

NOTES

Attachment of *Pneumocystis carinii* to Type I Alveolar Cells Studied by Freeze-Fracture Electron Microscopy

KOKICHI YONEDA^{1,2} AND PETER D. WALZER^{3,4*}

Lexington Veterans Administration Medical Center¹ and Department of Pathology, University of Kentucky,² Lexington, Kentucky 40506, and Cincinnati Veterans Administration Medical Center,³ and Division of Infectious Diseases, Department of Internal Medicine, University of Cincinnati College of Medicine,^{4*} Cincinnati, Ohio 45267

Received 13 December 1982/Accepted 17 February 1983

Pneumocystis carinii was tightly attached to host alveolar type I cells, as judged by freeze-fracture electron microscopy. In contrast to other organisms studied by this technique, no changes in the cell membranes of *P. carinii* or the host cells could be demonstrated. These data suggest that *P. carinii* attaches in an unusual manner.

Pneumocystis carinii is a frequent cause of pneumonia in immunosuppressed patients. Accumulating evidence indicates that the interaction of *P. carinii* with the alveolar type I epithelial cells occupies a central role in the host-parasite relationship in this infection (1, 5, 10). We have used the cortisone-treated rat model to conduct detailed sequential studies of the interaction of *P. carinii* with type I cells (12-14). The stages of this process include attachment of *P. carinii* to type I cells, proliferation of *P. carinii* within alveolar spaces, alterations in the permeability of the alveolar-capillary membrane, and degenerative changes in type I cells. The data suggest that *P. carinii* damages type I cells in heavy infection.

The mechanism of attachment of *P. carinii* to type I cells is poorly understood. Some authors using transmission and scanning electron microscopy have suggested that *P. carinii* attaches to the cell by cytoplasmic extensions termed filopodia (1, 8). On the other hand, we found with the glycocalyx stain ruthenium red that *P. carinii* and type I cell membranes are closely apposed to each other (13). Freeze-fracture electron microscopy has provided another approach to the study of cell surfaces. We have used freeze-fracture to study the developmental stages of *P. carinii* (15) and, in the present study, to examine the attachment of *P. carinii* to type I cells.

Fifty male adult Sprague-Dawley and Lewis rats (Harlan Industries, Indianapolis, Ind.) weighing about 250 g were used in the study. The protocols used to develop *Pneumocystis* pneumonia are similar to those used in previous

studies (12, 13) and are based on the mechanism of reactivation of latent infection by corticosteroid administration. In brief, the rats were administered the following medications: cortisone acetate (25 mg) injected subcutaneously twice weekly or dexamethasone (1 mg/1,000 ml) in their drinking water; a regular or low (8%) protein diet; and tetracycline (1 mg/ml) in their drinking water. Animals were sacrificed at various intervals from 4 to 12 weeks by an overdose of halothane anesthesia. The lungs were excised, sliced into small pieces (1 mm³), and immersed in the fixative (2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2) for 2 h at 4°C. After the fixation, the tissue was washed overnight by several changes of the cacodylate buffer. Half of the tissue specimens were processed for thin-section electron microscopy, and the other half were processed for freeze-fracture.

For thin-section electron microscopy, the tissue was postfixed in 2% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) for 2 h, stained en bloc with 0.5% uranyl acetate in 0.1 M barbital buffer (pH 5.4), dehydrated through graded ethanols, and embedded in epoxy resin. Sections were cut with a diamond knife, stained with lead citrate, and examined with a Phillips 300 electron microscope.

For freeze-fracture, the tissue blocks were infiltrated with glycerol (with concentrations increasing from 10 to 30%) in 0.1 M cacodylate buffer. The tissue was frozen in Freon 22 cooled in liquid nitrogen. Freeze-fracture was performed in a Polaron E7900 freeze-fracture device (Polaron Co., Doylestown, Pa.), at a stage temperature of -115°C in a vacuum of 10⁻⁶ torr.

Carbon-platinum replicas were made of the fractured surface, and the underlying tissue was dissolved with household bleach. The replicas were washed with distilled water, mounted on an uncoated copper grid, and examined with a Philips 300 electron microscope.

Initial experiments were performed on Sprague-Dawley rats sacrificed after 6 to 8 weeks of corticosteroid treatment. The results showed that *P. carinii* trophozoites were tightly attached to the alveolar type I cells (Fig. 1). As described previously with transmission electron microscopy (13), the cell membrane was closely apposed to that of the parasite. Under higher magnification, no changes in the structure of the membrane (i.e., the distribution of the intramembranous particles) were observed in the type I cell or in the trophozoite (Fig. 2). Rather, they were always separated by amorphous material which surrounded the surface of the trophozoite and appeared to anchor the trophozoite to the host cell.

