

Pneumocystis carinii and Specific Fungi Have a Common Epitope, Identified by a Monoclonal Antibody

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Because *Pneumocystis carinii* may be related to fungi, we evaluated the reactivities of monoclonal antibodies raised against *P. carinii* with a variety of fungi. Fifty-two fungi and six protozoa were evaluated by immunofluorescence. One of three monoclonal antibodies (MAbs) tested (Mab 7D7) reacted with 15 fungi but no protozoa. *Saccharomyces cerevisiae* showed the strongest reactivity by immunofluorescence. The reactive antigen was characterized for four fungi by the immunoblot technique. In all cases the antigen that was reactive with Mab 7D7 was larger than the *P. carinii* antigens that reacted with 7D7. In further studies with *P. carinii*, *Aspergillus* species, and *S. cerevisiae*, we found that Mab 7D7 reacted with a carbohydrate component in all organisms. The presence of an epitope that is common to *P. carinii* and a number of fungi further supports the fungal nature of *P. carinii*.

The protozoan versus fungus debate on the taxonomy of *Pneumocystis carinii* has existed for many years, dating back almost as far as the discovery of the organism (1, 17). Detailed ultrastructural studies by various investigators suggested that *Pneumocystis carinii* resembles fungi more than it does protozoa (22), but the organism has been maintained as a probable protozoan in most textbooks. More recent reports now support a closer relationship between *Pneumocystis carinii* and fungi on the basis of rRNA sequences (5, 19) as well as structural characteristics of dihydrofolate reductase (4) and thymidylate synthetase (6). It has also been reported that *Pneumocystis carinii* cross-reacts with polyclonal antisera prepared against *Aspergillus* spp. (2, 21).

We have previously reported the preparation and evaluation of three monoclonal antibodies (MAbs) for use in the diagnosis of human *Pneumocystis carinii* infections (8, 13, 14). Two of these MAbs (MAbs 2G2 and 6B8) were originally produced by using human *Pneumocystis carinii*, while one (Mab 7D7) was derived by using rat *Pneumocystis carinii* (11, 12). Specificity studies were done with these MAbs mainly to determine whether yeasts, particularly *Candida* and *Cryptococcus* spp., would cross-react with these MAbs and yield false-positive reactions when the MAbs were used for the diagnosis of *Pneumocystis carinii* infections. These original studies showed no cross-reactivity with several isolates of different species of *Candida* as well as *Cryptococcus* spp. We expanded those studies to include a wider variety of fungal and protozoal organisms to investigate the possible antigenic relationship between *Pneumocystis carinii* and either fungi or protozoa. The finding of cross-reactions between *Pneumocystis carinii* and fungi by using MAbs prepared against *Pneumocystis carinii* provides additional support for the fungal nature of this organism.

MATERIALS AND METHODS

Mab preparation. Ascites containing MAbs to human and rat *P. carinii* were prepared as described previously (11, 12).

Indirect fluorescent-antibody assay (IFA). Organisms were applied to a slide and air dried, fixed in cold acetone for 5 min, and then covered with Mab and incubated for 15 min at 37°C. Slides were then washed, air dried, covered with a 1:10 dilution of anti-mouse immunoglobulin G (IgG; Tago, Burlingame, Calif.), incubated for 15 min at 37°C, washed, and mounted. Organisms were first screened by using a combination of the following three MAbs at the indicated dilutions (previously determined to be optimal for the individual MAbs): 2G2, 1:150; 6B8, 1:60; 7D7, 1:90. If the pooled reaction was positive, the MAbs were tested individually at the same dilution used in the pool.

Organisms. All fungal isolates except *Penicillium marneffe* were obtained from clinical specimens and were previously identified by the Mycology Laboratory of the Microbiology Service, National Institutes of Health. *Penicillium marneffe* was obtained from June Kwon-Chung of the Mycology Division, National Institute of Allergy and Infectious Diseases. All isolates were grown on and tested from Sabouraud agar, using vegetative growth such as mycelial or yeast forms as well as specialized structures such as macro- or microconidia or various fruiting bodies. For dimorphic fungi, including *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Penicillium marneffe*, both yeast and mycelial forms were tested after autoclaving the test material. For *Histoplasma capsulatum*, time course studies were done to examine the variabilities in intensities of fluorescence during the transition from the yeast to the mold stage. The individual fungi that were tested are listed in Table 1.

