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Aspergillus fumigatus causes *in vitro* electrophysiological and morphological modifications in human nasal epithelial cells

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Summary. The role of the airway epithelium in the development of invasive aspergillosis in immunocompromised hosts has rarely been studied although patients at risk for this infection frequently have epithelial damage. We developed an in vitro model of primary culture of human nasal epithelial cells (HNEC) in air-liquid interface, which allows epithelial cell differentiation and mimics in vivo airway epithelium. We subsequently tested 7-day and 24-hour *Aspergillus fumigatus* filtrates on the apical side of HNEC to know whether *A. fumigatus*, the main species responsible for invasive aspergillosis, produces specific damage to the epithelial cells. The results were compared with those obtained with non-pathogenic filamentous fungi. Seven-day culture filtrates of *A. fumigatus* and *Penicillium chrysogenum* induced electrophysiological modifications whatever the fungus tested. In contrast, only 24-hour *A. fumigatus* filtrates induced a specific decrease in transepithelial resistance, hyperpolarization of the epithelium, and cytoplasmic vacuolization of HNEC compared with both *A. niger* and *Penicillium chrysogenum*. The inhibition of the *A. fumigatus* effects with amiloride suggests that the 24-hour fungal filtrate acts through sodium channels of HNEC. These early modifications of the epithelial cells could facilitate colonization of the airways by *A. fumigatus*. To know whether the molecules involved are specific to *A. fumigatus* or simply produced more rapidly than by other filamentous fungi warrants further investigation. In this perspective, the primary culture of HNEC represents a suitable model to study the interactions between airway epithelial cells and *A. fumigatus*.

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Introduction

The prevalence of invasive aspergillosis, mainly due to *Aspergillus fumigatus*, has increased in parallel with the number of immunocompromised patients (Denning, 1998). The mortality rate is very high, close to 80% in populations such as allogenic stem cell transplant recipients. The failure of innate immune cells to kill the organism is thought to be the main reason for the development of invasive aspergillosis (Schaffner et al., 1982; Latgé,1999). The role of the respiratory epithelium has been poorly studied although inhalation of spores, or conidia, is the main route of infection. The normal respiratory epithelium is very efficient in clearing the airways of the fungal spores (Latgé, 1999). In contrast, the respiratory epithelium is frequently altered by chemotherapy, radiation, or viral infections and is more sensitive to pathogen invasion (Cordonnier, 1996). The fungus itself could also contribute to its own colonization by altering the capacity of the respiratory epithelium to clear the conidia.

The *in vitro* models mainly used for studying the interactions between *A. fumigatus* and respiratory epithelial cells consist of either cell lines (DeHart et al., 1997) or human nasal or tracheal explants (Amitani et al., 1995, 1997; Pieckova and Jesenska, 1996). These models are either poorly differentiated or have a short life span, or both. Therefore, we developed a primary culture of human nasal epithelial cells (HNEC) with an

air-liquid interface to obtain cell differentiation. After one week of in vitro culture, the HNEC obtained are arranged in a pseudostratified epithelium with mucus and ciliated cells, similar to the i*n vivo* airway epithelium (Gruenert et al., 1995).

In the present study, we tested the ability of *A. fumigatus* culture filtrates to damage in vitro HNEC cultures, evaluating electrophysiological properties and morphological changes. We looked for a specific effect of *A. fumigatus* by comparing results to those obtained with *A. nige*r and *Penicillium chrysogenum* which are non- or poorly pathogenic filamentous fungi.

Material and methods

HNEC primary cultures

The HNEC cultures were adapted from a culture model originally developed with human tracheo-bronchial cells (Rennard et al., 1994). Nasal polyps were sampled in patients with polyposis during ethmoidectomy. Subjects were requested to stop any therapy such as steroids, antibiotics or anti-allergy drugs, at least one month prior to surgery. The transport medium, DMEM/F12, consisted of a mixture of Dulbecco's modified Eagle's nutrient medium (Life Technologies, France) and Ham's-F12 nutrient medium (Life Technologies, France), supplemented with antibiotics, (100 U/ml of penicillin, 100 mg/ml of streptomycin, 100 mg/ml of gentamycin and 2.5 μg/ml of amphotericin B). The polyps were immediately transported to the laboratory and stored at 4 °C for 2 hours. Because of their special features, polyps from patients with cystic fibrosis or primary ciliary dyskinesia were rejected. Informed consent was obtained from all patients, and permission was obtained from the Ethics Committee of our Institution.

