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Aspergillus fumigatus Forms Biofilms with Reduced Antifungal Drug Susceptibility on Bronchial Epithelial Cells[▽]

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Aspergillus fumigatus **is a leading cause of death in immunocompromised patients and a frequent colonizer of the respiratory tracts of asthma and cystic fibrosis (CF) patients. Biofilms enable bacteria and yeasts to persist in infections and can contribute to antimicrobial resistance. We investigated the ability of *A. fumigatus* to form biofilms on polystyrene (PS) and human bronchial epithelial (HBE) and CF bronchial epithelial (CFBE) cells. We developed a novel in vitro coculture model of *A. fumigatus* biofilm formation on HBE and CFBE cells. Biofilm formation was documented by dry weight, scanning electron microscopy (SEM), and confocal scanning laser microscopy (CSLM). The in vitro antifungal activities of seven antifungal drugs were tested by comparing planktonic and sessile *A. fumigatus* strains. *A. fumigatus* formed an extracellular matrix on PS and HBE and CFBE cells as evidenced by increased dry weight, SEM, and CSLM. These biofilms exhibited decreased antifungal drug susceptibility and were adherent to the epithelial cells, with fungi remaining viable throughout 3 days. These observations might have implications for treatment of *A. fumigatus* colonization in chronic lung diseases and for its potential impact on airway inflammation, damage, and infection.**

The saprophytic mold *Aspergillus fumigatus* is found worldwide and has a high sporulating capacity, which results in the ubiquitous presence of high concentrations of conidia in the air (14). The virulence of *A. fumigatus* is caused by the production of fungal proteins that promote mycelial growth into the lung parenchyma or by structural features of the conidia that confer resistance to the host's antifungal properties (13). *A. fumigatus* toxins have been shown to inhibit ciliary activity, and proteases can damage the epithelial tissue (1). *A. fumigatus* can cause invasive pulmonary aspergillosis, aspergilloma, immunoglobulin E-mediated allergic rhinitis and asthma, hypersensitivity pneumonitis, chronic necrotizing pneumonia, and allergic bronchopulmonary aspergillosis (ABPA). ABPA is a pulmonary disorder of cystic fibrosis (CF) and asthma patients arising from allergic response to multiple antigens expressed by *A. fumigatus* colonizing the bronchial mucus. *A. fumigatus* can produce cytotoxic and immunosuppressive proteins that enable it to persist for long periods in the respiratory tract, particularly in airflow obstructions and impaired clearance mechanisms (14). In neutropenic cancer patients, the conidia can germinate and produce a mycelium that invades the lung tissue (2, 14, 24). This can cause invasive pulmonary aspergillosis, with high mortality rates despite the use of antifungal drugs with potent in vitro activity. What all of these situations have in common is that the fungus encounters an aerial and static environment quite similar to the conditions found by the fungus during in vitro growth (14).

The preferred growth form of bacteria and yeast is in multicellular communities to colonize a substratum and resist external aggression. In addition to evolutionarily developed antifungal drug resistance, temporary resistance was observed inside yeast biofilms (13). Specific genes and quorum-sensing molecules are regulated in *Candida* biofilms: the CaACE2 gene affects morphogenesis, adherence, and virulence; NOT4 affects hyphal development; YWP1 affects attachment in *Candida albicans*; and farnesol is a quorum-sensing molecule affecting biofilm formation (7, 10, 11, 18). Persister cells, interactions with polysaccharides of the extracellular matrix (ECM), and ECM acting as a barrier can contribute to reduced antifungal drug susceptibility (17). In the case of yeast biofilms, hyphal growth is necessary, and mature *Candida* biofilms exhibit a complex three-dimensional (3D) structure and display extensive spatial heterogeneity (17). This complexity is thought to represent the optimal spatial arrangement to facilitate the influx of nutrients, the disposal of waste products, and the establishment of microniches throughout the biofilm.