Smears containing *Cryptosporidium* oocysts, *Isospora belli* oocysts, *Entamoeba histolytica* cysts and trophozoites, *Giardia lamblia* cysts and trophozoites, and *Blastocystis hominis* from stool specimens were obtained from the Parasitology Laboratory, National Institutes of Health. These smears, along with a preparation of a non-chlorophyll-containing alga, *Prototheca wickerhamii* (obtained from the Mycology Laboratory, National Institutes of Health), and *Toxoplasma gondii* tachyzoites from tissue culture, were

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TABLE 1. Fungi examined by IFA by using MAb 7D7 raised against rat *P. carinii*

| Test result and strain |
|---|
| Positive by IFA |
| <i>Aspergillus</i> spp. (<i>A. flavus</i> , <i>A. fumigatus</i> , <i>A. glaucus</i> , <i>A. nidulans</i> , <i>A. niger</i>) |
| <i>Candida</i> spp. (<i>C. krusei</i> , <i>C. lipolytica</i>) |
| <i>Histoplasma capsulatum</i> |
| <i>Penicillium</i> sp. |
| <i>Saccharomyces cerevisiae</i> |
| <i>Saccharomyces unisporus</i> |
| <i>Trichosporon beigeli</i> |
| Positive by IFA, conidia only, <i>Aspergillus</i> spp. (<i>A. clavatus</i> group, <i>A. ochraceus</i> , <i>A. terreus</i>) |
| Negative by IFA |
| <i>Absidia</i> sp. |
| <i>Alternaria</i> sp. |
| <i>Aspergillus ustus</i> |
| <i>Aureobasidium</i> sp. |
| <i>Beauveria</i> sp. |
| <i>Blastomyces dermatitidis</i> |
| <i>Candida</i> spp. (<i>C. albicans</i> , <i>C. glabrata</i> , <i>C. guilliermondii</i> , <i>C. kefyr</i> , <i>C. lambica</i> , <i>C. parapsilosis</i> , <i>C. rugosa</i> , <i>C. tropicalis</i>) |
| <i>Chaetominum</i> sp. |
| <i>Chrysosporium</i> sp. |
| <i>Circinella</i> sp. |
| <i>Cryptococcus neoformans</i> |
| <i>Cunninghamella</i> sp. |
| <i>Curvularia</i> sp. |
| <i>Exophiala</i> sp. |
| <i>Geotrichum</i> sp. |
| <i>Hansenula anomala</i> |
| <i>Malbranchea</i> sp. |
| <i>Microsporium canis</i> |
| <i>Microsporium gypseum</i> |
| <i>Mucor</i> sp. |
| <i>Penicillium marneffe</i> |
| <i>Rhizopus</i> sp. |
| <i>Schizosaccharomyces pombe</i> |
| <i>Sepedonium</i> sp. |
| <i>Sporothrix schenkii</i> |
| <i>Syncephalastrum</i> sp. |
| <i>Talaromyces bacillospor</i> |
| <i>Talaromyces flavus</i> |
| <i>Trichoderma</i> sp. |
| <i>Trichophyton rubrum</i> |

stained as described above. *Pneumocystis carinii* was obtained from clinical specimens or from immunosuppressed rats.

PAGE and immunoblot procedures. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblots were performed as described previously (7, 9, 12, 15, 20). Cultures of the molds to be tested were grown on Sabouraud agar slants until sporulation was evident. Growth was harvested and suspended in 250 μ l of 0.125 M Tris HCl (pH 6.8)-2% SDS, incubated overnight at 4°C, and pelleted. *Saccharomyces cerevisiae* was harvested from 1- to 2-day-old Sabouraud slants and treated as described above for the molds. In attempting to solubilize the antigen, *Saccharomyces cerevisiae* was also treated with Lyticase (β -1,3-glucanase; 5,000 U/ml; Sigma, St. Louis, Mo.). Samples were prepared for SDS-PAGE by heating them to 100°C for 15 min in the presence of 5% 2-mercaptoethanol and bromphenol blue and subsequently pelleting them for 5 min in an Eppen-

dorf centrifuge (14,000 \times g). The supernatants were run by using a 4% stacking gel (the buffer was 0.125 M Tris HCl [pH 6.8]) and a 10% running gel (the buffer was 0.475 M Tris HCl [pH 8.8]). The electrode buffer used was Tris glycine (pH 8.3; 0.25 M Tris, 0.192 M glycine, 0.1% SDS). A 40- μ l aliquot was applied to each lane, and gels were run at 10°C under 60 mA of constant current for 3.5 h.