Each nasal polyp was rinsed in PBS-antibiotics with 5 nM dithiothreitol and placed overnight at 4 °C in a PBS-antibiotics solution containing 0.1% pronase (Sigma, France). The sample then was rinsed in DMEM/F12 with 5% fetal calf serum (FCS). After centrifugation (500 g, 7 min), the cell pellets were suspended in a 0.25% trypsin solution (Life Technologies, France) diluted in DMEM/F12 with 5% FCS for 3 minutes, again centrifuged and then suspended in DMEM/F12-5% FCS. Finally, 10^6 cells were plated in 12-mm insert wells (Transwell, Costar, Dutscher, France) with microporous membranes coated with type IV collagen (Sigma, France) and incubated at 37 °C in 5% CO_2. After 24 hours, the liquid medium was removed from the apical compartment to obtain an air-liquid interface. The basal compartment was filled with 1 ml of the HNEC culture medium, consisting of DMEM/F12 with 2% ultroser G (Sigma, France). The HNEC culture medium was changed daily and cultures could be maintained at least 3 weeks.

The epithelial nature of the cultured cells has been previously characterized using a flow cytometric analysis of immunofluorescent labeling showing 99% cytokeratin-positive cells (Escudier et al., 1998). After intense proliferation during the first week, the HNEC reached the most differentiated state during the second week of culture with the presence of cells exhibiting cilia and secretory differentiation.

Preparation of fungal filtrates and challenge of HNEC

Fungal filtrates were obtained from one *A. fumigatus* reference strain (Pasteur Institute (IP) 2279.94), an *A. fumigatus* isolate from a patient with invasive aspergillosis, and two *A. fumigatus* isolates from the environment. For comparison, one strain of non-pathogenic (*Penicillium chrysogenum*, strain IP 1652.86) or poorly pathogenic (*A. niger*, strain IP 218.53) fungi were used.

All fungi were cultivated on 2% malt agar slants. Conidia were harvested after 7 days culture at 30 °C. Fifteen ml of HNEC culture medium without amphotericin B were spiked with 10^7 conidia of each fungus. Incubation was performed at 37 °C in a shaking incubator at 1600 rpm for either 7 days or 24 hours. After incubation, the fungus hyphae were removed by centrifugation (500 g, 15 min), and the supernatant sieved through 0.22 μm filters (Millex-GV, France). The fungal filtrates were aliquoted and stored at -20 °C. After thawing, each fungal filtrate was adjusted for pH and osmolarity to approximate the same range concentration as the HNEC culture medium.

All experiments were performed on 14-day HNEC cultures when cell differentiation was established and stable. The apical sides of HNEC cultures were exposed to 750 μl of fungal filtrates for 3 hours. Control wells consisted of HNEC exposed to HNEC culture medium alone. All tests were performed in duplicate and each experiment was repeated at least three times with different HNEC cultures.

Bioelectrical properties of HNEC cultures

Interactions between HNEC and fungi were evaluated by bioelectrical properties of HNEC cultures, i.e., transepithelial resistances and potential differences, which measure intercellular junctions and transcellular ionic transports (Boland et al., 1999). For 14-day cultures, the usual range of transepithelial resistances (Rt) and transepithelial potential differences (Vt), measured with a microvoltmeter with concentric electrodes (EVOM® World Precision Instruments, UK), is 600 to 800 ohms/cm^2 and -20 to -40 mV, respectively. The day before challenge with the fungal filtrates, referred to as T0, the HNEC inserts were immersed in 750 μl of HNEC culture medium. After 30 min to balance electrodes, the Rt and Vt were recorded. After the first measurement (T0), the culture medium was removed from the apical side to recover the air-liquid interface. Twenty-four hours later, 750 μl of fungal filtrates or culture medium alone were added to the apical side of HNEC cultures and the electrophysiological values were measured after 30

minutes, 1 hour, and 3 hours. Each result was expressed as a percentage of variation from the T0 measurements.

