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Immunocytochemical Detection of Chitin in *Pneumocystis carinii*

ANNA N. WALKER,[1] RONALD E. GARNER,[2] AND MICHAEL N. HORST[3]*

Department of Pathology,[1] and Immunology Section[2] and Biochemistry Section,[3] Division of Basic Medical Sciences, Mercer University School of Medicine, Macon, Georgia 31207

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Polyclonal antisera against chitin and chitin oligomers were used to stain *Pneumocystis carinii* by the immunoperoxidase technique in Formalin-fixed, paraffin-embedded sections of four human lung biopsies and in alcohol-fixed, paraffin-embedded cell blocks of two bronchioloalveolar lavage specimens from infected human patients. In all cases, the antisera bound *P. carinii* but did not bind the host tissue elements. Moreover, the antisera bound not only to the cyst forms of *P. carinii* but also to the intracystic bodies and to the trophic forms. Preadsorption of the anti-chitin antiserum with purified chitin abolished all staining of *P. carinii*. Our results indicate that *P. carinii* produces chitin at more than one stage of its life cycle in the infected human host.

Pneumocystis carinii has become an increasingly common opportunistic pathogen. Currently, an estimated 60 to 80% of patients with acquired immunodeficiency syndrome suffer from *P. carinii* pneumonia during the course of their illness (8). Many biological details of *P. carinii*, however, have eluded investigators, and much of its structural biochemistry has been inferred from histochemical observations (9).

Chitin, a polysaccharide composed of repeating units of *N*-acetyl-D-glucosamine (GlcNAc), is a major cell wall constituent of most fungi and is found to a lesser extent in the cyst forms of several protozoans (1, 18, 24). There have been conflicting reports regarding the presence of chitin in *P. carinii* (2, 9, 22). In an attempt to resolve this conflict, *P. carinii*-infected human lung tissue was probed with a polyclonal rabbit antiserum specific for macromolecular chitin and with a series of rabbit antisera specific for chitin oligomers. The results of these studies indicate that chitin is present in *P. carinii* at more than one stage of its life cycle.

MATERIALS AND METHODS

Sources of materials. Protein A-agarose was purchased from E-Y Labs, San Mateo, Calif. A mouse monoclonal antibody (DAKO-Pneumocystis M778, lot no. 118) that binds to an 82-kilodalton *P. carinii* protein (13) was purchased from DAKO, Inc., Santa Barbara, Calif. For use in negative controls, nonimmune rabbit and nonimmune mouse sera were purchased from DAKO, Inc. Biotinylated swine anti-rabbit immunoglobulin, streptavidin-peroxidase conjugate, rabbit anti-mouse immunoglobulin, and mouse immunoglobulin peroxidase conjugate were also purchased from DAKO, Inc. Indophan Blue and Indophan Red were obtained from Viomedics, Worcester, Mass. Reacetylated chitosan was prepared as described previously (7) and is macromolecular chitin that is free of amino acids and more than 90% reacetylated.

Antiserum production, purification, and characterization. Female New Zealand rabbits were immunized with reacetylated chitosan which was suspended in phospate-buffered saline (20 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl). After weekly subcutaneous injection (0.8 ml of phosphate-buffered saline containing 0.1 to 0.6 mg of reacetylated chitosan) for 4 weeks, animals were ear bled by using a vacuum cuff. The antiserum was removed and purified by affinity chromatography on protein A-agarose (10). Bound antiserum was eluted with 0.2 M glycine buffer, pH 2.9, neutralized, and dialyzed. In order to remove the fraction which bound di-*N*-acetylchitobiose, the antiserum was further purified by affinity chromatography on ovalbumin-agarose prepared by standard methods (10). The unbound material, which had no binding activity toward ovalbumin (undetectable by Western [immuno-] blot), was stored at −20°C until needed. Polyclonal antisera against the chitin oligosaccharides chitotriose (GlcNAc-3), chitotetraose (GlcNAc-4), and chitopentose (GlcNAc-5) were generously provided by Byron Anderson of Northwestern University (15).

