INFECTION AND IMMUNITY, Apr. 1997, p. 1510–1514 Vol. 65, No. 4
0019-9567/97/$04.00+0

Internalization of *Aspergillus fumigatus* Conidia by Epithelial and Endothelial Cells

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Received 15 October 1996/Returned for modification 31 December 1996/Accepted 14 January 1997

The internalization of conidia of the opportunistic fungus *Aspergillus fumigatus* by primary cell cultures of nonprofessional phagocytes was investigated. This study is the first to show that *A. fumigatus* conidia were able to be engulfed by tracheal epithelial, alveolar type II, and endothelial cells.

Aspergillus fumigatus is an opportunistic pathogen responsible for various respiratory diseases in normal hosts and severe invasive infections in neutropenic patients (2). The common route of infection is via inhalation of the infective particles, the conidia, from the environment. Due to their small size, the conidia can reach the lower parts of the lung, where they adhere to epithelial cells before infection develops. Alveolar macrophages and neutrophils are known to phagocytose and kill *Aspergillus* conidia (14) and are the main lung defense against *Aspergillus* infection. A mechanism for *Aspergillus* conidia to escape from professional phagocytes could be to be internalized by lung epithelial and endothelial cells and then to colonize the respiratory tissue and initiate vascular diffusion. In this paper, we show that incubation of *A. fumigatus* conidia with epithelial and endothelial cells results in internalization of conidia by both cell types.

Rabbit tracheal epithelial (TE) cells (1, 9), rat alveolar type II (ATII) cells (6, 16), and human umbilical cord endothelial cells (EC) (15) were obtained and cultured as previously described. The *A. fumigatus* strain used in this study was G10, a pathogenic spontaneous nitrate reductase mutant of strain CBS 144-89 (12). Conidia were collected from 1-week-old culture at 25°C on 2% malt agar. Then they were resuspended in cell culture medium without fetal calf serum, added to epithelial cell cultures to obtain a conidium/cell ratio of 5:1, and incubated for 0.5 to 6 h at 37°C (15, 16). At the end of the incubation, cells were washed free of nonadherent conidia, recovered, and processed for electron microscopy by standard techniques (1, 6, 15).

TE cells. The tracheobronchial epithelial (TE) cells are the first cells of the respiratory tract to encounter inhaled pathogens. Therefore, we examined the ability of *A. fumigatus* to enter epithelial cells of the trachea. Primary cultures of tracheal explants reconstituted an epithelial stratified outgrowth composed of basal cells, ciliated cells, and nonciliated cells; the nonciliated cells of the outgrowth did not contain secretory granules and do not secrete polysaccharides (mucins) (1). After 6 h of incubation, conidia did not adhere to the ciliated cells, since the ciliary beat removed them from the outgrowth surface, and most of the conidia were concentrated outside the culture on the collagen matrix. However, some conidia remained associated with epithelial cells located either at the periphery of the culture or in depressions of the outgrowth. Adherent as well as intracellular conidia were observed (Fig. 1). The cell walls of adherent conidia were in close contact with several microvilli of the nonciliated cells (Fig. 1A). Intracellular conidia were always enclosed in membrane-bound vacuoles (Fig. 1B and C). Small vesicles were often seen within the vacuole as in multivesicular bodies (Fig. 1B).

This is the first report of internalization of a microorganism by TE cells. So far pathogenic bacteria of the upper respiratory tract, like *Bordetella* or *Haemophilus* spp., have never been found inside cells. However, the nonciliated TE cells were shown to be able to internalize mineral particles (9), and Churg et al. (5) found that in vitro the uptake can be enhanced by exposure to active oxygen species. Moreover, the ciliary beat of the TE cells, which partially controls mucociliary transport efficiency, is inhibited by common polluting gases, viruses, or cytotoxic drugs used by patients who are prone to *Aspergillus* infections (3). Under these conditions of stress, internalization of conidia by nonciliated TE cells may play a role in vivo in the dissemination of the fungal inoculum.

ATII cells. The alveolar epithelium is a continuous cell layer (type I and type II cells) that lines the alveolus. ATII cells play a key role in the homeostasis of the alveolar space and are directly exposed to inhaled particles. ATII cells were used for the experiment 24 h after isolation. At that time, the cells had formed a confluent monolayer (6). In contrast to TE cells, where conidia were seldom found associated with cells because of the ciliary beat, adhesion of conidia to ATII cells was quantitatively important, and depending on the experiment, it could reach up to 45% of the conidial inoculum (16). Internalization of conidia was frequently observed. Figure 2A shows an adherent conidium in close contact with pseudopodia of the ATII cell. Internalization proceeds by invagination of the membrane (Fig. 2B and C). As observed with TE cells, the conidium is confined inside a membrane-bound vacuole (Fig. 2D).