After 3 hours, the filtrates were removed from the apical compartment. The HNEC culture medium was harvested from the basal compartment for cytotoxicity measurements based on determination of lactate deshydrogenase activity (LDH). Some wells were fixed for electronic microscopy. Other wells were washed three times with PBS and maintained in culture conditions with the air-liquid interface to test the reversibility of the effects observed. New electrophysiological measurements were performed after 24 hours on these wells as described above.

HNEC response to amiloride

To explore the mechanisms of Rt and Vt modifications observed between *A. fumigatus* and the other fungi (see below), and because airway cells exhibit *in vitro* dominant sodium ion apical absorption, the sodium ion channels at the apical side were blocked by the addition of amiloride hydrochloride according to Berstein et al (Bernstein et al., 1997). Briefly, transepithelial flux measurements were evaluated after the addition of amiloride (Sigma, France) as follows: 10^{-2} M amiloride was added to the apical side, immediately followed by the addition of either HNEC culture medium alone or *A. fumigatus* filtrate to achieve a final amiloride concentration of 10^{-4}M.

Morphological studies with electron microscopy

HNEC morphology was determined by transmission electron microscopy. Control and challenged HNEC cultures were immediately fixed in 2.5% glutaraldehyde (in 0.045 M cacodylate buffer at pH 7.4) for 2 hours at 4 °C. HNEC were then postfixed in buffered 1% osmium tetroxide for 90 min, stained in 2% uranyl acetate and dehydrated through graded solutions of ethanol. Cell culture filters were removed from the inserts and embedded in Epon. Semi-thin sections (1 μm) were stained with toluidine blue and examined by light microscopy to evaluate the specimen prior to thin sectioning. Ultrathin sections (80 nm) were examined on a transmission electron microscope (Philips EM 301) at an acceleration of 80 kV and a final magnification ranging from 2,500 to 30,000.

Data analysis

Statistical analysis was performed using EPI-INFO 6.04c computer software (Centers for Disease Control and Prevention, Atlanta, Georgia). Each value was derived from at least 3 experiments and was expressed as the mean ± SEM. Means of controls and fungal filtrates were compared using a Student's t test or Kruskall-Wallis tests. A p value less than 0.05 was considered statistically significant.

Results

Modifications of bioelectrical properties of HNEC cultures

Both *A. fumigatus* and *P. chrysogenum* 7-day fungal filtrates induced a significant decrease in Rt compared to controls at 30, 60 and 180 minutes (p= 0.005, 0.01, and