After separation by SDS-PAGE, antigens were electrophoretically transferred to nitrocellulose and evaluated by the immunoblot (Western blot) technique. For transfer, gels were preequilibrated for 30 min in Tris glycine (pH 8.3) in 20% methanol and transferred overnight in the same buffer at 4°C by using an electrophoretic blotting apparatus (Bio-Rad, Richmond, Calif.) set at 30 V. The nitrocellulose was blocked with 3% gelatin in 0.02 M Tris-0.5 M NaCl (TBS; pH 7.5), washed in TBS containing 0.05% Tween 20, incubated for 2 h at room temperature with MAb 7D7, washed again, and incubated for 2 h at room temperature with goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad). Specific bands were detected by incubating with 4-chloro-1-naphthol (Bio-Rad) in the presence of 0.0015 M H₂O₂. Parallel gels were stained for protein by using a silver stain (Bio-Rad).

Studies on stability of *Saccharomyces cerevisiae*, *Aspergillus* spp., and *Pneumocystis carinii* antigens reactive with MAb 7D7. *Saccharomyces cerevisiae* or specimens containing *Pneumocystis carinii* were treated by the following procedures and then stained by IFA with MAb 7D7: (i) autoclaving, (ii) treatment with up to 800 μ g of proteinase K (Sigma) per ml for 3 h, (iii) treatment with up to 5% trypsin (Sigma) for up to 3 h, (iv) treatment with up to 0.2% pronase (Calbiochem, Behring Diagnostics, San Diego, Calif.) for up to 30 min, (v) treatment with Sputolysin (6.5 \times 10⁻³ M dithiothreitol; Behring Diagnostics) at 35°C for 2 h, (vi) treatment with up to 5,000 U of Lyticase (β -1,3-glucanase; Sigma) for up to 3 h, and (vii) treatment overnight with 0.05 M periodic acid (pH 4.5) at 4°C. Cultures of *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus niger* were treated as described above with proteinase K, trypsin, pronase, Sputolysin, Lyticase, and periodic acid.

RESULTS

Screening of fungi with anti-*Pneumocystis carinii* MAbs 7D7, 2G2, and 6B8. None of the 52 fungal isolates showed reactivity by immunofluorescence with MAb 2G2 or MAb 6B8, and the majority showed little or no fluorescence with MAb 7D7. However, of the yeast strains tested, *Candida krusei*, *Candida lipolytica*, *Saccharomyces cerevisiae*, *Saccharomyces unisporus*, and *Trichosporon beigeli* showed substantial fluorescence with MAb 7D7. *Saccharomyces cerevisiae* demonstrated the strongest reactivity (Fig. 1). Of the other fungi tested, only *Histoplasma capsulatum*, *Penicillium* sp. and certain of the *Aspergillus* spp., including *Aspergillus nidulans*, *Aspergillus glaucus*, *Aspergillus niger*, *Aspergillus flavus*, and *Aspergillus fumigatus*, showed consistent and strong fluorescence. The site of reactivity of the *Aspergillus* spp. varied, in that for some species fluorescence occurred along the hyphae, while for others the reaction was confined to conidia, conidiophores, or vesicles.

IFA studies on *Histoplasma capsulatum*. Although both the yeast and mold phases of this organism showed good fluorescence with MAb 7D7, it was clear that the yeast phase was much less reactive than the mold phase (Fig. 2). As the organism progressed from the yeast to the mold phase, an increase in fluorescence was very apparent.

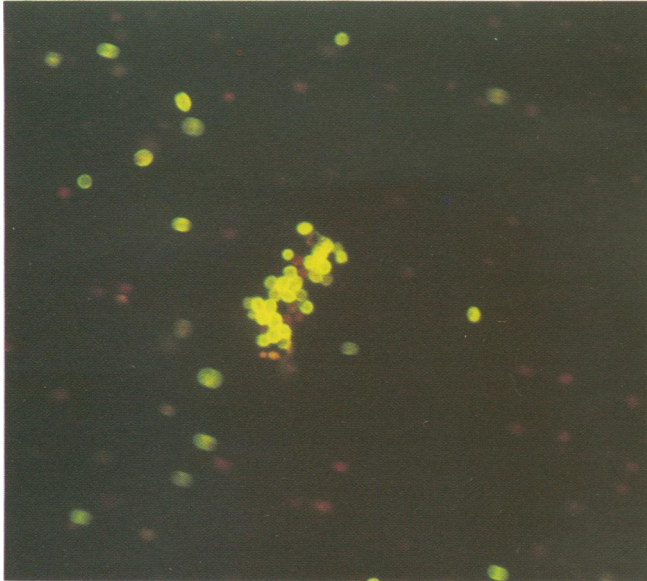


FIG. 1. Bright fluorescence of *Saccharomyces cerevisiae* obtained by using MAb 7D7.