Light microscopy and immunocytochemistry. Six cases of human *P. carinii* pulmonary infection were located by reviewing recent surgical pathology files. Relevant specimens were obtained. These consisted of open lung biopsy tissue in four cases and cell blocks prepared from bronchioloalveolar lavage in two cases. All six patients had acquired immunodeficiency syndrome. All specimens had been fixed in Formalin or alcohol, processed, and embedded in paraffin for routine histopathology. Microscopic sections (5 to 10 μm) of specimens from each case were stained with hematoxylin and eosin, Gomori silver methenamine (GMS) stain, and Giemsa stain. The slides were examined to confirm the diagnosis of *P. carinii* infection. Thereafter, additional sections from each case were deparaffinized, rehydrated, and stained with either the antisera or the monclonal antibody preparation. Representative sections to be stained with anti-chitin antiserum were pretreated with 0.1% trypsin in a 0.1% $CaCl_2$ buffer for 5 min; pretreatment enhanced staining, presumably by removing excess human protein adhering to the organisms. Binding of primary polyclonal antiserum was detected by biotinylated swine anti-rabbit immunoglobulin followed by streptavidin-peroxidase conjugate with aminoethylcarbazole, diaminobenzidene, Indophan Blue, or Indophan Red as the chromogen. Binding of the primary monoclonal antibody was detected by secondary rabbit anti-mouse immunoglobulin, which was then allowed to bind a tertiary mouse immunoglobulin peroxidase conjugate. Negative control slides were treated in an identical fashion except for the substitution of nonimmune rabbit serum (in the case of the rabbit antiserum) or nonimmune mouse

* Corresponding author.

serum (in the case of the monoclonal antibody) for the primary antibody.

Processed and paraffin-embedded sections of Formalin-fixed *Artemia* sp. (brine shrimp) were used as positive controls. In addition, to evaluate the binding of the anti-chitin antiserum to microorganisms of established taxonomic position, sections of Formalin-fixed, processed human tissue infected with the following organisms were also stained with the anti-chitin antiserum: *Aspergillus* sp., *Rhizopus* sp., *Blastomyces dermatitidis*, *Cryptococcus neoformans*, *Candida* sp., and *Toxoplasma gondii*.

To insure that the anti-chitin antiserum bound to chitin in the wall of *P. carinii*, 2 mg of that antiserum in phosphate-buffered saline was mixed with 1 g of reacetylated chitosan and allowed to bind for 1 h at room temperature. The mixture was then centrifuged, and the supernatant was removed; the preabsorption process was repeated two additional times. The final supernatant was used to stain sections of *P. carinii*-infected lung and *Artemia* sp.

RESULTS

Light microscopy and immunocytochemistry. The taxonomic position of *P. carinii* remains unresolved, and thus the most appropriate terms for the various forms of the organisms have not been established. We followed the example of ul Haque et al. (20) and designated the rounded, relatively thick-walled, GMS-positive structures cysts. The presumed progeny within the cysts were designated intracystic bodies, and the irregularly shaped, GMS-negative structures were designated trophic forms.

Examination of the hematoxylin and eosin-stained lung biopsy specimens revealed interstitial thickening with infiltrates of plasma cells. The alveolar-lining cells were enlarged and cuboidal. The intra-alveolar spaces were free of inflammatory cells but were filled with masses of frothy, amorphous eosinophilic material. GMS staining revealed the characteristic *P. carinii* cysts within this material. Giemsa staining visualized the intracystic bodies but did not allow for the recognition of the trophic forms or cysts. The lavage cell blocks contained alveolar cells and the eosinophilic masses; similar staining reactions with GMS and Giemsa stain were observed.

