

# *Aspergillus fumigatus* biofilm on primary human sinonasal epithelial culture

Deepti Singhal, M.S., Leonie Baker, B.Sc. Hon., Peter-John Wormald, M.D., and LorWai Tan, Ph.D.

## ABSTRACT

**Background:** Bacterial biofilms have been implicated in chronic rhinosinusitis (CRS). However, direct evidence in support of fungal biofilms in sinus disease is lacking in the literature. This study was designed to develop and characterize an *in vitro* *Aspergillus fumigatus* biofilm model on primary human sinonasal epithelial cell culture.

**Methods:** Sinonasal biopsy specimens harvested during endoscopic sinus surgery of six CRS patients and three pituitary tumor (control) patients were cultured in Dulbecco's modified Eagle media (DMEM; Invitrogen)/Hams F12 airway media to encourage epithelial cell proliferation. Epithelial cells separated by immunomagnetic beads were seeded in tissue culture-treated Y-shaped microslides. At confluence the primary cultures were inoculated with *A. fumigatus* spores. Fungus was allowed to germinate and form biofilms under two *in vitro* conditions: (1) static (no flow through of media) and (2) continuous flow coculture (continuous flow movement of media). At regular intervals cocultures were stained with FUN-1, concanavalin A-alexa fluor 488, and examined by confocal scanning laser microscopy. Comstat software was used to assess biomass and thickness.

**Results:** *A. fumigatus* formed three-dimensional biofilm structures with parallel-packed, cross-linked hyphae and channels/passages. Metabolically active hyphae showed orange-red fluorescing intravacuolar structures. Extracellular matrix (ECM) between/around the hyphae fluoresced intense green. *A. fumigatus* biofilms development occurred in five stages: (1) conidial attachment to epithelial cells, (2) hyphal proliferation, (3) ECM production, (4) hyphal parallel packing and cross-linking, and (5) channel/pores formation. Mature biofilms showed basal conidial, middle hyphal, and superficial ECM layers. Biofilms formed under flow conditions displayed more robust and faster growth kinetics when compared with that under static conditions, with a thick, stocky, wrinkly/undulating hyphal growth and extensive ECM production. The differences in biomass and average thickness of the cocultures under static and flow conditions were statistically significant after similar periods of incubation ( $p = 0.0002$ ;  $p < 0.0001$ , respectively).

**Conclusion:** To our knowledge, this is the first article of an *in vitro* model characterizing *A. fumigatus* biofilm formation using primary human sinonasal epithelium under different growth conditions.

(Am J Rhinol Allergy 25, 219–225, 2011; doi: 10.2500/ajra.2011.25.3622)

Over the past 5 years, mounting evidence has been presented to implicate bacterial biofilms in propagating the recalcitrant nature of chronic rhinosinusitis (CRS).<sup>1–5</sup> However, direct evidence for the existence of fungal biofilms and their role in the pathogenesis of CRS has been lacking. While studying bacterial biofilms on sinonasal tissue, Healy *et al.* incidentally detected fungal elements within bacterial biofilms but could not determine if these fungi produced biofilms.<sup>6</sup> Foreman *et al.*, using a pan-fungal fluorescent *in situ* hybridization probe to screen tissue biopsy specimens from CRS patients found nonspecific fungal elements showing biofilms-like structures in 11/50 (22%) patients.<sup>7</sup>

Fungi are now being increasingly recognized as able to adopt a biofilm phenotype both on biotic and on abiotic surfaces. To date, much of the work in fungal biofilm research has focused on *Candida* species<sup>8–11</sup> and these have been isolated from indwelling medical devices such as stents, shunts, implants, endotracheal tubes, pacemakers, and catheters. The fungi most commonly identified from CRS patients belong to the *Aspergillus*, *Alternaria*, *Bipolaris*, *Cucurularia*, and *Rhizopus* species. Villena and Gutierrez-Correa<sup>12</sup> reported that *Aspergillus niger* biofilms grown adhering to a surface had orderly hyphal distribution forming a surface and inner channeled structure with evidence of extracellular matrix (ECM) production. In contrast, *A. niger* pellets grown in a suspension form showed no such organization and was composed of highly intertwined superficial hyphae and densely packed deep mycelium.

Mowat *et al.*<sup>13</sup> standardized the first *in vitro* model of *Aspergillus fumigatus* biofilms and using LIVE/DEAD fluorescent stain with confocal scanning laser microscopy (CSLM) showed that *A. fumigatus* formed coherent filamentous multicellular structures exhibiting acute angled dichotomous branching, with parallel-packed and cross-linking hyphae. They also found antifungal agents were at least 1000 times less effective at reducing the metabolic activity of cells in biofilms when compared with the planktonic form. Seidler *et al.*<sup>14</sup> applied some of Mowat's techniques and grew *A. fumigatus* biofilm on human bronchial epithelial cell lines. Via scanning electron microscopy and CSLM they described the biofilm as consisting of filamentous multicellular structures exhibiting parallel-packed and cross-linked hyphae with exopolysaccharide (EPS) matrix in between.