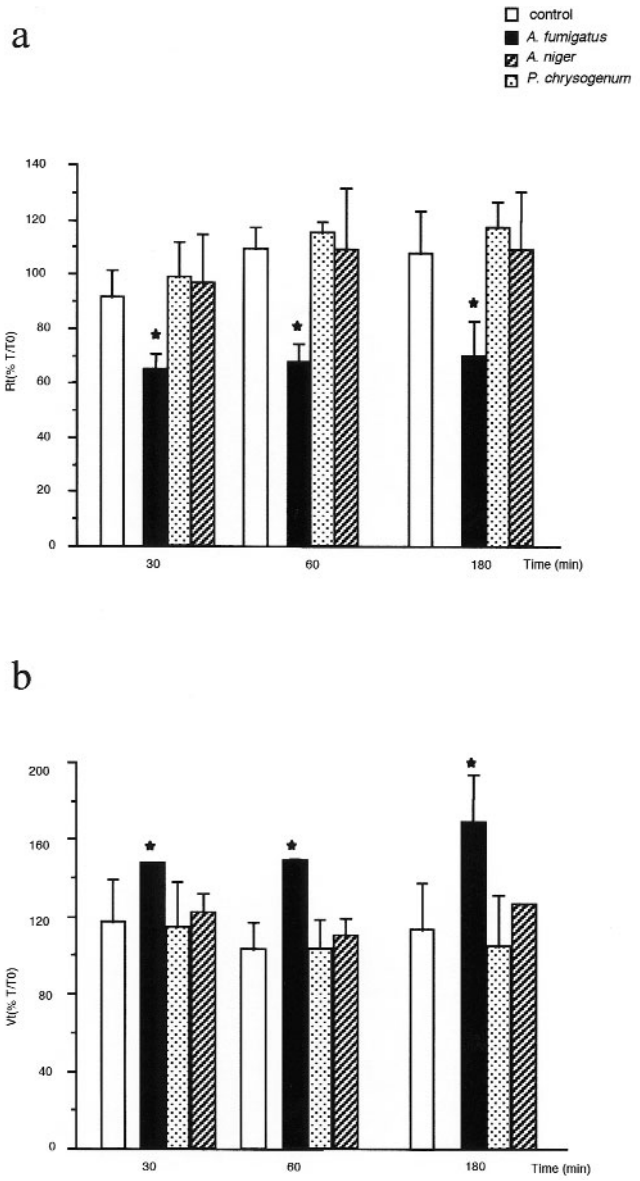

Fig. 1. Effect of culture filtrates of *Aspergillus fumigatus* (*A. fumigatus* reference strain IP 2279.94). *A. Niger* and *Penicillium chrysogenum* on human nasal epithelial cells (HNEC) when challenged with 24-hour culture filtrates for 3 hours. Each result is expressed as a percentage of variation from the T0 measurements. Calculation is performed on the mean ± SEM obtained from 5 different cultures. **a.** A significant decrease of transepithelial resistances (Rt) is observed only for *A. fumigatus.* **b.** An increase of transepithelial potential differences (Vt) is observed only for *A. fumigatus*. *: significant difference compared with controls (p<0.05).

0.06, respectively), and there were non-significant transepithelial hyperpolarization of cells between the two fungi and controls (data not shown). However, the 7-day fungal filtrates did not show any significant variations of the Rt and Vt between *A. fumigatus* and *P. chrysogenum*. Therefore, we decided to focus on fungal filtrates obtained with shorter incubations and to include another *Aspergillus* species, *A. niger*, as an additional control.