Screening of nonfungal organisms. No cross-reactivity was detected with *Prototheca wickerhamii*, a non-chlorophyll-containing alga which grows well on fungal media. In addition, all the protozoa tested, including *Toxoplasma gondii*, *Cryptosporidium* sp., *Isospora belli*, *Entamoeba histolytica*, *Giardia lamblia*, and *Blastocystis hominis*, were also nonreactive.

SDS-PAGE and immunoblot results. To identify the antigens that were reactive with MAb 7D7 and to evaluate their relationship with *Pneumocystis carinii*, immunoblot studies were undertaken. Immunoblots on *Aspergillus niger*, *Aspergillus flavus*, and a *Penicillium* sp. all showed bands

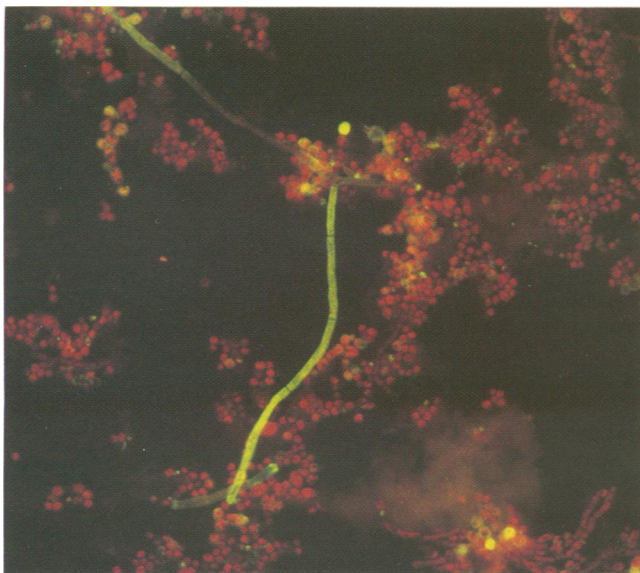


FIG. 2. Bright fluorescence of hyphae (mycelial phase) of *Histoplasma capsulatum* obtained by using MAb 7D7. The yeast phase in the background shows only minimal fluorescence.

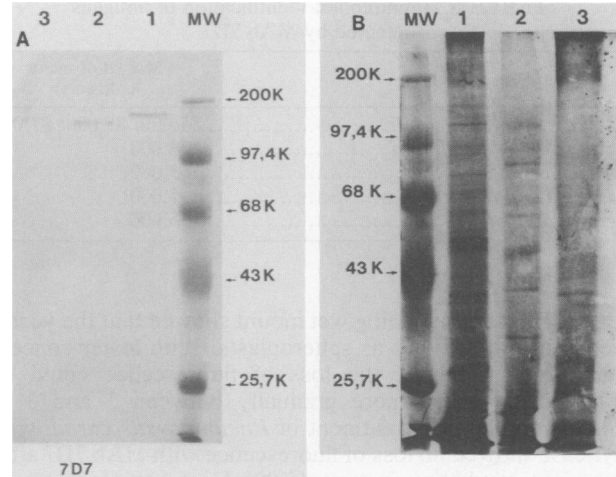


FIG. 3. (A) Immunoblot demonstrating reactivity of MAb 7D7 with antigens of three fungi. Lane 1, *Aspergillus niger*; lane 2, *Aspergillus flavus*; lane 3, *Penicillium* sp. Organisms were harvested from Sabouraud agar slants and processed for electrophoresis as described in the text. The molecular weight (MW) markers indicated on the right include myosin (200,000), phosphorylase *b* (97,400), bovine serum albumin (68,000), ovalbumin (43,000), and chymotrypsinogen (25,700). (B) Silver-stained SDS-polyacrylamide gel of extracted proteins. Lane numbers correspond to those in panel A.

which reacted with anti-*Pneumocystis carinii* MAb 7D7 (Fig. 3). As shown previously (11, 12), the antigens of *Pneumocystis carinii* that are reactive with MAb 7D7 are approximately 55,000, 85,000, and 87,000 Da. However, with *Aspergillus*, *Penicillium*, and *Histoplasma* spp., the molecular weights of the major antigens that were reactive with MAb 7D7 were all greater than 120,000 (Fig. 3 and 4; Table 2). Silver staining of parallel gels failed to demonstrate protein bands that corresponded to the antigens identified by immunoblot. Attempts to demonstrate the antigen in *Saccharomyces cerevisiae* by immunoblot were unsuccessful, possibly because of solubilization difficulties.