So far, most of the published research in the field of sinusitis has centered on bacterial biofilms. The aim of this study was (1) to characterize *A. fumigatus* biofilms grown on primary cell cultures from CRS patients using the aforementioned staining and imaging techniques<sup>13,14</sup> and (2) develop an *in vitro* model to better understand the role that fungi play in CRS pathogenesis.

## METHODS

### Primary Sinonasal Epithelial Cell Cultures

After obtaining ethics approval from the hospital Institutional Review Board and appropriate consents from patients, sinonasal tissue samples were collected from nine patients undergoing endoscopic sinus surgery (ESS) between April and June 2009. Six of the patients underwent ESS as a part of the surgical management for CRS, diagnosed based on criteria set by the Rhinosinusitis Task Force,<sup>15</sup> and tissue was harvested from their osteomeatal regions or their ethmoid sinuses. Three of the patients underwent ESS as a part of the approach during transsphenoidal hypophysectomy and their posterior ethmoid tissue was collected. All tissue specimens were transported on ice in Dulbecco's modified Eagle media (DMEM; Gibco products, Invitrogen Corp., Grand Island, NY). Tissue was dissected into 5 × 5 mm

From the Department of Surgery–Otorhinolaryngology Head and Neck Surgery, The Queen Elizabeth Hospital, and the University of Adelaide, Adelaide, South Australia Supported by The Queen Elizabeth Hospital Research Foundation

The authors have no conflicts to declare pertaining to this article

Address correspondence and reprint requests to Peter-John Wormald, M.D., Department of Otorhinolaryngology–Head and Neck Surgery, The Queen Elizabeth Hospital, 28 Woodville Road, Woodville South, South Australia 5011, Australia

E-mail address: peterj.wormald@adelaide.edu.au

Copyright © 2011, OceanSide Publications, Inc., U.S.A.

and grown in a nontreated 6-well dish (Nordic Cell; NUNC, Denmark) as explants, in airway media (DMEM/F12 with 10% fetal calf serum [FCS]). Primary epithelial cells derived from these explants were isolated using Dynabeads Epithelial Enrich (Invitrogen). These sinonasal epithelial cells were grown to 90% confluence on ibiTreat (tissue culture–treated y-shaped microslides; Ibidi GmbH, Munich, Germany).

## Organisms

*A. fumigatus* ATCC 204305 strain (source: Mycology unit, The Women's and Children's Hospital, Adelaide, Australia) was grown on Sabouraud Dextrose Agar at 37°C for 72 hours. Conidia were harvested by flooding the surface of the agar plates with 5 mL of phosphate buffer saline (PBS) on a rocking platform. The conidia in the suspension were counted using Neubauer hemocytometer and adjusted to a concentration of  $1 \times 10^6$ /mL in DMEM (with 10% FCS).

## In Vitro Fungal Coculture on Sinonasal Epithelial Cells

Two hundred microliters of the fungal-conidial solution was used to inoculate the primary cell cultures grown on the ibiTreat y-slides. These were incubated at 37°C and 5% CO<sub>2</sub> for 24 hours to allow adhesion of fungal elements to the cell surface. Each of the slides was then washed with PBS to remove nonadherent conidia, and fresh DMEM with 10% FCS was added. The slides were then incubated at 37°C to allow fungal biofilm formation, under two different *in vitro* conditions: (1) static coculture (StC; no surrounding media movement) and (2) CFC (continuous flow of media through the Y-shaped channels of the ibidi slides).

## Static Coculture

For the static *in vitro* model, nine fungal-epithelial cocultures (six using epithelial cells from CRS patients and three from non-CRS patients) were incubated without any flow movement of the media through the slide chambers. Two control experiments consisted of one with only fungal spores and the other with epithelial cell culture were included. Media was changed every 24 hours, the slides stained with fluorescent dyes and biofilm formation examined by CSLM.

## Continuous Flow Coculture

Seven pairs of fungal-epithelial cocultures (four primary epithelial cultures from CRS patients and three from non-CRS patients) were set up under continuous media flow conditions. One slide of each pair was incubated as an StC and the other was connected to a Continuous Flow Cell Apparatus (Stovall Life Science, Inc., Greensboro, NC) set to a flow rate of 10 mm/min. These flow conditions were maintained over 24 hours except for the brief period of time daily when the formation of biofilm was examined by CSLM. Two control ibidi slides were set up under similar flow conditions: one with fungal spores only and another with epithelial cell culture only.

## Confocal Scanning Laser Scanning Microscopy

At selected time periods, the cocultures were washed with PBS and incubated in dark for 60 minutes with 300  $\mu$ L of PBS containing the fluorescent stains: 10  $\mu$ M of FUN-1 (Live/Dead Yeast Viability Kit; Molecular Probes, Inc., Eugene, OR) and 25  $\mu$ g/mL of concanavalin A–alexa fluor 488 conjugate (ConA; Molecular Probes; Invitrogen). Excess stain was removed by washing in PBS before image capture and analysis using the Leica TCS SP5 (Leica Microsystems, Wetzlar, Germany).