Probing the tissues with the anti-*P. carinii* monoclonal antibody resulted in staining of both the cysts and trophic forms. The outlines of both stages were well visualized (Fig. 1A). There was no staining of the host tissue elements. Probing with the anti-chitin antiserum revealed a pattern of staining analogous to that of the monoclonal antibody (Fig. 1B). In many cysts, intracystic bodies were also stained (Fig. 1C). No staining of the host tissues occurred with the anti-chitin antiserum. Substitution of nonimmune rabbit serum for primary anti-chitin antiserum resulted in no staining of the organisms (Fig. 1D). The anti-chitin antiserum also stained the cell walls of *Candida* sp., *Rhizopus* sp., *Aspergillus* sp., *C. neoformans*, and *B. dermatitidis*. No staining of the tachyzooites or tissue cysts of *T. gondii* was observed (data not shown).

After preadsorption with reacetylated chitosan, the anti-chitin antiserum did not stain any *P. carinii* organisms, nor did it stain the positive control, *Artemia* sp. (data not shown).

The antisera to the chitin oligomers stained the *P. carinii* organisms in a pattern like that of the anti-chitin antiserum. No staining of host tissue elements was observed (data not shown). Substitution of nonimmune rabbit serum for primary antisera resulted in no staining of the organisms.

DISCUSSION

The results presented herein indicate that *P. carinii* produces chitin at more than one stage of its life cycle within the infected human host. The antiserum to macromolecular chitin and the antisera to chitin oligomers all bound the different forms of the organisms. Preadsorption of the anti-chitin antiserum with macromolecular chitin eliminated staining of all *P. carinii* tissue forms. In addition, none of these antisera bound avidly to host tissue elements, which contain N-linked oligosaccharides that exhibit both a di-*N*-acetylchitobiosyl core region and peripheral GlcNAc residues, as observed in polylactosamine-containing glycoproteins. Thus, we interpret these results as indicating that chitin, and not GlcNAc or chitobiose, bound the antisera. Moreover, the results support the rationale of using ovalbumin-agarose for prescreening the antisera to remove activity against chitobiose and peripheral GlcNAc residues.

The possibility that chitin is present in *P. carinii* has been discussed sporadically over the last four decades. In 1955, Bruns interpreted his histochemical results as indicative of the presence of either GlcNAc or chitin in the cyst walls of *P. carinii* (2). Subsequently, it was observed that pretreatment with chitinase diminished the staining intensity of *P. carinii* cysts (9). In 1979, however, Waldrop et al. published observations that they considered to be evidence against chitin being a constituent of the trophic form of the organism (22). The authors of a recent investigation used lectins as probes and concluded that GlcNAc, as well several other saccharides, is present on the surface of *P. carinii*; the presence of chitin was not addressed (4). Another related study revealed the presence of a GlcNAc-containing surface glycoprotein, the immunoreactivity of which was only slightly affected by chitinase (14). To our knowledge, no published work has either confirmed or refuted the expression of chitin itself by *P. carinii*.

The implications of demonstrating chitin in *P. carinii* are several. First, at the taxonomic level, a debate has continued for years over whether *P. carinii* is a fungus or a protozoan, with recent ultrastructural and nucleotide-sequencing data favoring the former (5, 6, 17, 19, 20, 25). The finding of chitin in more than one stage of *P. carinii* is consistent with a fungal classification as well. Fungi vary in their chitin content, but most contain some of the polysaccharide, and a correlation with virulence has been suggested in certain pathogenic species. For example, a decreased dry weight percentage of chitin has been observed in an avirulent isolate of *B. dermatitidis* compared with a virulent strain (3).

Fungi also produce other carbohydrate polymers. A possible physical linkage between glucan and chitin has been hypothesized from enzymatic digestion studies of *Histoplasma capsulatum* (18). Recent ultrastructural observations of glucan in *P. carinii* (16) combined with our findings of chitin may foster linkage investigations of *P. carinii* as well.

Certain protozoans, e.g., *Entamoeba* sp. and *Giardia lamblia*, produce cyst walls that contain chitin (1, 24). Chitin has not been found, however, in their trophic forms. We were unable to demonstrate chitin reactivity in the tissue cysts or tachyzooites of *T. gondii*, a member of the phylum *Apicomplexa* and class *Coccidia*, into which *P. carinii* has on occasion tentatively been placed. No documentation of chitin expression by other members of *Apicomplexa* or by trophic forms of other protozoan phyla that bear morphologic similarities to *P. carinii* could be found (11, 12, 23).