Biochemical characterization of the antigens reacting with MAb 7D7. *Saccharomyces cerevisiae* pretreated with proteinase K, pronase, trypsin, autoclaving, or dithiothreitol demonstrated no loss of fluorescence when it was stained with MAb 7D7 (Table 3). Exposure to 5,000 U of Lyticase per ml, however, resulted in the immediate loss of fluores-

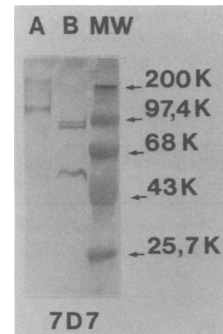


FIG. 4. Immunoblot showing reactivity of MAb 7D7 with *Histoplasma capsulatum* (lane a) and rat *Pneumocystis carinii* (lane b). The molecular weight (MW) markers indicated on the right are the same as those described in the legend to Fig. 3.

TABLE 2. Immunoblot identification of antigens detected by MAb 7D7

| Source of antigen | Mol wt of major antigen(s) |
|---------------------------------------|----------------------------|
| Rat <i>Pneumocystis carinii</i> | 55,000, 85,000, 87,000 |
| <i>Histoplasma capsulatum</i> | 120,000 |
| <i>Aspergillus niger</i> | 165,000 |
| <i>Aspergillus flavus</i> | 155,000 |
| <i>Penicillium</i> sp..... | 155,000 |

cence. The corresponding wet mount showed that the yeasts were still present, but as spheroplasts. With lower concentrations of Lyticase, the loss of fluorescence could be observed to occur more gradually between 1 and 3 h. Conversely, similar treatment of *Pneumocystis carinii* with Lyticase showed no loss of fluorescence with MAb 7D7 after overnight incubation in up to 5,000 U of Lyticase per ml. Fluorescence also remained intact after autoclaving as well as after dithiothreitol treatment. However, diminution or loss of fluorescence was readily demonstrable after treatment with proteinase K, trypsin, or pronase. *Aspergillus flavus*, *Aspergillus fumigatus*, and *Aspergillus niger* showed patterns of reactivity similar to that of *Pneumocystis carinii*. *Saccharomyces cerevisiae*, *Pneumocystis carinii*, and all three *Aspergillus* spp. showed a loss of fluorescence following treatment with periodic acid.

DISCUSSION

This study demonstrates that a MAb (MAb 7D7) generated against rat *Pneumocystis carinii* also reacts with a number of fungi but not with protozoa. This further supports the characterization of *Pneumocystis carinii* as a fungus and not a protozoan. Of note, two MAbs that reacted only with human and not with rat *Pneumocystis carinii* showed no cross-reactivity with fungi. It is interesting that MAb 7D7 is the only MAb of seven developed by our group that reacts with both human and rat *Pneumocystis carinii*, suggesting that the antigen identified by this antibody is common to all *Pneumocystis carinii* strains. The demonstration of this antigenic epitope among other organisms suggests that this epitope may be functionally important since it has been conserved between species. Of the 52 fungi tested by IFA, 15 strains were found to have a positive reaction with MAb 7D7; in 3 of the 15 strains, only conidia appeared to fluoresce

TABLE 3. Reactivity with MAb 7D7 following treatment of *Saccharomyces cerevisiae*, *Pneumocystis carinii*, *Aspergillus fumigatus*, *Aspergillus niger*, and *Aspergillus flavus* with enzymes, dithiothreitol, autoclaving, and periodic acid

| Treatment | Reactivity ^a | | |
|----------------|---------------------------------|-----------------------------|-------------------------|
| | <i>Saccharomyces cerevisiae</i> | <i>Pneumocystis carinii</i> | <i>Aspergillus</i> spp. |
| Lyticase | - | + | + |
| Pronase | + | - | - |
| Proteinase K | + | - | - |
| Trypsin | + | - | - |
| Autoclaving | + | + | ND |
| Dithiothreitol | + | + | + |
| Periodic acid | - | - | - |

^a-, diminution or loss of fluorescence; +, no diminution or loss of fluorescence; ND, not done.