When excited with the argon laser (excitation wavelength, 488 nm), FUN-1 stain fluoresces orange–red as a result of formation of cylindrical intravacuolar structures (CIVSc) inside metabolically active

cells. ConA binds to the fungal cell wall polysaccharides and produces intense green fluorescence. Stacked images at 20 $\times$ , 63 $\times$ , and 200 $\times$  magnification were obtained. Sections of the *xy*-plane were taken at 0.5-mm intervals along the *z*-axis to determine the depth of the biofilms and overall physical ultrastructure. Three-dimensional images were obtained using computer software. COMSTAT software (DTU Systems Biology, Lyngby, Denmark) was used to calculate the biomass and thickness of the cocultures.<sup>16–18</sup> Comstat software was preferred over other methods such as dry weight measurements because it allowed for a repeated temporal qualitative and quantitative assessment of the developing biofilm over a period of days, without removing it from the microslides. Such a removal of biofilms grown on cultured monolayer epithelium from within the channelled microslides was technically challenging, without a possible loss of some of the biofilm components. Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA). A value of  $p = 0.05$  was considered significant. Differences were analyzed using the Mann-Whitney *U* test.

## RESULTS

### Static Cocultures

*A. fumigatus* conidia incubated with no epithelial cell monolayer to adhere to (control) showed no hyphal packing or ECM production (Fig. 1 B). Almost all of the conidia were washed away with the first PBS flush after 24-hour incubation. Some residual conidia at the periphery of the slide channels produced the typical blue–green cotton–wool growth made up of a dense clump of conidiophores. Figure 1 B displays the hyphal clump of *A. fumigatus* ATCC 204305 produced under static nonbiofilm conditions after 48 hours at 37°C.

In contrast, all nine *A. fumigatus*–epithelial cell cocultures showed a different form of fungal growth when compared with the fungal only control slides and were similar to the *A. fumigatus* biofilms described in literature.<sup>13,14</sup> These biofilm structures of *A. fumigatus* ATCC 20305 on the primary sinonasal epithelial culture displayed a highly coordinated network of hyphae (Fig. 1, C and D), spreading in all directions, cross-linking each other and arranging in a parallel-packed manner. This led to the formation of an intricate network of channels and passages and represented a structure of high stability and protection. By running parallel as threads and crossing, the structure is known to form a meshlike arrangement and hence strengthens itself. The FUN-1 stain formed orange–red fluorescing CIVSc inside metabolically active fungal conidia and hyphae (Fig. 1, C and D). ECM was present between and surrounding the hyphae (Fig. 1, C and D). The polysaccharides of the ECM stained intense green by ConA (ConA–alexa fluor 488 conjugate).

The StCs were monitored over a period of 4 weeks and the formation of the *A. fumigatus* biofilm was observed to occur in stages. After inoculation of the *A. fumigatus* conidia to the primary culture on the ibidi slides, the spores were seen to attach to the epithelial monolayer within 24 hours, because they were not flushed away by subsequent daily media changes. The conidia then produced horizontal hyphae along the surface of the epithelial cells and in between the cells. The hyphae also grew vertically over the monolayer of cells and proliferated rapidly over the next 10 days until they formed a dense network of cross-linked and parallel-packed hyphae. There was evidence of ECM production at days 3 and 4 in between the hyphae and it appeared to ensheath the hyphae and at the same time bound the hyphal network in an organized manner. By days 6 and 7, a well-organized hyphal network with channels and pores in between them was observed. During this time, the epithelial cells slowly began to disintegrate and were undetectable in this fungal mesh by 20th–25th day. By days 25–28, this mesh of hyphae and cellular debris completely filled and blocked the Y channels. This thick mat of hyphae and cells appeared to be encased within a thick gel-like substance that became gradually very difficult to wash with media or stain with the

**Figure 1.** (A and B) Confocal scanning laser microscopy (CSLM) images using fluorescent stains FUN-1 and concanavalin A (ConA) of (A) monolayer of cells on ibidi slides (60× magnification) and (B) *Aspergillus fumigatus* only on ibidi slides (20× magnification). (C and D) *A. fumigatus* biofilm structure on a monolayer of sinonasal epithelial cells on CSLM (panel C, 60× magnification; panel D, 200× magnification). Red arrows indicate orange–red fluorescence due to FUN-1 staining of cytoplasmic intravacuolar structures in metabolically active fungal cells. Yellow arrows indicate intense green fluorescence from ConA stain binding to fungal cell wall polysaccharides and extracellular matrix (ECM). (E) Side (xz) view on CSLM showing the structure of a fully formed *A. fumigatus* biofilm at 200× magnification on day 25 of coculturing showing the basal conidial layer, middle hyphal layer, and superficial polysaccharide matrix.

