THE ROLE OF TUBULAR PROJECTIONS OF *PNEUMOCYSTIS CARINII* IN PATHOGENICITY OF *P. CARINII* PNEUMONIA

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Abstract: The aim of this research is to clarify the role of tubular projections of *Pneumocystis carinii* in *P. carinii* pneumonia. Rats, mice and cats with *P. carinii* infections were investigated by electron microscopy and semithin section light-microscopy. We found that the tubular projections adhered closely to the type I alveolar epithelial cells in the same manner as the body of trophozoites attached to the type I epithelium. Numerous tubular projections were observed between the type I epithelium and small clusters of trophozoites, and some of these projections adhered closely to the type I epithelium. In the heavily infected alveoli, many tubular projections were found around the trophozoites. These projections are likely to link the organisms, creating a big mass of organisms too big to be discharged from the lung by the surfactant alveolar clearance mechanism. We think that the tubular projections may play a role in the adherence of *P. carinii* to the type I epithelium as well as in formation of large masses of these organisms that cannot be cleared from alveoli. This can then give rise to an increasingly severe respiratory failure.

INTRODUCTION

The development of *Pneumocystis carinii* pneumonia is significantly affected by the host defense mechanisms. It is generally considered that impaired cellular immunity is more important than impaired humoral immunity in predisposing to *P. carinii* infection. On the other hand, the parasitic behavior of *P. carinii* is also thought to be critical for the manifestation of its pathogenicity. *P. carinii* has been reported to be adherent to type I alveolar epithelial cells and form a significantly large mass in the alveoli. This parasitic behavior seems to be related to interference with gas diffusion and failure of ventilation. The pellicle of *P. carinii* trophozoite carries tubular projections, called tubular expansions, that show the same structure as the pellicle from which they arise (Vavra and Kucera, 1970; Ham *et al.*, 1971; Vossen *et al.*, 1978). Freeze-fracture replication studies of *P. carinii* revealed that the projection could be classified morphologically into four types as follows: tubular, lobopodia, branching and beaded structures (Yoshikawa *et al.*, 1987). However, the role of tubular projections in the pathogenicity of *P. carinii* pneumonia is not clear.

In this study, we show that the tubular projections

are one of the specialized structures which allow *P. carinii* to fix themselves to type I epithelial cells of alveoli that expand and contract during respiration. Furthermore, the projections around *P. carinii* may also adhere to each other giving rise to a big mass of *P. carinii* that hinders respiratory air exchange.

MATERIALS AND METHODS

The methods of provoking pulmonary alveolus filling with *P. carinii* in cortisone-treated rats, conventionally raised nude mice and corticosteroid-treated cats have been described (Frenkel *et al.*, 1966; Shiota *et al.*, 1986, 1990). For the electron microscopic investigations the lungs were removed and cut into small pieces. These tissue fragments were then put in a specially designed plastic syringe containing 2.5% glutaraldehyde in cacodylate buffer (0.05M, pH 7.4), and air was removed from the alveoli by negative pressure produced by withdrawal of the plunger (Ebe *et al.*, 1968; Takeuchi, 1980). The tissues were immersed in the same fixative for 3 hours at 4°C and washed with the same buffer containing 8.2% sucrose. Then the tissues were dehydrated through a series of acetone and embedded in epoxy resin. Ultrathin sections were cut with a diamond

Figure 1 JB-4 plastic-embedded 1 μ m thick section of lightly infected alveoli from the lung of a rat (after five weeks of corticosteroid treatment). In this figure, about 80 trophozoites (T) and 2 cysts (C) are observed along the surface of epithelial cell. A, air space of alveoli; Cp, capillary; Am, alveolar macrophage; *, a small cluster of *P. carinii*; Scale bar, 10 μ m. (Giemsa).

knife and placed on uncoated copper grids. The ultrathin sections were stained with uranyl acetate and lead citrate, and observed under a JEOL 100S electron microscope. The procedures for light microscopic investigations of Giemsa stained 1 μ m sections embedded in JB-4 plastic were briefly as follows. The sections were stained with Giemsa (diluted 20 times with pH 7.2 PBS). More than three hours staining in an incubator at 37°C was required to obtain satisfactory results because the sections were so thin (Shiota, 1984).