The rationale for this study was that it is most likely that *A. fumigatus* can grow as a biofilm in vivo, as well. To demonstrate *Aspergillus* biofilms in vivo, tissue removals and examinations have to be conducted. However, autopsy is not routinely performed in patients who have died due to invasive aspergillosis, and lung biopsies are rarely performed in chronic *Aspergillus* infections (aspergilloma, ABPA, etc.). So far, only indirect evidence for *A. fumigatus* biofilm in patients is available: the high mortality in neutropenic cancer patients suffering from invasive aspergillosis and chronic *Aspergillus* infections and the possible resistance to in vitro potent antifungal drugs, leading to the unavoidable necessity of surgery to cure the disease. Mowat et al. showed that *A. fumigatus* can form coherent multicellular biofilm structures that are resistant to the effects of antifungal drugs (15). Beauvais et al. have reported recently that the colony surface of *A. fumigatus* revealed the presence of an extracellular hydrophobic matrix that acted as a cohesive linkage binding hyphae into a contiguous sheath. The ECM

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was composed of galactomannan, α-1,3-glucans, monosaccharides and polyols, melanin, and proteins, including major antigens and hydrophobins. Additionally, they reported that *A. fumigatus* colonies were more resistant to polyenes than submerged shake mycelium (3). These observations require further in vitro and in vivo studies to investigate the putative role of *A. fumigatus* biofilm formation in this high-risk patient population (17).

The aim of this study was to produce biofilms of *A. fumigatus* on human bronchial epithelial (HBE) cells and CF bronchial epithelial (CFBE) cells to mimic the in vivo situation.

(Some of the results of this work have been previously reported in the form of abstracts [21, 22].)

MATERIALS AND METHODS

Organisms and bronchial epithelial cells. *A. fumigatus* ATCC 9197 from glycerol stocks that had been stored at −80°C was streaked out on fresh potato dextrose agar (PDA) plates until sporulation. 16HBE14o− (wild-type) and CFBE41o− (CFBE, homozygous for the delF508 mutation) cells, kindly provided by D. C. Gruenert, were cultured on collagen/fibronectin/bovine serum-coated flasks in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) as described previously (19).

An in vitro coculture model of *A. fumigatus* biofilm on HBE and CFBE cells. Polystyrene (PS) and glass were coated with a fibronectin (10-mg/ml)/collagen (30-mg/liter) solution. The HBE and CFBE cells were grown to confluence (8). Confluence was confirmed by microscopy as having 90% of the surfaces covered in cells. *A. fumigatus* on PDA plates was overlaid with 0.1% Tween 20 and removed gently. The solution was harvested with a syringe and passed through a 5.0-μm syringe-driven filter (Millipore GmbH, Schwalbach, Germany) to remove the hyphae. The conidia were washed, and the pellet was resuspended in phosphate-buffered saline (PBS). The conidia were adjusted to a final concentration of 10^6 conidia/ml in two different media: the medium for biofilm formation was MEM (c.c.pro, Oberdorla, Germany) plus 10% FCS. The control medium for nonbiofilm growth was MEM plus 10% PBS. Serum was added to the biofilm media as the main carbon source because it is present in vivo and promotes the growth of a hyphal network, one main component of fungal biofilms (4).The fungal-conidium solution was added to the cells and incubated at 37°C and 5% CO_2 for 2 h to let the fungus adhere to the cell surface. After the cells were washed with PBS to remove nonadherent conidia, new MEM (containing FCS or PBS) was added and the fungus was left to produce biofilm for up to 3 days. MEM, and not RPMI, was used in this study due to the recommendation for its use with bronchial epithelial cells (19). Hughes et al. also reported good reproducible results when testing MEM for *Aspergillus* spp. in comparison to RPMI (9). All experiments were repeated three times, and the mean and standard error (SE) were calculated.

Biofilm growth kinetics (dry weight). For an initial proof of principle, i.e., whether *A. fumigatus* is able to produce biofilm at all, biofilm formation was tested on PS and the dry weight was calculated. Later, biofilm was formed on confluent cells in PS 24-well plates (Corning Inc., New York) using the coculture model. The ECM produced was removed by scraping after 4 h, 1 day, 2 days, and 3 days, respectively. The ECM was collected on preweighed cellulose nitrate filters (0.45-μm pore size; 25-mm diameter) and dried to a constant weight at 80°C.

Safranin staining. The ability of safranin to stain the polysaccharide structure was recently reported by our group (20). Biofilm was produced in 96-well plates on confluent cells (Corning Inc., New York) using the coculture model. The ECM was stained with 50 μl safranin solution after 1 day, 2 days, and 3 days. After 5 min, the wells were washed carefully until the supernatant was clear. The optical density was measured at 492 nm with a spectrophotometer (Tecan Sunrise, Crailsheim, Germany).