Our results show that ATII cells were able to internalize *A. fumigatus* conidia in vitro. Internalization of exogenous molecules by ATII cells has been previously observed: in vivo ATII cells actively internalize surfactant proteins and lipids from the alveolar space as a part of the recycling pathway of lung surfactant (17). Moreover, carbon particles have been observed in ATII cells after intratracheal instillation in rats (4). However, very little data with regard to infectious agents are available.

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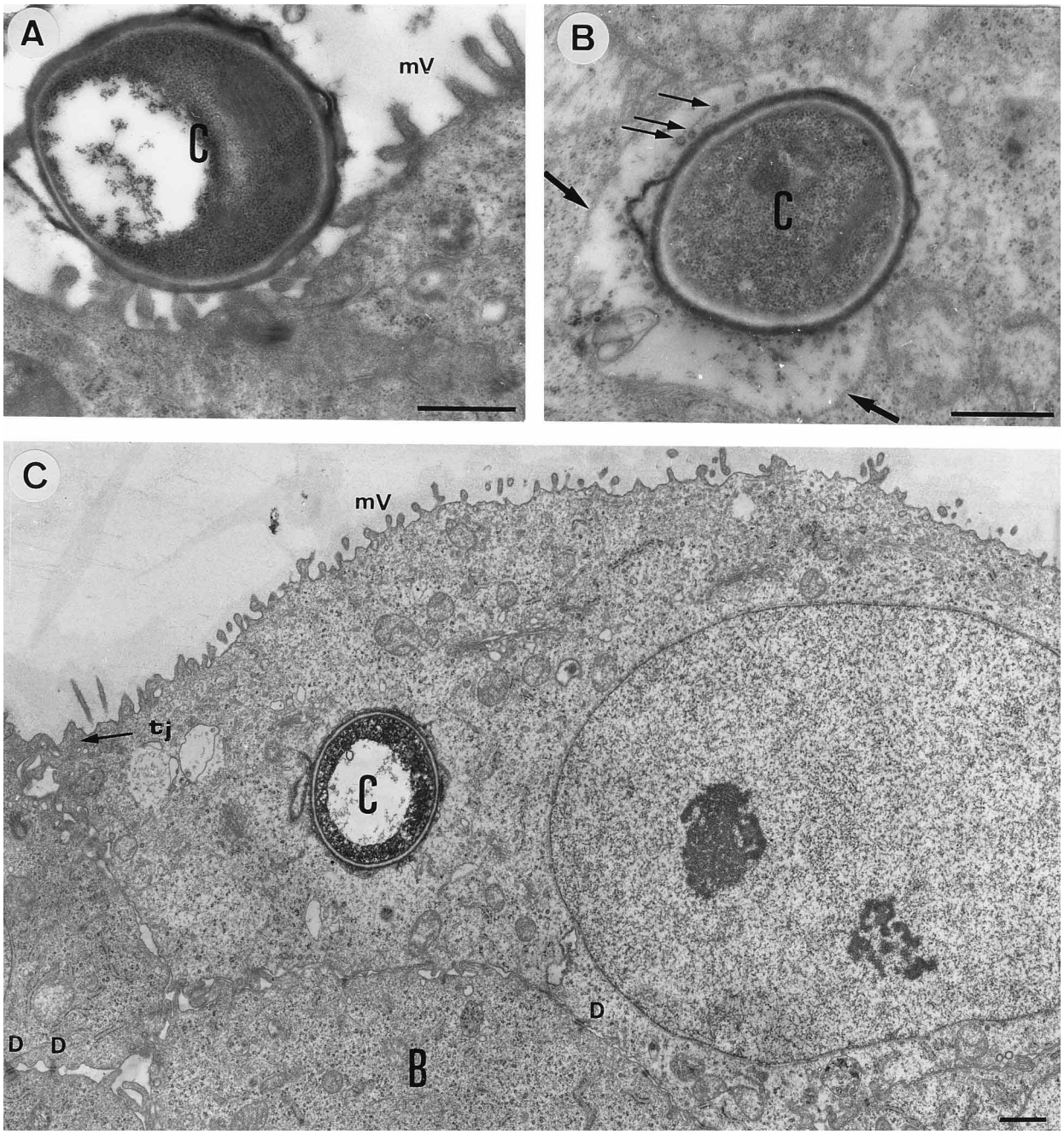