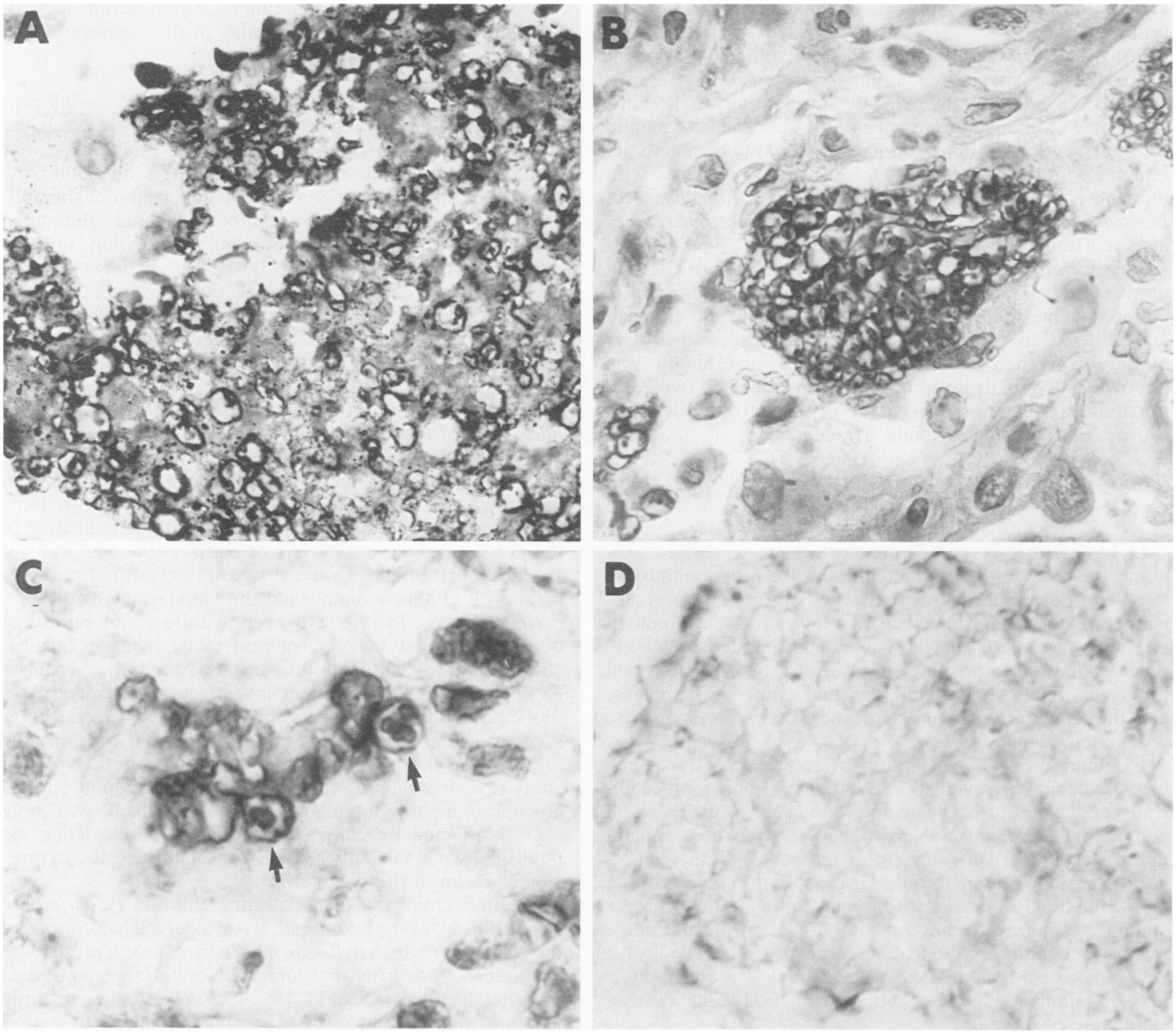

FIG. 1. (A) Intra-alveolar mass of trophic and cyst forms of *P. carinii* stained with the anti-*P. carinii* monoclonal antibody (hematoxylin counterstain; original magnification, ×1,000). (B) Intra-alveolar mass of trophic and cyst forms of *P. carinii* stained with the anti-chitin antiserum. The surrounding alveolar cells have undergone secondary reactive changes (hematoxylin counterstain; original magnification, ×1,000). (C) Small intra-alveolar collection of *P. carinii* stained with the anti-chitin antiserum. Intracystic bodies can be seen within the two cyst forms (arrows). No counterstain was used in this preparation, and thus the surrounding alveolar elements are not visualized. This underscores the absence of binding by the anti-chitin antiserum to host tissue (original magnification, ×1,000). (D) Intra-alveolar mass of *P. carinii* in the negative control slide; normal rabbit serum was substituted for the primary anti-chitin antiserum. The slide was otherwise identical to the slide in panel C (original magnification, ×1,000).

The presence of chitin in *P. carinii* has potential therapeutic implications. Approved anti-*P. carinii* therapy currently is limited to pentamidine and to trimethoprim sulfate and related compounds. Both drug regimens are associated with increased adverse reactions in acquired immunodeficiency syndrome patients (8). Hence, there is an ongoing search for additional effective antimicrobial agents. Drugs which disrupt chitin synthesis might offer another approach to *P. carinii* infections. Our finding of chitin in more than one stage of the organism suggests that such drugs could interrupt the *P. carinii* life cycle within the infected human host. Moreover, since chitin is not a component of human tissue, such agents would not directly interfere with normal host structure and function.

Finally, we also found the anti-chitin antiserum useful in visualizing the various opportunistic and pathogenic fungi included in the study. The traditional GMS stain might be said to have done the same, but it only stained the cyst walls of *P. carinii*. Lectins have been used to characterize the saccharide contents of various microorganisms (4, 24). In our experience, the inherent high background produced by their binding to host tissue elements limits the usefulness of lectins in the histopathologic evaluation of infected tissue. We believe that the anti-chitin antiserum may be helpful in

the microscopic screening of tissue specimens from immunocompromised hosts, as it stains *P. carinii* as well as other fungi and highlights their morphologic characteristics.