Scanning electron microscopy (SEM). In a six-well plate on round glass coverslips, submerged biofilm was produced using the coculture model. After biofilm production, the coverslips were transferred without special treatment, metalized with gold, and examined with a scanning electron microscope (Zeiss Novascan, Jena, Germany).

Confocal scanning laser microscopy (CSLM). On ibiTreat plastic (tissue culture-treated μ-dishes; Ibidi GmbH, Munich, Germany) and on confluent cells on ibiTreat plastic (tissue culture-treated μ-slides with a Y shape; Ibidi GmbH, Munich, Germany), biofilm was produced for 3 days using the coculture model. Staining was performed as previously described (13). A Nikon C1Si spectral imaging confocal laser scanning system on a Nikon TE2000-E inverted microscope with Nikon EZ-C1 Gold software was used for all confocal-microscopy experiments (Nikon GmbH, Düsseldorf, Germany). Forty stacked images at ×60 magnification were obtained, and average images were used. The 3D images were selected randomly from an apical surface area (23). Intense green fluorescence, resulting from concanavalin A binding to polysaccharides, framed the fungus cell walls. The red color of the FUN 1 cell stain was localized in dense aggregates in the cytoplasm of metabolically active cells. Thus, areas of red fluorescence represented metabolically active cells, and green fluorescence indicated cell wall-like polysaccharides, while yellow areas represented dual staining (6). The biofilm was carefully discarded, and the remaining cells were stained with trypan blue for cell viability.

Antifungal drug susceptibility testing. MICs of planktonic cells were tested with amphotericin B (AMB) (Sigma-Aldrich Co.), liposomal AMB (LAMB) (Gilead Sciences Inc.), voriconazole (VRC) (Pfizer Pharma GmbH), posaconazole (POS) (Schering-Plough Co.), itraconazole (ITC) (Janssen Pharmaceutica), caspofungin (CSP) (Merck & Co., Inc.), and micafungin (MFG) (Astellas Pharma GmbH) by following the Clinical Laboratory Standards Institute (CLSI) M38-A broth microdilution method with reading of end points at 24 h and 48 h (16). The concentrations used for the antifungal drugs were as follows (in μg/ml): AMB, 0.06 to 32; LAMB, 0.03 to 16; VRC, 0.03 to 16; POS, 0.03 to 16; ITC, 0.03 to 16; CSP, 0.03 to 16; and MFG, 0.03 to 16.

Biofilm was produced in PS 96-well plates using the coculture model. Sessile MICs were determined after 24-h and 48-h drug incubations at 50% inhibition of metabolic activity (MIC-2) and at 90% inhibition of metabolic activity (MIC-1) compared to a drug-free control using the XTT reduction assay (12). AMB and ITC activities were tested against *A. fumigatus* biofilms on PS without the influence of cells.

In addition to the microdilution method and the XTT reduction assay, the CFU were determined. Therefore, the plates were shaken vigorously to loosen attached cells. One hundred counted cells were struck out on PDA plates. After 24 h of incubation at 35°C, the colonies were counted.

RESULTS

Biofilm growth kinetics (dry weight). The biofilm dry weight on PS (data not shown) was 2.2 mg (±0.1 mg) after 4 h, 4.4 mg (±0.2 mg) after 1 day, 5.4 mg (±0.3 mg) after 2 days, and 8.3 mg (±0.3 mg) after 3 days. The biofilm in the control flask, containing MEM plus 10% PBS instead of MEM plus 10% FCS, was 0.12 mg (±0.01 mg) after 2 days of incubation. On the confluent cell layer, the dry weight of the ATCC 9197 control flask increased from 0.002 mg (±0.001 mg) to 0.1015 mg (±0.0005 mg) between 4 h and 3 days (Fig. 1). The dry weight of the biofilm produced significantly exceeded that of the control, with an ECM of 7.4 mg (±0.5 mg) ($P < 0.005$) on the HBE and 7.7 mg (±0.6 mg) ($P < 0.005$) on the CFBE cells after 3 days of biofilm production (Fig. 1). There was no significant difference in dry weight increase between the two cell lines (Fig. 1). Finally, both biofilm and planktonic cells of *A. fumigatus* displayed an increase in dry weight due to FCS-induced growth, but in the biofilm, significantly more ECM was produced, as evidenced by a dry-weight increase.