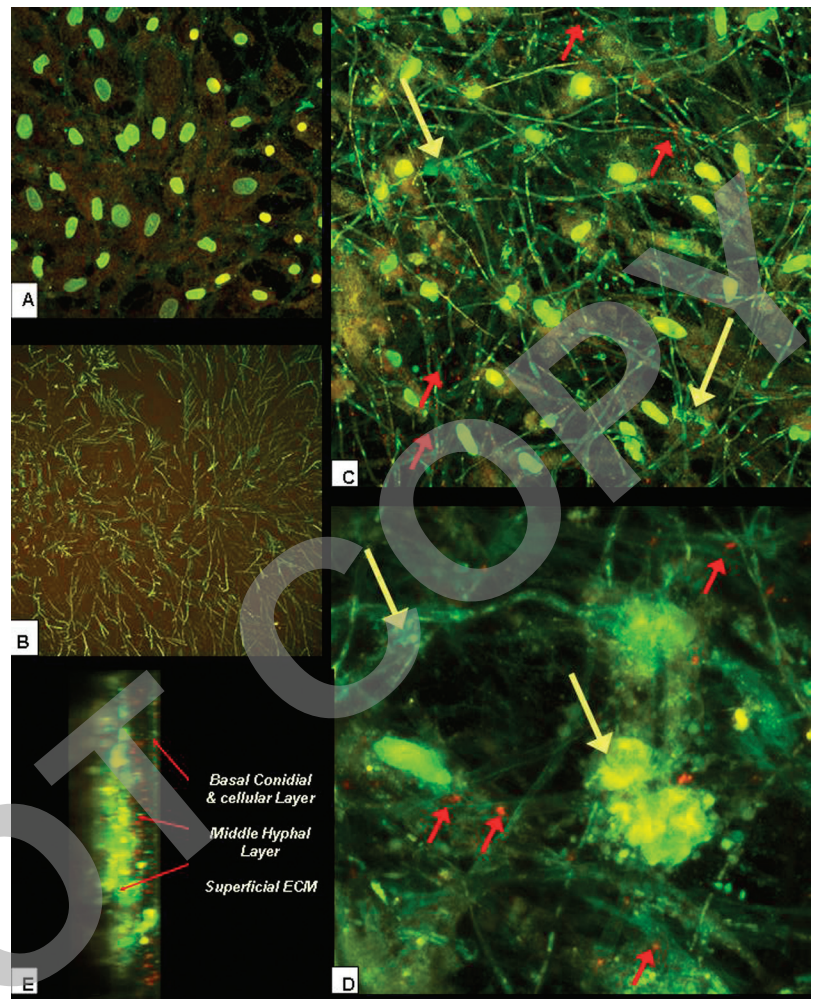


**Table 1** Biomass, mean thickness, and roughness coefficient of the fungal-epithelial cocultures grown under static conditions over a 25-day incubation period

|                                           | Days of Incubation |                 |                 |                 |                 |                  |                 |                 |
|-------------------------------------------|--------------------|-----------------|-----------------|-----------------|-----------------|------------------|-----------------|-----------------|
|                                           | D2                 | D4              | D6              | D8              | D 10            | D 15             | D 20            | D 25            |
| Biomass ( $\mu\text{m}^3/\mu\text{m}^2$ ) | $7.5 \pm 2.1$      | $10.3 \pm 0.1$  | $12.9 \pm 0.7$  | $24.9 \pm 1.1$  | $20.7 \pm 0.8$  | $24.4 \pm 1.4$   | $27.8 \pm 1.7$  | $46.4 \pm 3.1$  |
| Average thickness ( $\mu\text{m}$ )       | $13.3 \pm 1.5$     | $13.6 \pm 0.9$  | $16.3 \pm 0.9$  | $27.9 \pm 1.1$  | $23.9 \pm 0.3$  | $26.4 \pm 1.6$   | $32.8 \pm 1.1$  | $52.2 \pm 3.8$  |
| Roughness coefficient                     | $0.36 \pm 0.02$    | $0.36 \pm 0.01$ | $0.32 \pm 0.01$ | $0.17 \pm 0.01$ | $0.19 \pm 0.01$ | $0.15 \pm 0.004$ | $0.11 \pm 0.01$ | $0.04 \pm 0.02$ |

Each of the values is means of data calculated from 32 z-stacks (4 random image stacks recorded from 8 cocultures). D = day.

dyes. Staining and changing media beyond this time period was not possible and additional experiments were stopped after 4 weeks of incubation. A fully formed biofilm on the monolayer of epithelial cells showed well-defined structure (Fig. 1 E), composed of basal conidial and epithelial layers, and then a hyphal layer, and the superficial ECM.