To rule out sampling error, further experiments were conducted with Lewis rats, and the protocol was modified to include different times

of sacrifice. Findings in these experiments were identical to those of the experiments with Sprague-Dawley rats.

The data presented here are of considerable interest when considered in light of the general phenomenon of organism attachment. Other authors have found that freeze-fracture electron microscopy is a valuable technique in examining the attachment of a variety of types of microorganisms to host cells (2-4, 7, 9). These studies have all demonstrated some morphological changes in the membrane (e.g., fusion or rearrangement of intermembranous particles) of the organism or the host cell or both in relation to the attachment process. In some cases these changes correlated with the ability of the organism to invade or maintain an existence within the host cell; at the very least, the changes suggested that the organism or host cell obtained some benefit from the attachment.

The lack of ultrastructural changes in *P. carinii* or host alveolar type I cells in the present study suggests an unusual mechanism of attachment, perhaps involving the surface-coating material of the trophozoite. Little is known about



FIG. 1. Alveolar wall in a Sprague-Dawley rat at 6 weeks after cortisone treatment. *P. carinii* trophozoites (T) in the alveolar space (ALV) are lined up against the cell membrane of the alveolar type I cells (I). The capillary lumen (CAP) is shown in the upper portion of the figure. Notice the tight apposition of the type I cell membrane (arrow) and the cell membrane of the trophozoite (double arrow). $\times 26,000$.

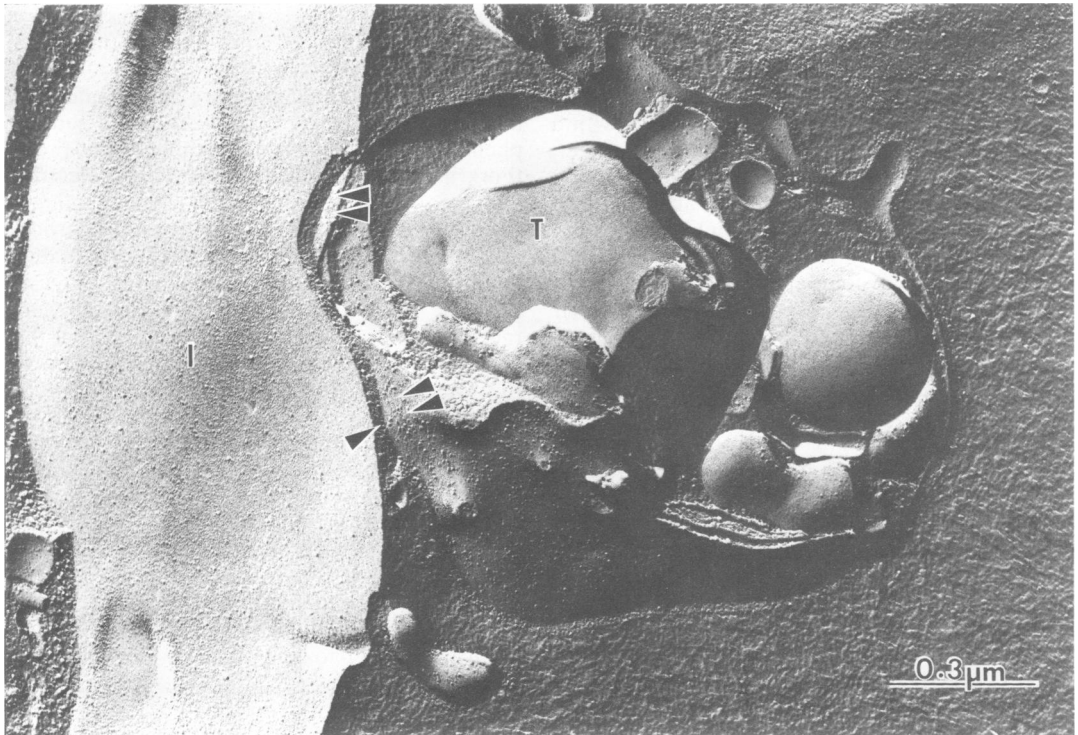


FIG. 2. Higher magnification of the cell membranes at the attachment of the trophozoite (T) to the alveolar type I cell (I). Double arrows show the cell membrane of the trophozoite. The single arrow indicates the extracellular coating of the trophozoite. Notice the random distribution of intramembranous particles on the cell membranes of the type I cell and the trophozoite. $\times 69,000$.

the nature of this material except that it is rich in polysaccharide as determined by histochemical staining (13).

