosuppressed mice, LY307853 at the same dosage, 2.5 mg/kg/day, reduced infection so that the mean Giemsa stain infection score was 1.3 ± 0.4, whereas the L3T4$^+$-immunosuppressed untreated mice had a mean Giemsa stain infection score of 4.6 ± 0.1, a >99% reduction. The infection scores of the L3T4$^+$-immunosuppressed mice are presented in Table 1. Since Giemsa stain demonstrates all life cycle forms (trophozoites, precysts, and cysts), infection scores are always higher than those demonstrated by methenamine-silver nitrate stain. Heavily infected animals always have many more trophic forms than cyst forms. The decrease in total Giemsa-stained organisms reflects a decrease in both trophozoites and cysts.

Transmission electron micrographs demonstrated the early effects of LY307853 on *P. carinii* trophic forms in culture. Within the first day of culture, cell wall-like material appeared in the cytoplasm of LY307853-treated trophic forms (Fig. 2A). By day 4, treated trophozoites had numerous internal structures in their cytoplasms. These structures ultrastructurally looked like the tubular elements that normally extend from the plasma membranes of untreated organisms (Fig. 2B). The ultrastructural changes noted in LY307853-treated trophic forms were not observed in trophic forms from control cultures (Fig. 3), cultures treated with brefeldin A, or cultures treated with both brefeldin A and forskolin (data not shown).

Trophic forms of *P. carinii* examined in sections of rat lungs obtained following four to eight daily treatments (2.5 mg/kg intravenously) with LY303366 had ultrastructural changes (Fig. 4) similar to those described for trophic forms in cultures treated with LY307853. After 4 days of treatment with LY303366, cysts were practically eliminated from the lungs. The cytoarchitectural changes described above were not observed in trophic forms of *P. carinii* in lung tissue obtained from untreated rats.

Immune-specific staining with antibody directed to surface glycoproteins demonstrated binding to the plasma membrane and external tubular elements and to the tubular elements apparently confined in the cytoplasm of LY307853-treated trophic forms (Fig. 5).

DISCUSSION

Compounds of the echinocandin class of antifungal agents are noncompetitive inhibitors of (1,3)-β-D-glucan synthase, an