Safranin staining. The density of the polysaccharides increased significantly in comparison to the control wells. After 3 days of biofilm production, the control displayed an absorbance maximum at 492 nm of 0.0141 (±0.0005), while the absorbance maximum increased significantly to 0.23 (±0.01) ($P < 0.005$) on HBE cells and to 0.26 (±0.01) ($P < 0.005$) on CFBE cells (Fig. 2). The safranin staining clearly indicated that the significant increase in dry weight mainly resulted from the production of ECM.

SEM. Figure 3a displays the hyphal network of *A. fumigatus* ATCC 9197 produced under nonbiofilm conditions after 2 days of coculturing. Even at a much higher resolution, no hyphal

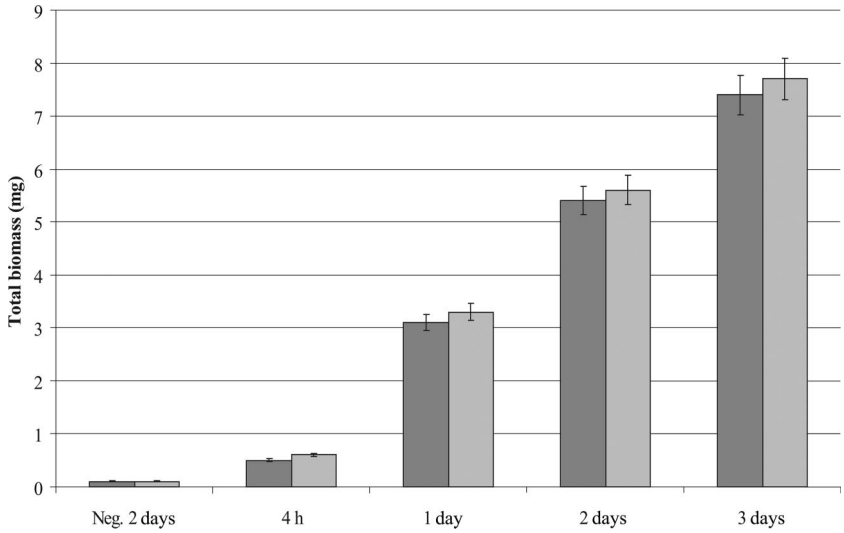

FIG. 1. Quantification of *A. fumigatus* biofilms in coculture (dark-grey bars, 16HBE; light-gray bars, CFBE41o). Each bar represents the mean biomass of three independent experiments with dry-weight measurement, and the error bars indicate the standard errors of the mean. Neg., PBS control containing MEM plus 10% PBS after 2 days of incubation.

packing or ECM production was observed. In the biofilm of *A. fumigatus* ATCC 9197 after 2 days of coculturing, the metalized surface displayed a highly coordinated network of hyphal structures (Fig. 3b). Parallel-packed hyphae were observable (Fig. 3b). These threads run in every direction, often crossing each other. By running parallel as threads and crossing, the structure strengthens itself. Self-produced ECM was observable between the hyphae (Fig. 3b). The colony surface of the *A. fumigatus* biofilm revealed the presence of an ECM that was produced between packed threads of hyphae and represented a structure of high stability and protection.

CSLM. The polysaccharides of the ECM were stained by CAAF (concanavalin A-Alexa Fluor 488 conjugate) and surrounded conidia, hyphae, or free-floating *Aspergillus* cells. Using this differential staining, it was possible to observe *Aspergillus* biofilm on confluent cells consisting of a basal conidium layer (see Fig. 5), a hyphal layer (Fig. 4b), and the self-produced ECM (Fig. 4a). The red fluorescence of the FUN 1 cell stain in Fig. 4 and 5 displays the metabolically active sites. The biofilm thickness after 2 days was determined to be >50 μm (Fig. 4). As shown in Fig. 5B and G, conidia and small colonies of *Aspergillus* were initially scattered over the apical surfaces of the epithelia. The cocultured *A. fumigatus* produced hyphae and early ECM in 1 day (Fig. 5C and H). The regions of the ECM proliferated after 2 days of coculture as regions of bright-green fluorescence without clear edges (Fig. 5D and I). Figure 5E and K show a 3D reconstruction of *Aspergillus* on epithelial cells to demonstrate the viability of the cells after 3 days of