(Table 1). Cross-reactivity between *Aspergillus* spp. and *Pneumocystis carinii* has been reported previously, using rabbit serum raised against the *Aspergillus fumigatus* cell wall (2, 21). It is unclear on the basis of the available data whether the previously identified cross-reactive antigens are the same as those identified in this study.

To identify and compare the reactive antigens, we used the immunoblot technique to evaluate four fungi that were positive by IFA. Again, only MAb 7D7 showed reactivity by immunoblot with the fungal antigens. The sizes of the antigens reacting with MAb 7D7 are summarized in Table 2. The antigens identified in *Aspergillus* and *Penicillium* spp. were approximately the same size, suggesting that MAb 7D7 recognizes homologous antigens in these fungi. The cross-reacting antigen of *Histoplasma capsulatum* had a lower molecular weight than the *Aspergillus* and *Penicillium* antigens, but this weight was still substantially higher than those of the *Pneumocystis carinii* antigens. The finding that the mycelial phase of *Histoplasma capsulatum* showed greater cross-reactivity than the yeast phase corresponds to earlier findings that the *Histoplasma* mycelial-phase cell wall is more similar to the *Saccharomyces cerevisiae* cell wall than is the *Histoplasma* yeast-phase cell wall. The *Saccharomyces cerevisiae* cell wall and the *Histoplasma* mycelial-phase cell wall, both of which cross-reacted with MAb 7D7, have lower chitin and higher mannose and amino acid contents than the *Histoplasma* yeast-phase cell wall (3). The only fungus tested that did not react by immunoblot was *Saccharomyces cerevisiae*, which gave the brightest reaction by IFA. This appeared to result from a difficulty in solubilizing the antigen, despite attempts at solubilization with a number of detergents.

For *Pneumocystis carinii* as well as three *Aspergillus* spp., loss of fluorescence was seen after treatment with proteinase K, trypsin, and pronase, while for *Saccharomyces cerevisiae*, loss of fluorescence was seen with Lyticase treatment only. Lyticase is a dual enzyme consisting of a β -1,3-glucanase as well as an alkaline protease that is capable of yeast cell lysis (18). It is speculated that the protease acts on the protein portion of an outer layer of mannoprotein, which then allows the glucanase to attack yeast cell glucan, which is responsible for cell integrity and rigidity. Since Lyticase removed the MAb 7D7-binding site on *Saccharomyces cerevisiae*, while autoclaving and proteolytic enzymes alone did not, it is likely that the antigen that reacts with MAb 7D7 is intimately associated with a carbohydrate, possibly a glucan, within the yeast cell wall. On the other hand, the Lyticase treatment did not affect the binding of MAb 7D7 to *Pneumocystis carinii* or *Aspergillus* spp., while trypsin, proteinase K, and pronase did, suggesting that the MAb 7D7-reactive antigen on *Pneumocystis carinii* and *Aspergillus* spp. has an important protein component. The loss of reactivity following treatment with periodic acid in all organisms tested clearly demonstrates, however, that the reactive epitope has an important carbohydrate component. Thus, for *Pneumocystis carinii* and *Aspergillus* spp. it is likely that the reactive epitope is a carbohydrate component of a glycoprotein.

Several previous reports support the idea that *Pneumocystis carinii* is a fungal organism. The nucleotide sequence of *Pneumocystis carinii* rRNA corresponds most closely with those of the rRNAs of *Saccharomyces cerevisiae* in particular and other fungi in general (5, 19). Comparisons of rRNA sequences of various *Aspergillus* spp. and *Histoplasma capsulatum* were not included in the rRNA studies, but this would be of interest since we have shown that these

fungi cross-react with MAb 7D7. Two separate genes encode for dihydrofolate reductase and thymidylate synthetase in *Pneumocystis carinii*, as is the case for fungi, whereas a single gene encodes both activities in protozoa (4, 6, 16). Ultrastructural findings also support the idea that *Pneumocystis carinii* is a fungus (22). On the other hand, *Pneumocystis carinii* is susceptible to sulfonamides and trimethoprim and, thus, has a folate metabolism that resembles those of the protozoan *Toxoplasma gondii* and species that cause malaria (10, 23).

The controversy on the taxonomy of *Pneumocystis carinii* is ongoing. More studies, particularly those that look at DNA homologies between *Pneumocystis carinii* and specific fungi, as suggested by the results of this study, are needed to finally resolve this issue.

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