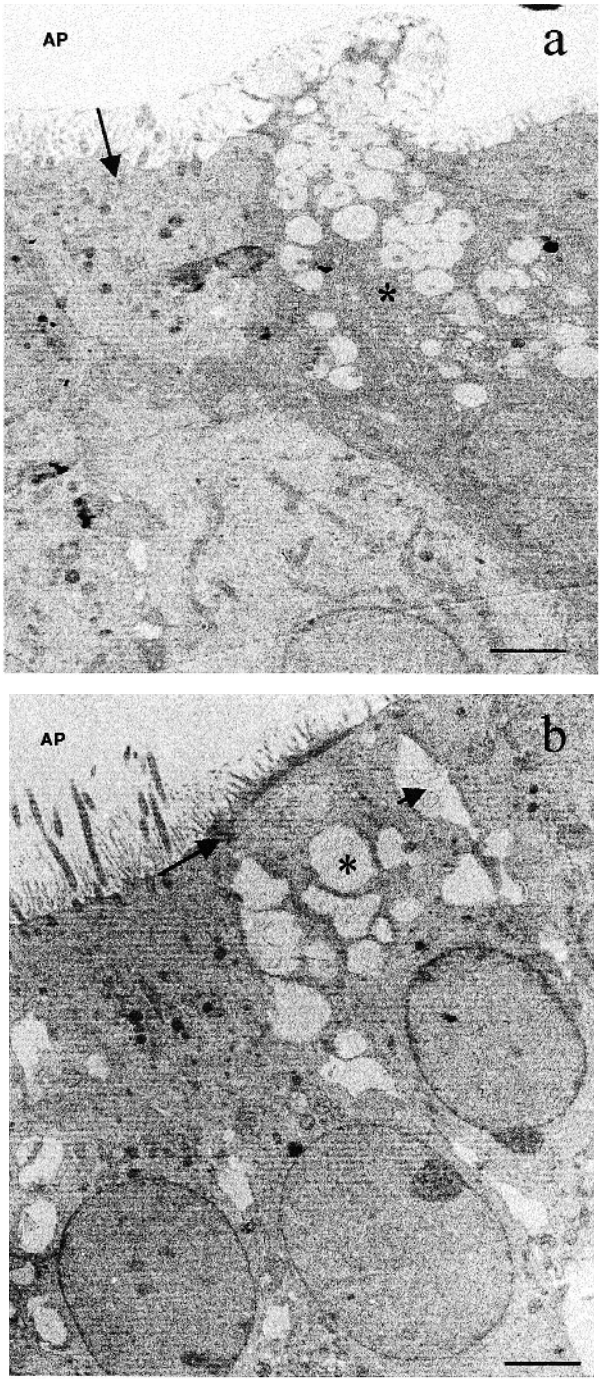

Fig. 2. TEM analysis. **a.** HNEC control with ciliated cell (arrow) and goblet cell (*) **b.** Presence of cytoplasmic vacuolization (*) and increased intercellular spaces (arrow head) with normal tight junctions (arrow) are detected in HNEC epithelium challenged with *A. fumigatus* culture filtrate for 3 hours. AP: apical pole. Bar: 2 μm.

The 24-hour fungal filtrates gave different results depending on the fungus species. Whereas *P. chrysogenum* and *A. niger* did not differ from the controls, *A. fumigatus* induced significant modifications in HNEC cultures (Fig. 1). Rt decreased by 40 % at 30 min, 60 min, and 180 min ($p<0.001$). Vt increased by 40% at 30 min ($p=0.04$), at 60 min ($p=0.001$), and at 180 min ($p<0.001$). The same effects were obtained with each the three other *A. fumigatus* isolates tested (data not shown). These electrophysiological differences disappeared after recovery for 24 hours in air-liquid culture conditions, returning to values of T0 with no differences between the wells challenged with different fungal filtrates and control wells. The pH and osmolarity of the filtrates were within the same values for each filtrate and for the cell-culture medium (i.e., pH 7.4 and 310-315 mosm/l). Determination of cell cytotoxicity by LDH activity in the cell-culture medium did not detect any differences between control wells and wells exposed to fungal filtrates. The results were similar when using unfrozen filtrates or heated filtrates 100 °C for 5 min C (data not shown). The 24-hour filtrates obtained at 30 °C instead of 37 °C showed the same differences in Rt and Vt between *A. fumigatus* and *P. chrysogenum* (data not shown).

Morphological studies

Optical microscopy detected no differences between controls and cell cultures exposed to the different fungal filtrates. In contrast, TEM analyses showed cytoplasmic vacuolization and increased intercellular spaces in HNEC cultures exposed to 24-hour *A. fumigatus* filtrates only (Fig. 2) but the tight-junctions and desmosomes were present. The modifications disappeared when the cell cultures were fixed 24 hours after the air-liquid interface was recovered.

HNEC response to amiloride

The effects of amiloride hydrochloride are shown in Figure 3. As expected, amiloride hydrochloride treatment of HNEC cultures blocked apical sodium absorption and induced a hyperpolarisation in controls. After amiloride pretreatment of HNEC, the decrease of transepithelial resistance observed with the 24-hour *A. fumigatus* filtrate was no longer observed.

Discussion

Our results showed, for the first time in an air-liquid interface model, that 24-hour filtrates of *A. fumigatus* can specifically alter human nasal epithelial cells (HNEC) compared with the non pathogenic filamentous