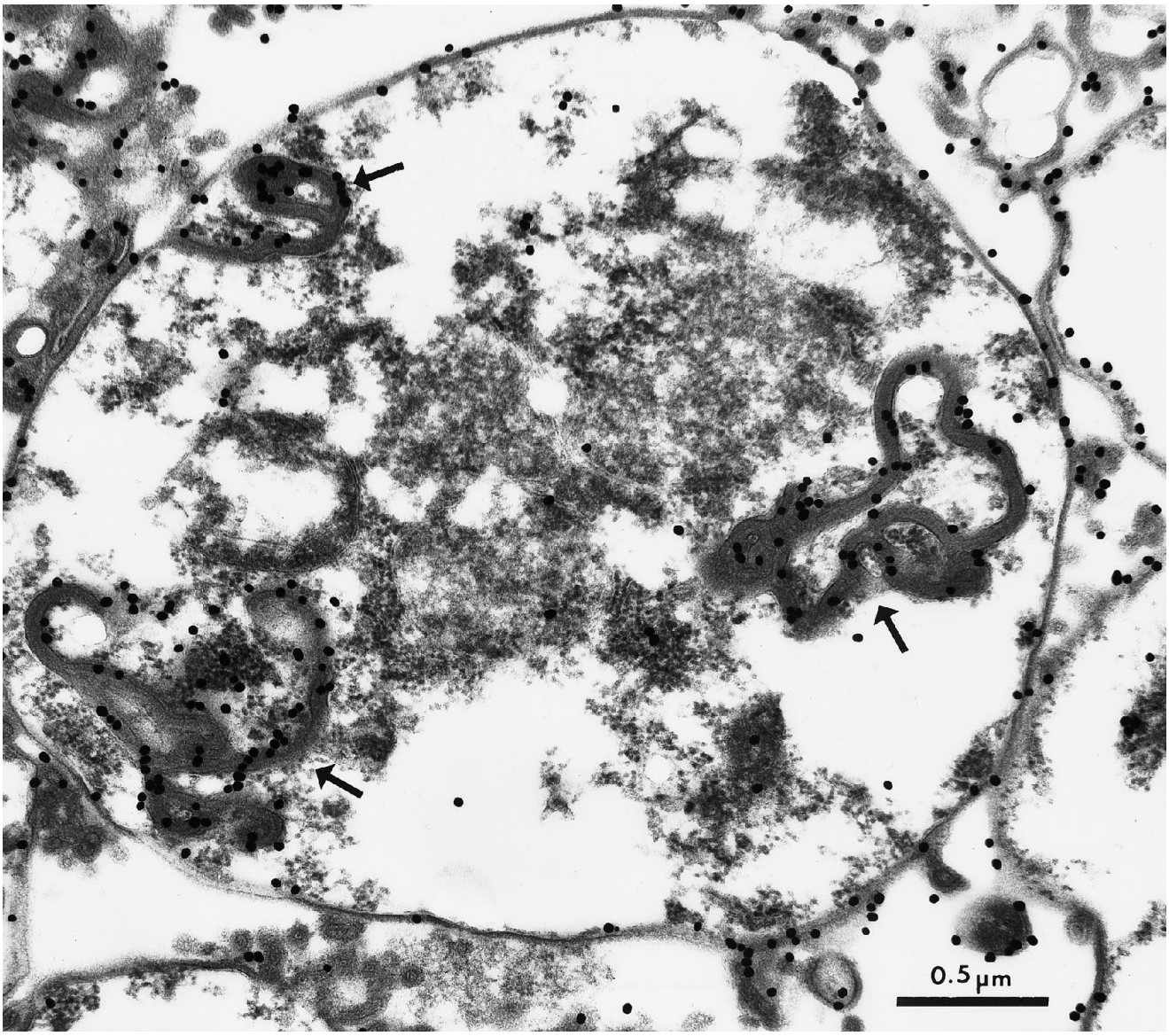


FIG. 5. Immunogold labeling of *P. carinii* isolates from culture with antibodies to a major surface glycoprotein of the organism demonstrates that the internalized material contains surface glycoprotein (arrows) normally found only on the external cell wall and tubular extensions. This organism was exposed to the compound LY307853 for 7 days.

enzyme complex that polymerizes UDP-glucose into (1,3)-β-D-glucan polymers that comprise a major component of the fungal cell wall. Because (1,3)-β-D-glucan has been demonstrated in the cyst wall of *P. carinii* cysts and because treatment of infected rats (25) and mice (1) with echinocandin analogs rapidly eliminates cyst forms, it has been suggested that these compounds act only on cysts to erradicate *P. carinii* infections (25). In the present study, the effects of LY303366 and its water-soluble phosphate analog on trophic stages of *P. carinii* were demonstrated. The apparent cytoplasmic accumulation of material that stains with antibody directed to surface glycoprotein suggests that LY303366 and its water-soluble phosphate analog LY307853 may interfere with the export of surface glycoprotein by trophic forms of *P. carinii*. After 1 day of exposure to LY307853 in culture, surface glycoprotein was observed within trophic forms. After 4 days of exposure to the compounds, surface glycoprotein was associated with internalized tubular elements, suggesting that LY307853 also interferes with the formation of the external tubular elements characteristic of trophic forms of *P. carinii*. Similar cytoarchitectural changes were observed in trophic forms of *P. carinii* in the lungs of rats treated for 4 to 8 days with LY303366, demonstrating that such cellular changes are not an artifact of culture.

Although a Golgi-like apparatus has been reported (20), the existence of this organelle in *P. carinii* isolates has not been confirmed. Brefeldin A is a compound known to inhibit protein secretion and to cause breakdown of the Golgi complex (16, 17). We included brefeldin A in short-term *P. carinii* cultures to see if it would cause the same morphologic changes as those produced by the echinocandins. Had brefeldin A caused the same distinctive morphology, it might have been hypothesized that the glycoproteins were transported through the Golgi apparatus. Since forskolin blocks the activity of brefeldin A, we included brefeldin A to see if blocking occurred. Forskolin and

the combination of forskolin and brefeldin A were included in cultures to see if blocking could be reversed. The electron micrographs in this report failed to demonstrate the morphologic changes caused by the brefeldin A or the blocking of brefeldin A activity by forskolin. Early examination times including examination times of 5 min and 1 h would have resulted in examination before the occurrence of the reversible changes that normally occur with brefeldin A at 6 h. Although these negative data do not rule out the possibility that LY307853 is acting on a Golgi-like organelle, the experiments that were repeated with various concentrations of brefeldin A and forskolin failed to cause these changes, while the echinocandin LY307853 always produced the distinctive morphologic changes. Morphologic changes induced by another echinocandin B analog, LY302146, have been reported (12). We hypothesize that echinocandins inhibit the replication of *P. carinii* by altering the transport and deposition of cell wall components.

ACKNOWLEDGMENT

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