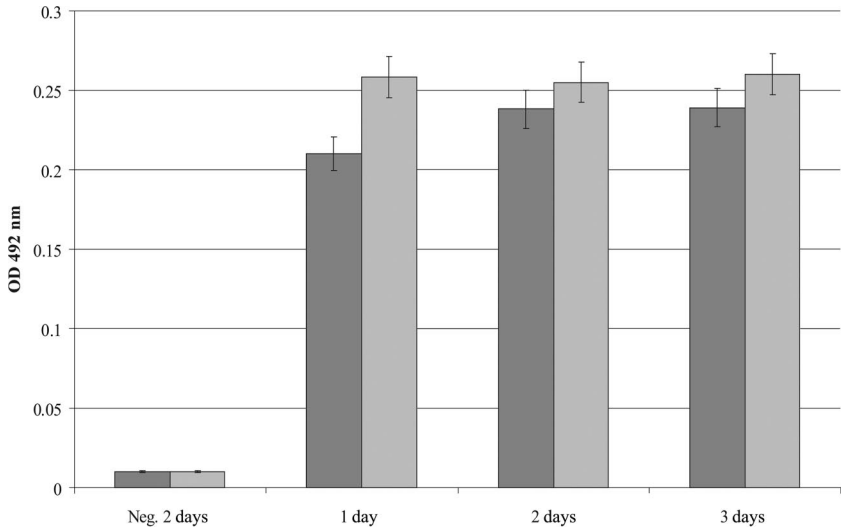

FIG. 2. Densities of *A. fumigatus* biofilms in coculture (dark-grey bars, 16HBE; light-grey bars, CFBE41o−) were quantified in triplicate by safranin staining. The error bars indicate the standard errors of the mean. Neg., PBS control containing MEM plus 10% PBS after 2 days of incubation.

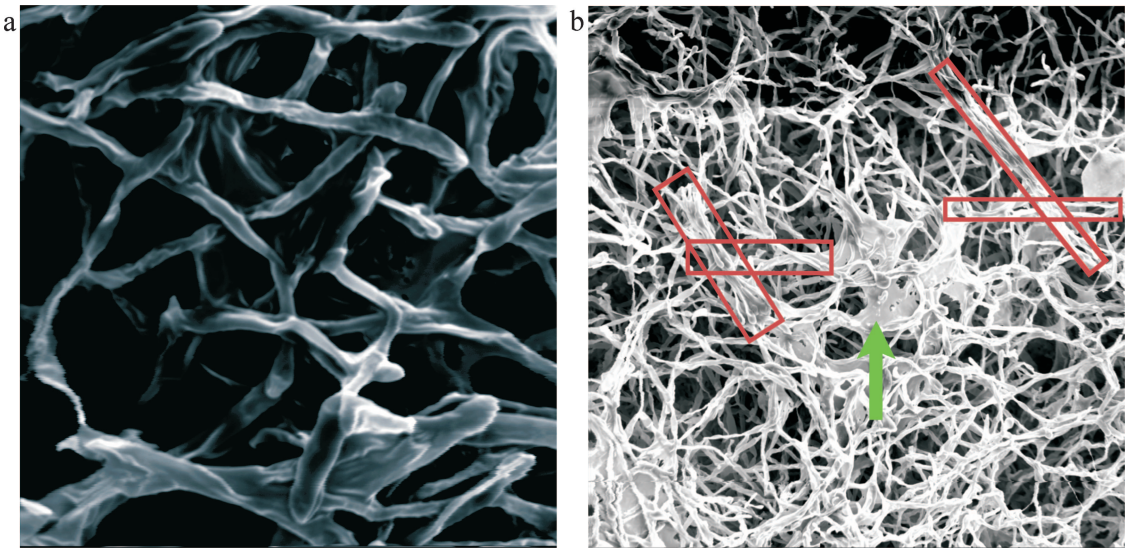

FIG. 3. (a) SEM under nonbiofilm conditions at 48 h. (b) SEM of biofilm surface at 48 h. The boxes indicate parallel-packed hyphae. The arrow indicates ECM embedded in the hyphal network.

coculturing. Additionally, there were highly active (red-stained) conidia and hyphae, as well as green-stained areas without clear edges between and around the hyphal structures. The trypan blue stain showed that almost all infected cells were viable while the cells were attached to the surface. However, of the attached cells that were invaded by hyphae, only 50% were viable. These results show by CSLM that the ECM surrounds conidia and hyphae. Additionally, the fungus attacks the cell layer, leading to 50% cell death.