On quantitative assessment, the biomass of the control cultures (epithelial cells) increased marginally from  $4.8 \pm 2.2 \mu\text{m}^3/\mu\text{m}^2$  at day 2 to  $13 \pm 1.1 \mu\text{m}^3/\mu\text{m}^2$  at day 13, after which the biomass began declining as these sinonasal epithelial cells were only viable for 14–19 days. The biomass of the StCs continued to increase from  $7.5 \pm 2.1 \mu\text{m}^3/\mu\text{m}^2$  at day 2 to  $46.4 \pm 3.1 \mu\text{m}^3/\mu\text{m}^2$  at day 25 in a fully formed biofilm (Table 1). This increase in biomass of the cocultures was thus the cumulative effect of both the epithelial cells and the fungal elements in the initial 5–7 days. Subsequently, the epithelial cells appeared to disintegrate within the fungal mesh as their viability de-

clined. Thus, the increase in biomass of the cocultures after the initial 7–10 days was predominantly as a result of the hyphal proliferation and ECM matrix production in the biofilm. The average thickness of the cocultures also increased fourfold from  $13.3 \pm 1.5 \mu\text{m}$  at day 2 to  $52.2 \pm 3.8 \mu\text{m}$  in a fully formed *A. fumigatus* biofilm (Table 1; Fig. 2). The roughness coefficient (*Ra*) provided a measure of biofilm thickness variability, and a defacto indicator of biofilm heterogeneity. This decreased over the observation period from an average value of 0.36 to 0.04 for the StCs and indicated the formation of the uniformly dense mass of fungal biofilm with the ECM.

### Continuous Flow Coculture

After understanding, defining, and reproducing the experimental conditions required for biofilms to form under static conditions, only

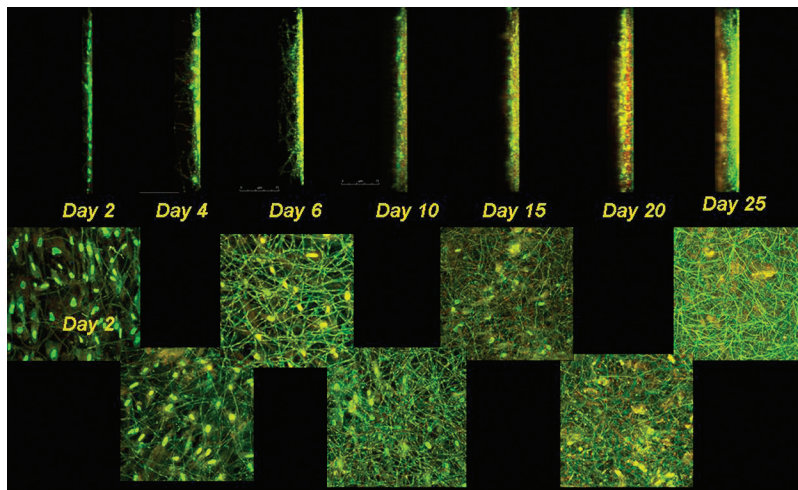


**Figure 2.** Development of the *Aspergillus fumigatus* biofilm on the epithelial monolayer over a period of 25 days, under static conditions showing the increasing thickness and biomass of the coculture. The top line shows side views of sections taken on confocal scanning laser microscopy (CSLM) and the bottom line shows xy images of those sections on CSLM.

seven of the nine collected samples were found suitable for study under continuous flow conditions. The nine samples initially collected from the patients were frozen and stored in liquid nitrogen to be retrieved for each set of the static and continuous flow experiments. Two of the frozen and stored samples did not produce confluent epithelial cultures after 3–4 months of the storage period and hence could not be used for the flow culture experiments set up.

The seven cocultures inoculated under continuous flow conditions also formed *A. fumigatus* biofilms on the monolayer of sinonasal epithelial cells. These fungal biofilms formed under flow conditions underwent the same stages in formation of the biofilm as seen in the StCs and showed the same structural organization, *i.e.*, they had cross-linked and parallel-packed hyphae, with surrounding ECM, and formation of internal pores and channels (Fig. 3, A–C). The *A. fumigatus* hyphae that proliferated under flow conditions were wrinkly and undulating in appearance, which was possibly a result of the continuous flow motion of the surrounding media. As seen in Fig. 3, D–G, hyphae that proliferated under flow conditions measured 3–5 mm thick when compared with 1–2 mm when formed under static conditions. These undulating, thicker, stockier hyphae with intensely green fluorescing walls showed structural organization by cross-linking and arranging in parallel manner. There was more extensive and intense green fluorescence seen in these cocultures, as early as 24 hours after culturing in the flow model, indicating more ECM production under flow conditions.