FIG. 1. Transmission electron micrographs of TE cells after 6 h of incubation with *A. fumigatus* conidia. (A) Adhesion of a conidium (C) to microvilli (mV) of TE cell. Magnification, ×15,000. Bar = 1 μm. (B) Conidium internalized in a membrane-bound vacuole. The thick arrows point to the membrane, and the thin arrows point to the intravacuolar vesicles. Magnification, ×16,000. Bar = 1 μm. (C) Low-power micrograph of a typical apical nonciliated TE cell after 6 days of culture, with an internalized conidium. Note the tight junctions between adjacent cells (tj), the apical side with microvilli, the desmosomes (D), and the basal cell (B). No secretory granules were observed. Magnification, ×7,000. Bar = 1 μm.

Merkel and Cunningham (11) mentioned that rat pulmonary epithelial cells are able to internalize *Cryptococcus neoformans* yeasts in vitro, but the type of epithelial cell studied was not specified, and no lamellar bodies characteristic of ATII cells could be seen on their pictures. On the other hand, *Streptococcus pneumoniae* (7) and *Pneumocystis carinii* (13) have been shown to adhere to alveolar epithelial cells without any report of internalization.

EC. In pathological conditions such as viral infections, airway epithelial lesions are often present (10) and conidia may gain direct access to vascular endothelium. This could play a critical role in the initiation of the invasive disease. As up to 45% of *A. fumigatus* conidia adhere in vitro to EC (15), internalization of *A. fumigatus* conidia by EC was also investigated. When conidia from *A. fumigatus* were incubated with EC monolayers, adherent and internalized conidia were seen after