RESULTS

Lightly infected alveoli:

Lightly infected alveoli were examined in 1 μ m section embedded in JB-4 plastic and stained with Giemsa. As shown in Figure 1, the alveolar septa were almost normal, and contained much air. The alveolar capillary membrane (respiratory membrane) and blood capillaries were well preserved. Trophozoites and cysts were found to be closely attached to the type I epithelium. Measurements of the respiratory membrane in Figure 1 showed that about 20% of the entire membrane was covered with the organisms.

In TEM sections, fine structures of *P. carinii* were similar to those reported previously (Vavra and Kucera, 1970). A membrane to membrane attachment was observed between the trophozoite of *P. carinii* and type I epithelium (Figure 2). A few tubular projections

Figure 2a-b Cat, corticosteroid treated for five months. 2a: A few tubular projections (arrows) are found at the peripheral attachment surface of a small trophozoite (T) adhering to the respiratory membrane. 2b: Higher magnification of the area in the square in Figure 2a shows that the trophozoite is attached to the respiratory membrane. A, air space of alveolus; P, pellicle of the trophozoite; Ep, cytoplasm of type I epithelium; B, basement membrane; Ed, endothelial cell; Rbc, red blood cell. Scale bar, 100 nm.

Figure 3a-b Nude mouse, five months old, conventionally raised. 3a: Many tubular projections developing between a small cluster of trophozoites (T) and the respiratory membrane. 3b: Higher magnification of the area in the square of Figure 3a showing some of the projections (arrows) adhered closely to the type I epithelium. A, air space of alveolus; Rbc, red blood cell; Cp, capillary; Ep, cytoplasm of type I epithelium; B, basement membrane; Ed, endothelial cell. Scale bar, 100 nm.

were found peripheral to the attachment surface of trophozoites (Figure 2). Small clusters of trophozoites frequently showed much better developed tubular projections than the solitary single trophozoite. Tangles of

Figure 4 Cat, corticosteroid treated for five months. Some tubular projections (arrows) invaginated in the type I epithelium. T, trophozoite; Ep, cytoplasm of type I epithelium; B, basement membrane; Ed, endothelial cell; Rbc, red blood cell. Scale bar, 100 nm.

Figure 5 Cat, corticosteroid treated for five months. A few tubular projections (arrow) are found on the side of the cyst (C) adhering to the respiratory membrane. An empty trophozoite (ET) with many tubular projections is seen next to the cyst. Rbc, red blood cell. Scale bar, 1 μ m.

Figure 6 Rat, corticosteroid treated for five weeks. Many trophozoites (T) and cysts (C) are forming a big mass of organisms in heavily infected alveoli. The alveolar air space (A) was almost completely occupied with the clumped trophozoites and cysts. Cp, capillary. Scale bar, 15 μ m. (Giemsa).

Figure 7 Nude mouse, five months old, conventionally raised. Many tubular projections are found around the trophozoites filling the heavily infected alveolus. Scale bar, 1 μ m.

numerous projections were observed between the trophozoites and type I epithelium (Figure 3a). Some of the projections adhered closely to the type I epithelium in the same manner as the trophozoites attached to its surface. That is, the surfaces of the organism and the host cells were closely opposed without fusion of the cell membranes (Figure 3b). Some tubular projections invaginated into the type I epithelium so that the epithelial cells became wider (Figure 4). A few tubular projections were also found peripheral to cysts adhering to the type I epithelium (Figure 5). The cyst adhered

closely to the type I epithelium in the same manner as the single trophozoite shown in Figure 2a. An empty trophozoite with many tubular projections was often seen beside such cysts (Figure 5).