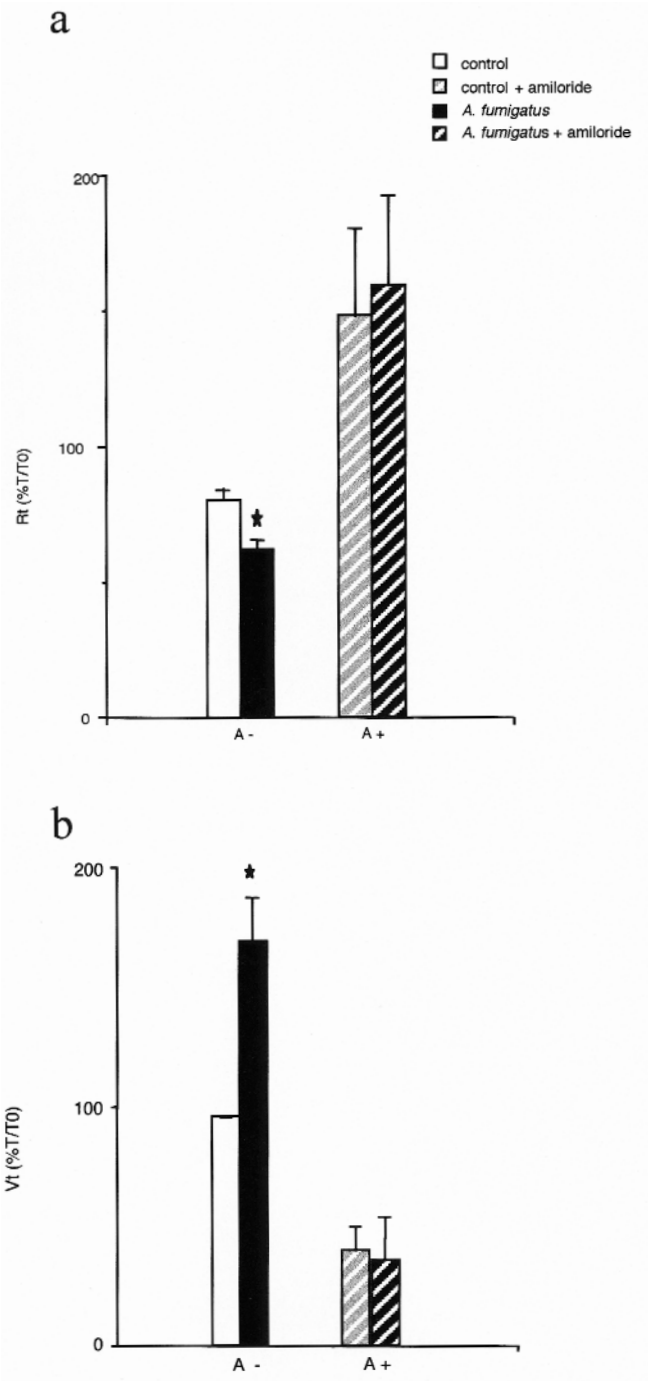

Fig. 3. Results of the challenge of HNEC with 24-hour culture filtrates of *Aspergillus fumigatus* after pretreatment of HNEC with 10^{-4} M of amiloride hydrochloride. (A+): with amiloride; (A-): without amiloride. Each result is expressed as a percentage of variation from the T0 measurements. Calculations are performed on the means ± SEM obtained from 3 different cultures. **a.** The decreased transepithelial resistances (Rt) observed with the 24-hour *A. fumigatus* filtrate disappear when the HNEC cultures are previously treated with amiloride hydrochloride (10^{-4} M). **b.** Amiloride hydrochloride treatment produces a decrease in transepithelial hyperpolarization (Vt = transepithelial potential differences) by blocking apical sodium absorption.*= significant difference compared with controls ($p<0.05$).

fungi, *A. niger*, and *P. chrysogenum*. These effects consisted in Rt and Vt modifications and cytoplasmic vacuolization. These modifications were reversible when the filtrates were removed from the apical side of the cells. The results obtained with HNEC can reasonably be extended to the whole airway epithelium, since the nasal epithelium is very similar to one of the lower airway (Gruenert et al., 1995). It has been shown that morphological differentiation and functional properties, such as ion transport, are similar in cultured cells from nasal polyps or tracheobronchial mucosa. As HNEC are differentiated in vitro, this model reproduces more physiological conditions than do explants or cell lines (Gruenert et al., 1995)