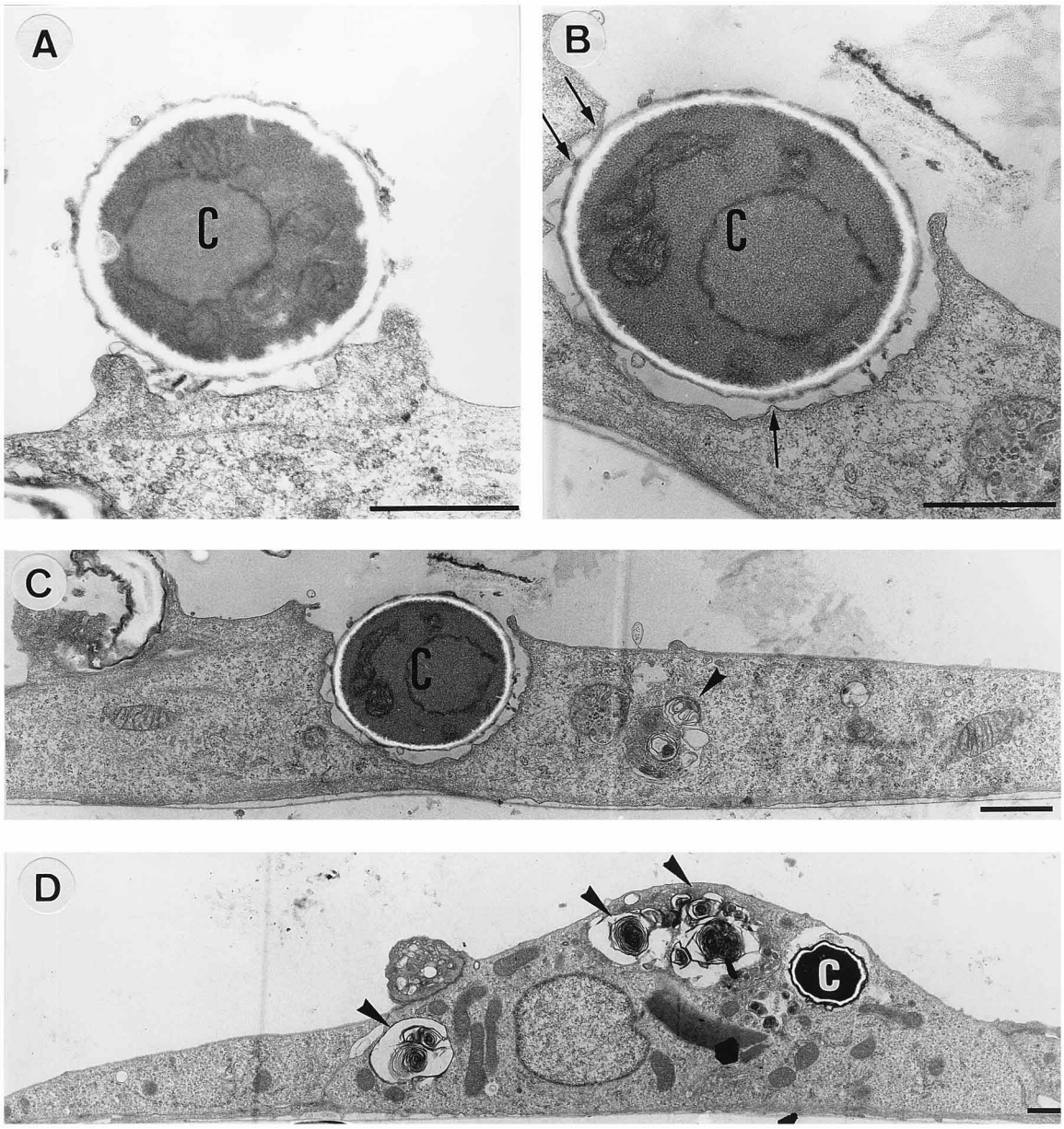

FIG. 2. Transmission electron micrographs of ATII pneumocytes following 6 h of incubation with *A. fumigatus* conidia. (A) Adhesion of a conidium (C) to prominent pseudopodia of the type II pneumocyte. Magnification, ×22,000. Bar = 1 μm. (B and C) Intermediary stage of internalization of a conidium. (B) Magnification, ×19,000. Bar = 1 μm. Note the adhesion sites (arrows) of the plasma membrane of the cell with the wall of the conidium. (C) Magnification, ×10,000. Bar = 1 μm. The arrowhead points to a lamellar body. (D) Low-power micrograph of a type II pneumocyte in culture with an internalized conidium. Note the flattened appearance of the cell and the lamellar bodies (arrowheads) characteristic of ATII cells in primary culture. Magnification, ×5,000. Bar = 1 μm.

2 h of incubation (Fig. 3). Figure 3A shows a conidium adherent to the plasma membrane of EC. Internalized conidia were surrounded by endocytic membrane; numerous particles and vesicles were present between the conidium and the membrane (Fig. 3B). The EC displayed a normal morphology and intact organelles. As EC were detached and collected by centrifugation for fixing and embedding, the apical side of the cell could not be shown, but numerous cytoplasmic extensions are seen between two adjacent cells (Fig. 3C).

Phagocytosis of pathogens by EC is a relatively common finding: EC have been found to ingest a variety of bacteria and fungi, including the pathogenic yeast *Candida albicans* (8). It is interesting that both *A. fumigatus* and *C. albicans*, which are responsible for invasive infections in humans, are able to be internalized by EC. This could be a mechanism through which the microorganisms gain access to the circulation and spread into the tissues. The recent observation by one of us (15) that EC become activated and produce cytokines during adhesion and internalization of *A. fumigatus* conidia suggests that a local inflammatory reaction may take place and be either beneficial or detrimental to the host, depending on fungus load and the immune status of the patient.

In this paper we show that *A. fumigatus* conidia could be internalized by three types of cells. The cells (TE, ATII, and EC) were from different organisms essentially because of the availability of the models. The fact that *A. fumigatus* conidia