Heavily infected alveoli:

In the JB-4 plastic embedded sections, many alveoli were filled with trophozoites, cysts, debris of the host cells and occasionally with foamy macrophage (Figure 6). In TEM sections, many tubular projections were found around the many trophozoites filling the alveolus

(Figure 7). These tubules showed some variation in diameter and were often filled with dense granular materials. However, they contained no microtubules, intermediate filaments or microfilaments. The tubular projections were not found on the surface of intracystic bodies (daughter cells) in the mature cysts. The structure of tubular projections and the mode of adhesion to the type I epithelium was not different among rats, nude mice and cats.

DISCUSSION

The pellicle of *P. carinii* trophozoites consists of a 25–30 nm thick layer in which strata can be distinguished: at the inner side a unit membrane as the boundary of the cytoplasm and on the outside a 20–25 nm thick layer of moderately electron-dense material. This electron-dense outer layer contains a considerable amount of carbohydrates which bind to Concanavalin A and a lectin from *Macura ponrifera* (Yoshikawa *et al.*, 1988). These carbohydrates are known to play many roles in cell to cell adhesion. In *P. carinii* cyst walls, glucose, mannose and galactose were found and these sugars may mediate *P. carinii* host interaction (De Stefano *et al.*, 1990). A major surface glycoprotein (gp 120), which was of high-mannose type, was detected in *P. carinii*, and a possible role for the adherence of *P. carinii* through this glycoprotein to host mucin components in lung tissue was indicated by Radding *et al.* (1989).

Numerous reports have demonstrated that *P. carinii* attach themselves to a specific alveolar lining cell, the type I epithelium (Barton and Campbell, 1969; Vavra and Kucera, 1970; Ham *et al.*, 1971; Lanken *et al.*, 1980; Yoneda and Walzer, 1980, 1981; Yoshida *et al.*, 1984; Henshaw *et al.*, 1985). *P. carinii* is tightly attached to the type I epithelium, as judged by freeze-fracture electron microscopy (Yoneda and Walzer, 1983). The interaction of *P. carinii* with this cell plays a central role in the host-parasite relationship in the infection (Walzer, 1986). Tubular projections have been known for a long time (Yoneda and Walzer, 1980; Yoshida *et al.*, 1984; Henshaw *et al.*, 1985). *P. carinii* can alter its morphology to fit the contour of its immediate surroundings. Indeed, the analogy of a rock climber tenaciously grasping the side of a steep incline may well reflect the situation with *P. carinii* (Long *et al.*, 1986). Some authors clearly show these structures, but do not explain the process.

It seemed that *P. carinii* depends on the adherence of the pellicles to the type I epithelium for its par-

asitism in the alveoli. The type I epithelium extends and contracts when alveoli expand and contract with breathing. Thus, it seems that the tubular projections may help to fix the organisms to the motile type I epithelium, especially when the number of organisms had increased to fill the alveoli and many organisms had contact with type I epithelium.

In the lightly infected lungs, gas diffusion through the respiratory membrane may be interfered with by the membrane to membrane attachment of *P. carinii* and by limiting the functional area of the respiratory membrane. Furthermore, the large tangles of tubular projections, which were often found between the clusters of trophozoites and respiratory membrane, may also interfere with efficient ventilation locally.

In heavily infected lungs almost all alveoli are filled with trophozoites, cysts, debris of the host cells and occasionally with foamy macrophages (Shiota, 1984). In the present study, we found that many tubular projections developed around the trophozoites and filled the alveoli. These projections seem to link the organisms together, creating a large mass of these organisms in the alveoli anchored to the type I epithelial cells. Because clearance of alveoli normally depends on macrophages which are inhibited by immunosuppression, these masses cannot be cleared. The mixture of organisms and macrophages would need to advance into the bronchioles before becoming subject to removal by ciliary action and coughing.

In conclusion, tubular projections may play an important role in the adhesion of *P. carinii* to the type I epithelium and to one another preventing their being cleared.

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