Using filtrates of 7-day fungal cultures, Rt and Vt modifications were observed but the differences between *A. fumigatus* and *P. chrysogenum* disappeared. Therefore, the specificity of *A. fumigatus* disappeared with filtrates obtained after several days. Indeed, 5- or 10-day filtrates from more than 20 fungal species, even though never encountered in human invasive pathology, have been shown to reduce the ciliary beat frequency in a tracheal explant model (Pieckova and Jesenska, 1996; Cody et al., 1997). Similarly, proteases produced by stationary cultures of *Alternaria alternata*, *Cladosporium herbarum*, and *A. fumigatus* induce morphological changes in the A549 cell line (Kauffman, 2000). Other authors have also observed some effects of a 5-day culture *A. fumigatus* filtrate on the ciliary beat frequency of nasal explants (Amitani, 1995). However, as they used *Candida albicans* and *Cryptococcus neoformans* as controls, the specificity of *A. fumigatus* compared with other filamentous fungi cannot be evaluated. Therefore, the pathogenicity of *A. fumigatus* probably lies in its capacity to produce molecules able to damage epithelial cells more rapidly than other fungi. The diffusible component from the conidia of *A. fumigatus* has already been shown to have detrimental effects on neutrophils (Hobson, 2000) and macrophages Mitchell , 1997)

To know whether the molecules responsible for the effects observed in our experiments are *A. fumigatus*-specific or simply produced more rapidly by *A. fumigatus* than by other filamentous fungi warrants further investigation. The differences in Rt and Vt observed between *A. fumigatus* and *P. chrysogenum* with 24-hour filtrates obtained at 30 °C were similar to those observed with cultures at 37° C. This suggests that a better growth at 37 °C for *A. fumigatus* cannot be the only explanation. Moreover, the molecules involved in the toxic effects of 24-hour and 7-day *A. fumigatus* filtrates could also be different. Among the candidate molecules (Mitchell et al., 1997, Yang et al., 2000) gliotoxin has been proposed to play an important role in the pathogenesis of aspergillosis (Mullbacher and Eichner, 1984; Sutton et al., 1994, 1996; Amitani et al., 1997; Belkacemi et al., 1999). Depending on the concentration, gliotoxin has an antiphagocytic action on human alveolar macrophages and polymorphonuclear

leucocytes (Murayama et al., 1996) and slows the ciliary beat frequency of nasal explants (Amitani et al., 1997). In our model, gliotoxin is probably not responsible for the effects observed since heating of the filtrates at 100 °C for 10 min did not change the *A. fumigatus* effects although gliotoxin is inactivated under these conditions.

Several mechanisms can explain the *A. fumigatus*-specific effects observed. Cell detachment can be excluded since TEM analysis showed integrity of the cell junctions. However, increased intercellular spaces were detected after exposure to *A. fumigatus* filtrates. These morphological modifications could expose some *A. fumigatus* receptors present at the basolateral surface of epithelial cells, as was demonstrated for viruses (Wang et al., 2000). The electrophysiological modifications observed led us to hypothesize that the changes observed in treated cells were due to an increased sodium absorption by the apical cell membrane. Normal human nasal epithelium and in vitro HNEC exhibit dominant and active sodium absorption (Bernstein et al., 1997). These actions, coupled with active chloride ion secretion, result in water absorption that maintains efficient mucociliary clearance. As the apical deposit of amiloride hydrochloride, a specific blocker of the apical sodium channel, inhibits the bioelectrophysiological modifications due to *A. fumigatus* 24-hour filtrates, *A. fumigatus* probably acts, at least in part, through these sodium apical channels. The *A. fumigatus* effects may result in interstitial edema, modifications of the superficial lining fluid of the airways, and impairment of ciliary functions. These modifications may render the respiratory epithelium more susceptible to *A. fumigatus* colonization or invasion.

We conclude that 24-hour filtrates of *A. fumigatus*, but not those of *A. niger* or *P. chrysogenum*, induce specific and reversible functional and morphological changes in human nasal epithelial cells. The target of *A. fumigatus* is at least in part the sodium channels, which could act through modifications of the superficial lining fluid, helping the fungus to colonize the airways. The precise identification of the molecules involved warrants further studies. A search for other factors responsible for the pathogenic potential of *A. fumigatus* is necessary as virulence is probably multifactorial (Latgé, 1999). In this perspective, the in vitro model we have developed with a well differentiated airway epithelium in air-liquid interface will be very helpful.

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