The biofilms grown using the continuous coculture also displayed more robust and faster growth kinetics. The growth and structure of a flow biofilm at day 2 of incubation was comparable with a static biofilm at 5–6 days of incubation. The biomass of the flow cocultures increased very rapidly to an average of  $18.09 \pm 0.6 \mu\text{m}^3/\mu\text{m}^2$  on the 2nd day of incubation, which was more than double the biomass of a StC at the same time. Subsequent quantitative assessments also showed that the biomass in the flow cocultures on 3rd and 5th days of incubation was comparable with the biomass of an 11- and 15-day StC, respectively (Fig. 4 A). The thickness of the flow coculture also rapidly increased two to three-folds in the first 48–72 hours of incubation under flow when compared with static conditions (Fig. 4 B). The differences in biomass and average thickness of the cocultures under static and flow conditions were statistically significant ( $p = 0.0002$ ;  $p < 0.0001$ , respectively). This increased biomass and thickness of the flow cocultures was the cumulative effect of more numerous, rapidly proliferating fungal hyphae and increased EPS seen surrounding these hyphae as evident by the increased green fluorescence on CSLM.

Thus, the biofilm developed much more rapidly under flow conditions when compared with the StCs, and the stage of the thick

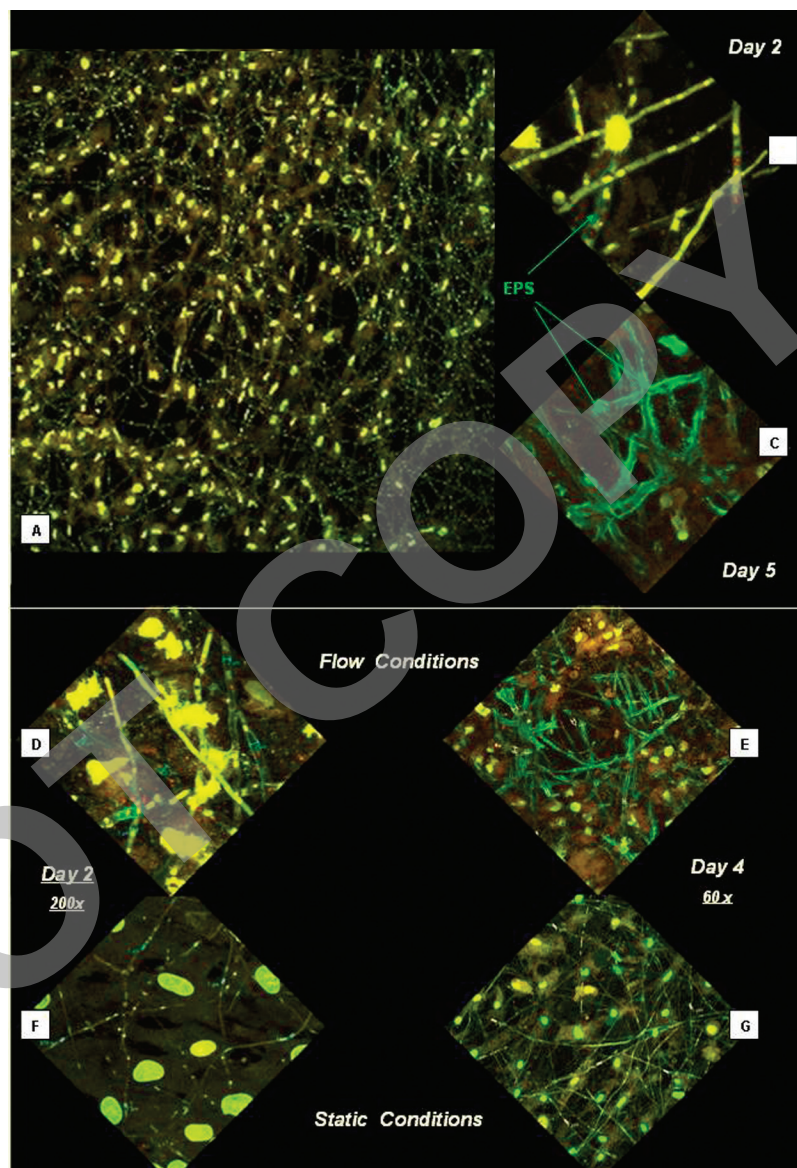
mat of fungal hyphae and cells bound together by the matrix, impermeable to the media or fluorescent dyes, was reached within 5 days under flow conditions when compared with 3–4 weeks in an StC. The epithelial cells were viable until the ~3rd and 4th day of the coculture under flow conditions after which they also started disintegrating and appearing less viable. There was no difference in the type of *A. fumigatus* biofilm formation seen on either the CRS or the non-CRS primary epithelial cell cultures, under both static and continuous flow cocultures (CFC).

## DISCUSSION

To our knowledge, this is the first report of an *in vitro* model of *A. fumigatus* biofilms on primary culture of sinonasal epithelial cells. Our model provides evidence that *A. fumigatus* can develop biofilms as a defense mechanism against host immune responses and topical antifungal therapy on sinonasal epithelial surfaces.