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LITERATURE CITED

1. **Avron, B., R. M. Deutsch, and D. Mirelman.** 1982. Chitin synthesis inhibitors prevent cyst formation by *Entamoeba* trophozooites. Biochem. Biophys. Res. Commun. **108**:815–821.
2. **Bruns, G.** 1955. Der Formwechsel bei *Pneumocystis carinii*. Acta Histochem. **2**:222–227.
3. **Cox, R. A., and G. K. Best.** 1972. Cell wall composition of two strains of *Blastomyces dermatitidis* exhibiting differences in virulence for mice. Infect. Immun. **5**:449–453.
4. **DeStefano, J. A., M. T. Cushion, L. S. Trinkle, and P. D. Walzer.** 1989. Lectins as probes to *Pneumocystis carinii* surface glycocomplexes. J. Protozool. **36**:65S–66S.
5. **Edman, J. C., J. A. Kovacs, H. Masur, D. V. Santi, H. J. Elwood, and M. L. Sogin.** 1988. Ribosomal RNA sequence shows *Pneumocystis carinii* to be a member of the Fungi. Nature (London) **334**:519–522.
6. **Edman, J. C., J. A. Kovacs, H. Masur, D. V. Santi, H. J. Elwood, and M. L. Sogin.** 1989. Ribosomal RNA genes of *Pneumocystis carinii*. J. Protozool. **36**:18S–20S.
7. **Hirano, S., Y. Ohe, and H. Ono.** 1976. Selective N-acetylation of chitosan. Carbohydr. Res. **47**:315.
8. **Hughes, W. T.** 1987. *Pneumocystis carinii* pneumonia, vol. 2, p. 30 and p. 73–93. CRC Press, Inc., Boca Raton, Fla.
9. **Hughes, W. T.** 1987. *Pneumocystis carinii* pneumonia, vol. 1, p. 20. CRC Press, Inc., Boca Raton, Fla.
10. **Johnstone, A., and R. Thorpe.** 1982. Immunochemistry in practice, p. 332. Blackwell Scientific Publishers, Boston.
11. **Levine, N. D.** 1982. Taxonomy and life cycles of Coccidia, p. 1–33. *In* P. L. Long (ed.), The biology of the Coccidia. University Park Press, Baltimore.
12. **Levine, N. D., J. O. Corliss, F. E. G. Cox, G. Deroux, J. Grain, B. M. Honigberg, G. F. Leedale, A. R. Loeblich III, J. Lom, D. Lynn, E. G. Merinfield, F. C. Page, G. Poljansky, V. Sprague, J. Vavra, and F. G. Wallace.** 1980. A newly revised classification of the Protozoa. J. Protozool. **27**:37–58.
13. **Linder, E., L. Lundin, and H. Vorma.** 1987. Detection of *Pneumocystis carinii* in lung-derived samples using monoclonal antibodies to an 82 kDa parasite component. J. Immunol. Methods **98**:57–62.
14. **Linke, M. J., and P. D. Walzer.** 1989. Analysis of a surface antigen of *Pneumocystis carinii*. J. Protozool. **36**:60S–62S.
15. **Makhlpur, S. E., L. E. Davis, D. Paul, and B. Anderson.** 1986. Immunochemical characterization of antisera reactivities to *N*-acetyl-D-glucosamine oligosaccharides with the β(1-4)-glycosidic linkage. Glycoconjugate J. **3**:351–362.
16. **Matsumoto, Y., S. Matsuda, and T. Tegoshi.** 1989. Yeast glucan in the cyst wall of *Pneumocystis carinii*. J. Protozool. **36**: 21S–22S.
17. **Matsumoto, Y., and Y. Yoshida.** 1984. Sporogony in *Pneumocystis carinii*: synaptonemal complexes and meiotic nuclear divisions observed in precysts. J. Protozool. **31**:420–428.
18. **Reiss, E.** 1986. Molecular immunology of mycotic and actinomycotic infections, p. 14–20. Elsevier Science Publishing, Inc., New York.
19. **Stringer, S. L., K. Hudson, M. A. Blase, P. D. Walzer, M. T. Cushion, and J. R. Stringer.** 1989. Sequence from ribosomal RNA of *Pneumocystis carinii* compared to those of four fungi suggests ascomycetous affinity. J. Protozool. **36**:14S–16S.
20. **ul Haque, A., S. B. Plattner, R. T. Cook, and M. N. Hart.** 1987. *Pneumocystis carinii*. Taxonomy as viewed by electron microscopy. Am. J. Clin. Pathol. **87**:504–510.
21. **Vavra, J., and K. Kucera.** 1970. *Pneumocystis carinii* Delanoe, its ultrastructure and ultrastructural affinities. J. Protozool. **17**:463–483.
22. **Waldrop, F. S., T. D. Younker, and H. Puchtler.** 1979. Histochemical observations on *Pneumocystis carinii*: selective demonstration of honeycomb forms. Histochemistry **63**:1–6.
23. **Wang, C. C.** 1982. Biochemistry and physiology of Coccidia, p. 167–216. *In* P. L. Long (ed.), The biology of the Coccidia. University Park Press, Baltimore.
24. **Ward, H. D., J. Alroy, B. I. Lev, G. T. Keusch, and M. E. A. Pereira.** 1985. Identification of chitin as a structural component of *Giardia* cysts. Infect. Immun. **49**:629–634.
25. **Watanabe, J. I., H. Hori, K. Tanabe, and Y. Nakamura.** 1989. 5S ribosomal RNA sequence of *Pneumocystis carinii* and its phylogenetic association with Rhizopoda/Myxomycota/Zygomycota group. J. Protozool. **36**:16S–18S.