Antifungal drug susceptibility testing. All MICs were elevated in the presence of biofilm compared to planktonic conditions. For the polyenes (AMB and LAMB), the MICs ranged from 0.25 to 1 μg/ml. In the presence of biofilm, the MICs ranged between 2 and >8 μg/ml. All tested azoles (VRC, POS, and ITC) displayed MICs between 0.125 and 0.25 μg/ml, and in biofilm, between 1 and 2 μg/ml. The echinocandins (CSP and MFG) were active against planktonic *A. fumigatus* (0.5 to 1 μg/ml); for *A. fumigatus* embedded in biofilm, the MICs were >8 μg/ml. The increases in the MICs in the presence of biofilm were not significantly different on PS or on HBE and CFBE cells (Table 1). There was no difference between the visual, the XTT, and the CFU results, and therefore, the data are not shown (SE = 0). Elevated MICs were observed for all antifungal drugs tested under biofilm conditions.

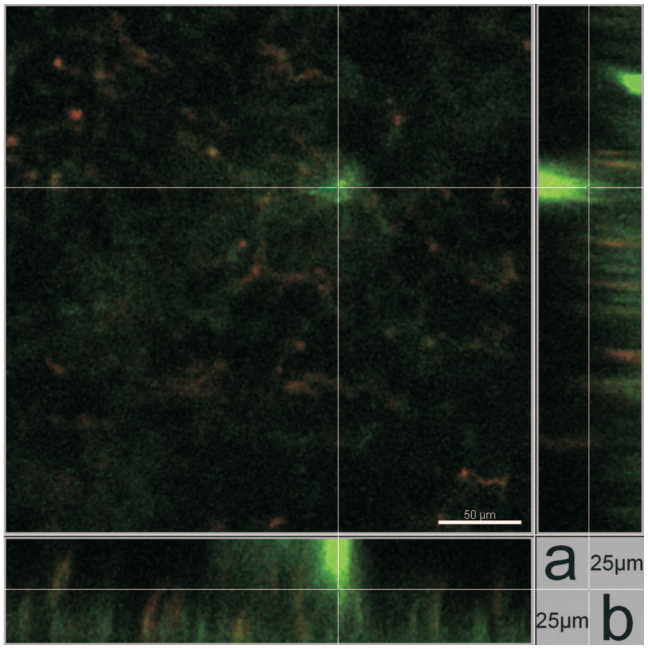

FIG. 4. CSLM image of a lateral view of an *Aspergillus* biofilm on PS.

DISCUSSION

To our knowledge, this is the first report of biofilm production by *A. fumigatus* on bronchial epithelia. The in vitro coculture results suggest that *A. fumigatus* can adhere and form an environment protected against antifungals on the surfaces of HBE and CFBE cells.

Aspergillus biofilm was evidenced by an increase in the dry weight, and the microstructure of the hyphal network was observable by SEM. The ECM is produced between the hyphae and surrounds them. Parallel-packed hyphae strengthen the structure in one direction, while crossing hyphae further stabilize the structure. Beauvais et al. recently reported that the hyphae are covered by an extracellular slime. The ECM is also seen between hyphae, where it apparently glues together the hyphal threads of the network (3). Covering of the hyphal structures, and in particular ending flow tubes, is an indicator of biofilm production.

Biofilm on HBE and CFBE cells was stained with safranin. The results indicate that polysaccharides are produced within the first 24 h, as has been reported for *Candida* biofilms (20). Biofilm on plastic surfaces was stained by safranin from *Staphylococcus* biofilms (6). Fessia and Griffin reported that coagu-