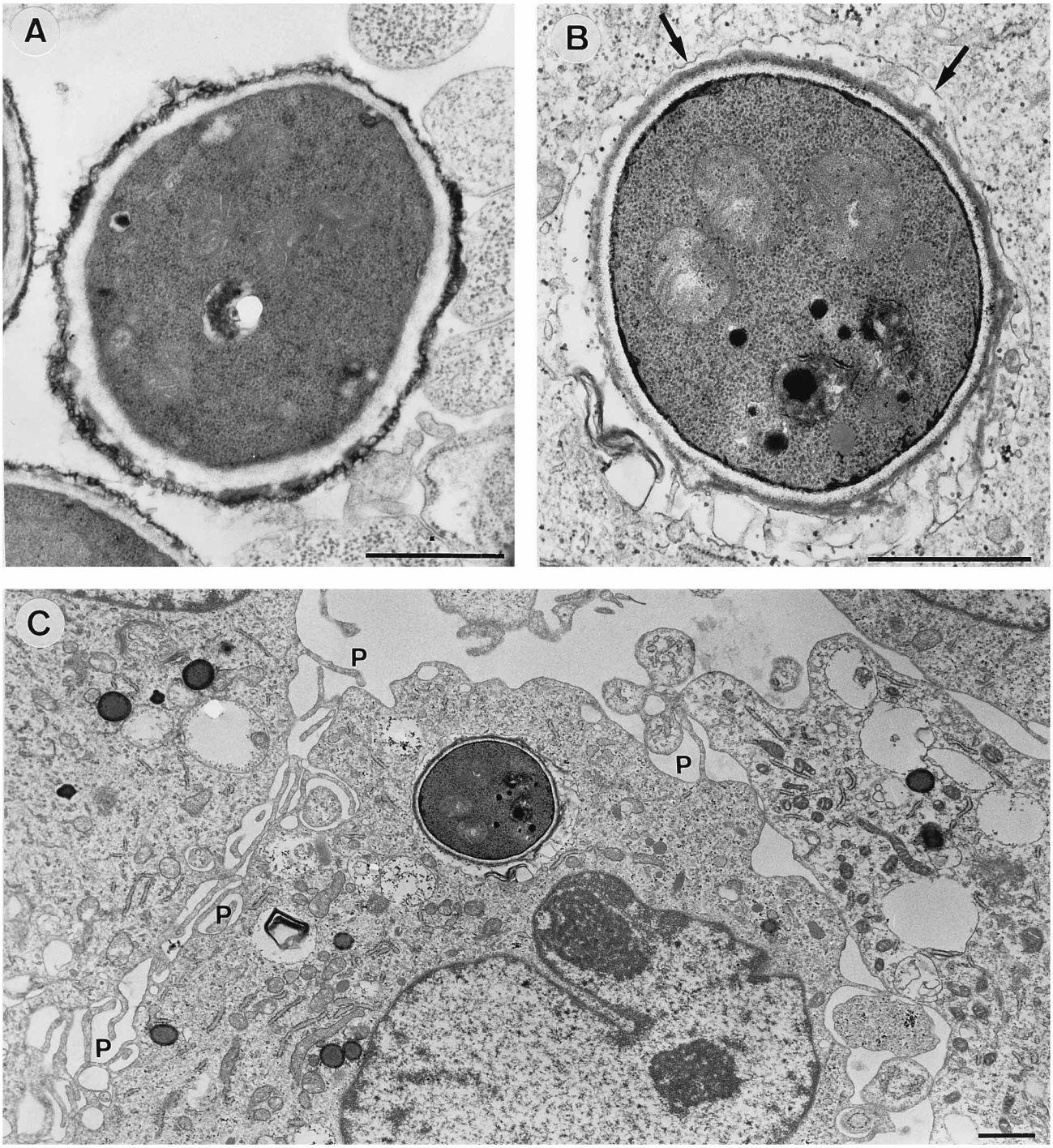

FIG. 3. Transmission electron micrographs of human umbilical vein EC incubated with *A. fumigatus* conidia. (A) Adhesion of a conidium to EC cytoplasmic projections after 30 min of incubation. Magnification, ×23,000. Bar = 1 μm. (B) Enlargement of an internal conidium after 2 h of incubation. The membrane of the vacuole is clearly visible (arrows). Magnification, ×27,000. Bar = 1 μm. (C) Low-power micrograph showing EC with abundant cytoplasmic projections (P) and an internalized conidium. Magnification, ×9,000. Bar = 1 μm.

were phagocytosed by the three types of nonprofessional phagocytic cells suggests that this capacity of the conidia to be internalized might be important in the development of the disease. Two questions remain to be addressed. (i) Does internalization favor progression of the infection by sequestration of the microorganism in a relatively protected site? In other words, are epithelial and endothelial cells a reservoir for *A. fumigatus* away from the macrophages? (ii) Do TE cells, ATII cells, or EC kill the conidium or are the cells damaged by the conidium? These questions have to be answered in order to know if the internalization of *A. fumigatus* conidia by epithelial and endothelial cells is a mechanism of defense of the host or

a mechanism for the conidium to escape the professional phagocytes, which could help dissemination of the pathogen. So it is of interest to further investigate the intracellular fate of *A. fumigatus* conidia following internalization.

D. Taramelli's research was financially supported by the Italian Association for Cancer Research (AIRC) and MURST.

We thank Guiliana Sala for her valuable help in growing EC monolayers and Simona Frigerio and Alain Grodet for their assistance with electron microscopy.

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Editor: T. R. Kozel