The spectrum of fungal disease in the sinuses (fungal rhinosinusitis [FRS]) extends from benign colonization (fungal ball), allergic FRS, to potentially life-threatening invasive disease.<sup>19</sup> Despite these recognized forms of FRS, the role of fungus in CRS<sup>20–22</sup> has been questioned, because different studies have shown similar fungal prevalence rates in diseased and control groups, with similar types of species isolated, and similar fungal load in the two groups. The fungal yield results in those studies were based on standard microbiology collection and culture techniques that possibly identify only the ubiquitous nonbiofilm forms of the fungi isolated from nasal swabs, secretions, and lavages. In our study we found that the appearance of *A. fumigatus* attached to the epithelial cell monolayer differed markedly from the smoky gray–green woolly–cottony textured colonies described when grown on potato dextrose agar. On microscopy the “cottony” foci of growth from agar slopes displayed a dense clump of branching hyphae radiating from a central clump of spores with no matrix produced around them. In contrast, the biofilm form of growth described in the aforementioned study was a coordinated network of hyphae spreading in all directions, in between the host cells, cross-linking with each other to form a parallel-packed matrix. This led to the formation of a structure of high stability and protection with an intricate network of channels and passages. ECM was observed between and surrounding the hyphae and stained intense green by ConA. To date, evidence supporting the presence of such fungal biofilms in CRS patients is lacking. Universal fluorescent *in situ* hybridization probes<sup>6,7</sup> are sensitive tools in identifying nonspecific attached fungal elements rather than embedded biofilms containing the possible pathogenic fungi. We have been able to apply the aforementioned *in vitro* model to the standardized sheep model for CRS in

**Figure 3.** (A–C) *Aspergillus fumigatus* biofilm on a monolayer of sinonasal epithelial cells formed under continuous flow conditions (panel A, 20× magnification; panels B and C, 200× magnification). Orange-red fluorescence is caused by FUN-1 stain forming cylindrical intravacuolar structure (CIVS) in metabolically active fungal cells. Intense green fluorescence is caused by ConA stain binding to fungal cell wall polysaccharides and extracellular matrix (ECM). (D–G) Comparison of an *A. fumigatus* biofilm formed under (D and E) flow conditions and (F and G) static conditions, after (D and F) 2 days of incubation, after (E and G) 4 days of incubation.



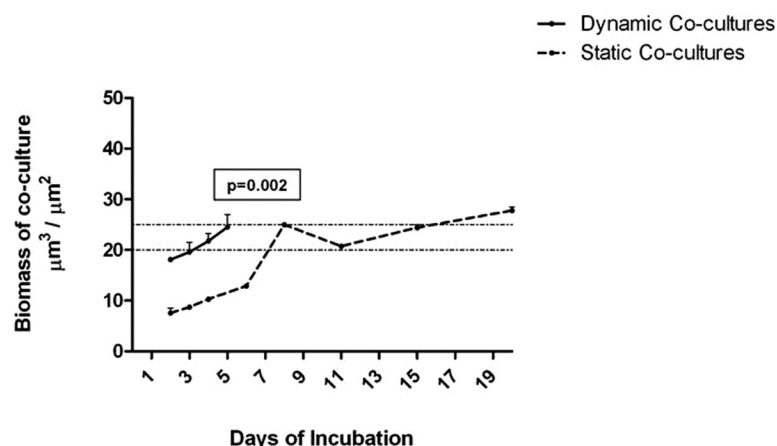
our department and shown the formation of *A. fumigatus* biofilms in sheep frontal sinuses.<sup>23</sup> We are presently conducting studies to identify similar *A. fumigatus* biofilms in CRS patients to better understand the role of fungi in CRS.

*A. fumigatus* biofilm on the monolayer of sinonasal epithelial cells was seen to form structurally and developmentally similar to those described in other *Aspergillus* biofilm models.<sup>12–14</sup> The spores of *A. fumigatus* first attached to the surface of the cells followed sequentially by colonization *via* hyphal proliferation, ECM production, and parallel and cross-linked hyphal arrangement, finally forming pores and internal channels. As described by Mowat<sup>13</sup> the seeding density of the fungal spores on the primary cell culture was the critical factor in defining the form of growth, with the biofilm forming by overlaying the cells with conidia at a concentration of  $1 \times 10^5$ /mL of PBS. The mature multilayered *A. fumigatus* biofilm formed a thick mat of hyphae and cells, which appeared to be bound by a thick gel-like substance that became gradually very impermeable to culture media and staining agents. *In vivo* this ECM may act as a physical barrier between the fungal cells and clinically used antifungal agents, thus leading to the ongoing colonization of fungi in the sinuses despite maximal treatment.

Loussert *et al.*<sup>24</sup> have recently described galactosaminogalactan and galactomannan as the major polysaccharides forming the ECM in *A. fumigatus* biofilms, which may be a possible target for antifungal treatments directed at *Aspergillus* biofilms.