The attachment characteristics of *P. carinii* must be interpreted in relation to other factors in the host-parasite relationship in this infection. The primitive cytoplasmic organelle system in *P. carinii* suggests that the organism has unusual metabolic pathways, although endocytosis may also occur (15). Both type I cells and alveolar fluid have been considered as potential sources of nutrients. *P. carinii* does not have a known intracellular phase in its life cycle and is not capable of resisting digestion after being phagocytosed by alveolar macrophages (6, 11). The first host changes during *P. carinii* infection involve alterations in the alveolar-capillary membrane permeability. The organism remains within the alveolar space throughout most of the course of the infection. It is only later, after damage to type I cells, that *P. carinii* may advance to the pulmonary interstitium. Thus, the slow and subtle changes in the dynamics of the alveolar microenvironment induced by *P. carinii* attachment may play a role in the pathogenesis of the disease.

In summary, this study has shown that *P. carinii* possesses unusual attachment characteristics. Further studies are needed to elucidate the nature of these characteristics and to determine whether they are found with other organisms.

This work was supported by the Medical Research Service, Veterans Administration, and a research grant from the American Cancer Society.

LITERATURE CITED

1. Barton, E. G., and W. G. Campbell. 1969. Pneumocystis carinii in the lungs of rats treated with cortisone acetate. *Am. J. Pathol.* **54**:209-236.
2. Bowers, B. 1980. A morphological study of plasma and phagosome membranes during endocytosis in *Acanthamoeba*. *J. Cell Biol.* **84**:246-260.
3. Caulfield, J. P., G. Korman, A. E. Butterworth, M. Hogan, and J. R. David. 1980. The adherence of human neutrophils and eosinophils to schistosomula: evidence for membrane fusion between cells and parasites. *J. Cell Biol.* **86**:46-63.
4. Caulfield, J. P., G. Korman, A. E. Butterworth, M. Hogan, and J. R. David. 1980. Partial and complete detachment of neutrophils and eosinophils from schistosomula: evidence for the establishment of continuity between fused and normal parasite membrane. *J. Cell Biol.* **86**:64-76.

5. **Lanken, P. N., M. Minda, G. G. Pietra, and A. P. Fishman.** 1980. Alveolar response to experimental *Pneumocystis carinii* pneumonia in the rat. *Am. J. Pathol.* **99**:561-578.
6. **Masur, H., and T. C. Jones.** 1978. The interaction in vitro of *Pneumocystis carinii* with macrophages and L-cells. *J. Exp. Med.* **147**:157-170.
7. **McLaren, D. J., L. H. Bannister, P. I. Trigg, and G. A. Butcher.** 1979. Freeze fracture studies on the interaction between the malaria parasite and host erythrocyte in *Plasmodium knowlesi* infections. *Parasitology* **79**:125-139.
8. **Murphy, M. J., L. L. Pifer, and W. T. Hughes.** 1977. *Pneumocystis carinii* in vitro. *Am. J. Pathol.* **88**:387-402.
9. **Neutra, M. R.** 1980. Prokaryotic-eukaryotic cell junctions: attachment of spirochetes and flagellated bacteria to primate large intestinal cells. *J. Ultrastruct. Res.* **70**:186-203.
10. **Vavra, J., and K. Kucera.** 1970. *Pneumocystis carinii* Delanoë, its ultrastructure and ultrastructural affinities. *J. Protozool.* **17**:463-483.
11. **Von Behren, L. A., and E. L. Pesanti.** 1978. Uptake and degradation of *Pneumocystis carinii* by macrophages in vitro. *Am. Rev. Respir. Dis.* **118**:1051-1059.
12. **Walzer, P. D., R. D. Powell, K. Yoneda, M. E. Rutledge, and J. E. Milder.** 1980. Growth characteristics and pathogenesis of experimental *Pneumocystis carinii* pneumonia. *Infect. Immun.* **27**:928-937.
13. **Yoneda, K., and P. D. Walzer.** 1980. Interaction of *Pneumocystis carinii* with host lungs: an ultrastructural study. *Infect. Immun.* **29**:692-703.
14. **Yoneda, K., and P. D. Walzer.** 1981. Mechanism of pulmonary alveolar injury in experimental *Pneumocystis carinii* pneumonia in the rat. *Br. J. Exp. Pathol.* **62**:339-346.
15. **Yoneda, K., P. D. Walzer, C. S. Richey, and M. G. Birk.** 1982. *Pneumocystis carinii*: freeze-fracture study of stages of the organism. *Exp. Parasitol.* **53**:68-76.