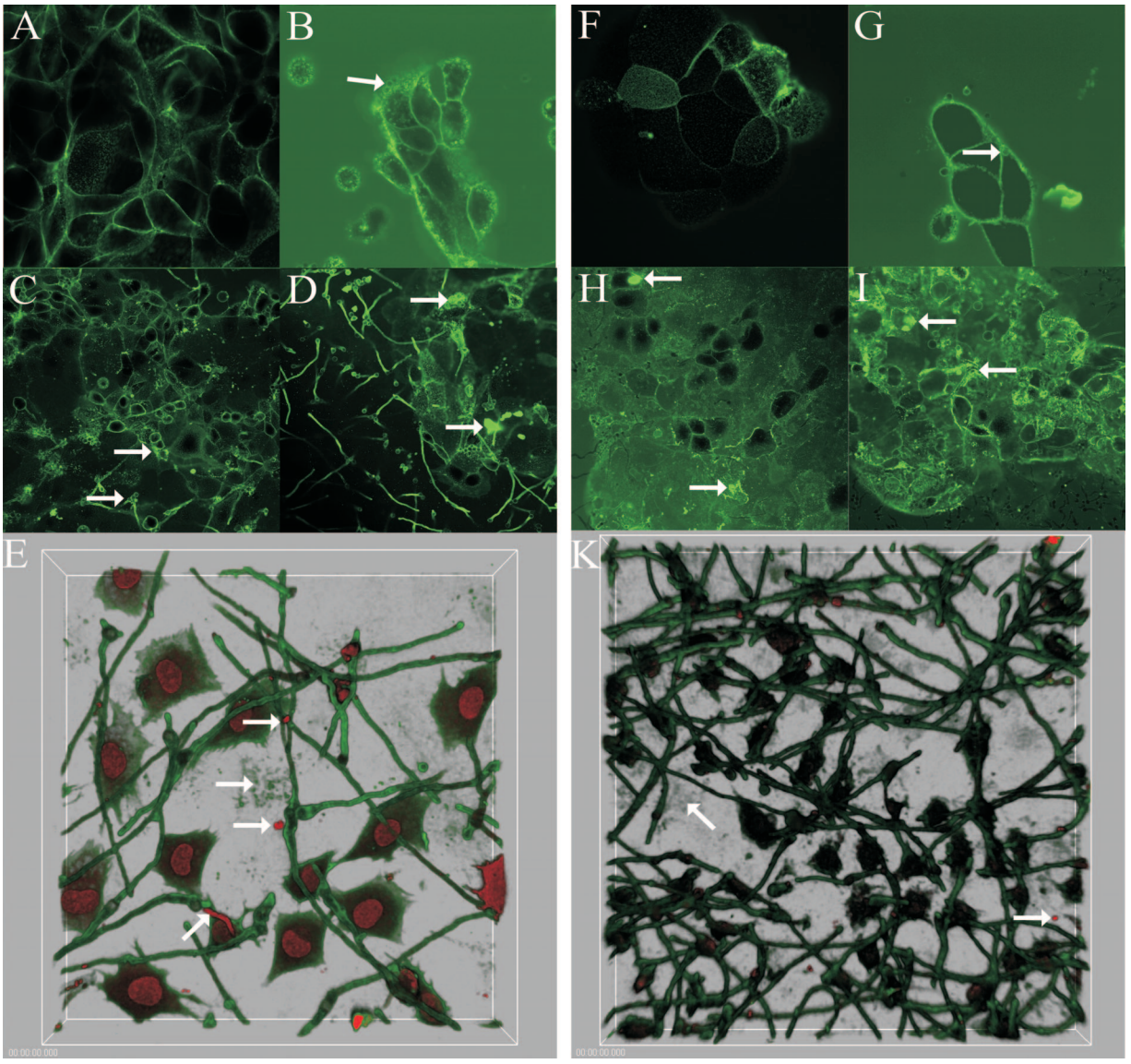

FIG. 5. CSLM images. (A to E) Biofilm formation on 16 HBE cells (panel A without infecting conidia). (F to K) Biofilm formation on CFBE41o− cells (panel F without infecting conidia) after 4 h (B and D), 1 day (C and H; the arrows indicate early ECM), and 2 days (D and I; the arrows indicate ECM proliferation as regions of bright-green fluorescence without clear edges) and a 3D reconstruction after 3 days (E and K; the arrows indicate highly active [red-stained] conidia and hyphae, as well as green-stained areas without clear edges between and around the hyphal structures).

lase-negative staphylococci appeared as a film of dull-red bacteria compared to the yellowish background seen in slides incubated with coagulase-negative staphylococci that did not form a biofilm (6).

For further visualization of the biofilm on viable HBE/CFBE cells, CSLM was used with two different stains. The polysaccharides in the cell wall were labeled green with CAAF and showed a highly organized multicellular structure after 3 days of coculturing. Mowat et al. demonstrated on PS complex multicellular aggregates of *Aspergillus* biofilm (15). Beauvais et al. reported that epifluorescence microscopy revealed the presence of ECM at the surfaces of mycelial mats under submerged shake conditions (3). Our findings on HBE/CFBE cells resemble the multicellular structure and the ECM production reported by Mowat and Beauvais.

The cytoplasts of the metabolically active fungi were stained red with FUN 1. Figure 5E and K illustrate highly active conidia and hyphae. Under all in vitro conditions on PS and bronchial epithelia, *A. fumigatus* biofilm develops through an early phase with germ tube formation and adherence to the surface, an intermediate phase during which the parallel-packed hyphae grow vertically to increase the depth of the network, and the maturation phase, including the stationary phase, during which crossing hyphae further stabilize the biofilm with formation of the ECM.

All seven antifungal drugs demonstrated good in vitro activ-

TABLE 1. Antifungal susceptibility testing of planktonic and biofilm-embedded *A. fumigatus* on PS and bronchial epithelial cells

Drug	*A. fumigatus*	16HBE		CFBE41o−		PS	
		MIC-2 (μg/ml)	MIC-1 (μg/ml)	MIC-2 (μg/ml)	MIC-1 (μg/ml)	MIC-2 (μg/ml)	MIC-1 (μg/ml)
AMB	Biofilm	2	4	4	8	8	16
	Planktonic	0.5	1	0.5	1	1	1
LAMB	Biofilm	2	4	>8	>8		
	Planktonic	0.25	0,5	0.25	0.25		
VRC	Biofilm	1	1	1	2		
	Planktonic	0.125	0.125	0.125	0.125		
ITC	Biofilm	1	1	1	2	2	4
	Planktonic	0.125	0.25	0.125	0.25	0.5	1
POS	Biofilm	1	1	1	2		
	Planktonic	0.125	0.125	0.125	0.125		
CSP	Biofilm	>8	>8	8	>8		
	Planktonic	0.5	1	0.5	1		
MFG	Biofilm	>8	>8	>8	>8		
	Planktonic	0.5	1	0.5	1		

ity under planktonic conditions at the MIC breakpoint of <1 μg/ml on HBE/CFBE cells. For none of the antifungals was a MIC of ≥1 μg/ml observed. In addition to the recent reports by Beauvais and Mowat of *A. fumigatus* biofilm resistance on PS (3, 15), development of antifungal drug resistance against all tested antifungals on HBE/CFBE cells was observed in this study.

In vitro susceptibility testing of the *A. fumigatus* biofilm on HBE/CFBE cells demonstrated that AMB and LAMB were active but reached the resistance breakpoint, as reported by Espinel-Ingroff et al. (5).

In general, the azoles were slightly more active than the polyenes and echinocandins against *Aspergillus* biofilm on HBE/CFBE cells, but they also reached the resistance breakpoint of 4 mg/liter at 48 h (5). CSP and MFG were ineffective in this study; at least AMB and the azoles were able to inhibit the metabolic activity of *Aspergillus* embedded in a biofilm on HBE/CFBE cells by over 50% at relatively low concentrations. A poor overall activity of the echinocandins against *Aspergillus* biofilms was previously reported by Mowat et al. (15). As echinocandins are fungistatic against molds and active on growing hyphae, it is likely that the drug cannot penetrate to the fungus due to the reduced permeability of the ECM, and therefore, the mold can develop antifungal resistance (2).

However, due to the differences for planktonic and biofilm growth of *A. fumigatus*, the MIC results can be influenced. As previously reported for *Candida* biofilms, XTT allows us to rapidly assess the concentration of an antifungal required to kill or inhibit the growth of *A. fumigatus* (15).

The initial conidium inocula in both the CLSI protocol and the XTT assay were the same. Under planktonic conditions (CLSI protocol), the drugs were added immediately, and the MICs were determined after 48 h. Under biofilm conditions (XTT assay), the drugs were added after 24 to 48 h of biofilm production, and the decrease in the metabolic activity was determined by XTT after another 24 to 48 h. A planktonic control was not carried along due to the hyphal growth after 48 h. Nonetheless, hyphal growth is present during an *Aspergillus* infection. Thus, in vitro antifungal susceptibility testing using the XTT assay allows some conclusions for the in vivo situation.

The results of the antifungal drug susceptibility testing in *A. fumigatus* biofilm formation suggest that higher doses or antifungal combination therapy should be considered for a better penetration of the drugs to the fungal cells.

A. fumigatus can produce a biofilm in vitro on PS and bronchial epithelial cells with typical characteristics. Future studies will have to demonstrate in vivo growth of *A. fumigatus* biofilm in chronic lung diseases and its potential impact on airway inflammation, damage, and infection.

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