Woodworth *et al.*<sup>25</sup> grew *Pseudomonas aeruginosa* biofilms supported on mouse nasal septal epithelium in air–liquid interfaces, to develop an *in vitro* model for trialing novel antibiofilm treatments for CRS. In the aforementioned study, we used primary cultures of sinonasal epithelial tissue taken from patients undergoing ESS, so that the substratum on which the biofilms grew was as similar as possible to those *in vivo* conditions in CRS patients. The CFCs were set up to mimic mucociliary clearance and to evaluate the formation of the fungal biofilm in conditions similar to the sinonasal physiology. As described in the results, not only was it possible for the *A. fumigatus* to grow in biofilm form in the dynamic environment, the fungal biofilms displayed much more robust and faster growth kinetics, as a result of continued replenishment of nutrients and waste removal. The hyphal complexity and thickness under the dynamic flow culture conditions is a possible response of the fungal biofilm to the shear stresses that it has to withstand as a result of the continuous surrounding fluid media movement. *Can-*

## Biomass of static vs continuous-flow co-cultures



## Thickness of static vs continuous-flow cocultures

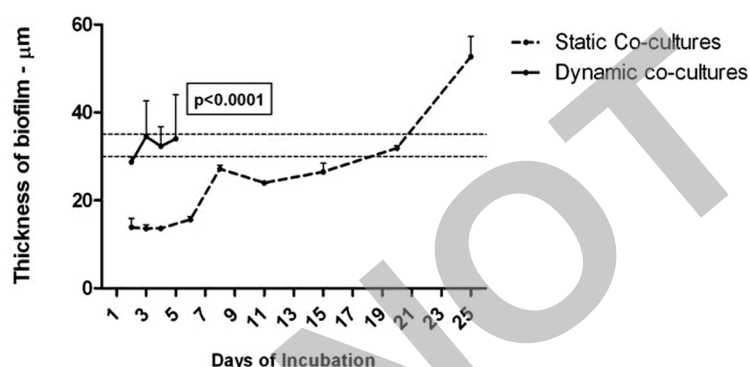


Figure 4. Biomass and average thickness of static and flow cocultures.

*didiasis albicans* biofilm formed in a simple flow biofilm model has also been shown to grow rapidly, form twofold thicker biofilms with extremely compact and wrinkled surfaces, and increased architectural complexity compared with statically grown biofilms.<sup>26</sup>

It has been suggested that the symbiotic interactions of bacteria and fungi augment biofilm survival by assisting surface adherence and improving the protection provided by the EPS matrix.<sup>27,28</sup> Some researchers have described the coexistence of fungal elements and bacterial biofilms in CRS patients<sup>6,7</sup> and in the sheep model for CRS<sup>23</sup> we have found a synergism between *Staphylococcus aureus* infection and *A. fumigatus* biofilm formation. Thus, the possible existence of bacteria and fungi in biofilm form in CRS patients would also indicate a symbiotic coexistence, better protected from the host defenses and antimicrobial therapies, thus playing a key role in CRS pathogenesis. Villena *et al.*<sup>12</sup> found that *A. niger* biofilm forms were more productive and metabolically efficient entities as their specific lignocellulolytic enzyme activities (endoglucanase, glucosidase, *etc.*) were fivefold higher than submerged cultures, showing highly coordinated enzyme synthesis. *In vitro* studies have shown *A. fumigatus* cultures can release gliotoxin and other uncharacterized high-molecular weight factors, which if produced in a sufficient quantity *in vivo*, can contribute to airway pathology by damaging epithelial cells and reducing ciliary beat frequency.<sup>29</sup> The biofilm form of *A. fumigatus* can be a possible efficient source of these factors in the sinonasal tissues contributing toward pathogenesis of CRS.

*A. fumigatus* secretes proteins after conidia have germinated into hyphae, which function as antigens, evoking both a protective (TH1-TH17) or a destructive allergic (TH2) immune response.<sup>30</sup> How these currently identified *Aspergillus* allergens (Asp f proteins) participate in the development of the differential immune processes remains unknown. Studies on antigenic and protein expression of fungi-like *Candida*<sup>31,32</sup> have shown differential protein expression in fungal biofilm forms when compared with planktonic forms. The biofilm form of *A. fumigatus* may thus prove to be the still unidentified source of the differential antigen expression favoring the initiation of protective/allergic immune processes. *A. fumigatus* biofilms were formed both on CRS and on non-CRS primary epithelial cell cultures under *in vitro* conditions, indicating that the immune system may possibly play a significant role *in vivo* in determining whether the ubiquitous spores are eliminated or established as biofilms within the sinuses *via* its still unclear interplay between the preferentially expressed antigens.

## CONCLUSION

The present study shows that *A. fumigatus* can form biofilms on primary of sinonasal epithelial cells, with highly organized three-dimensional hyphal structures with a more robust biofilm growth under continually replenishing media conditions. additional studies are required to establish the *in vivo* growth of *A. fumigatus* in biofilm form and to investigate its possible role in sinonasal inflammation, allergy, and infection in CRS patients.

## REFERENCES

- Cohen MK, Nayak J, Palmer JV, et al. Biofilms in chronic rhinosinusitis: A review. *Am J Rhinol Allergy* 23:255–260, 2009.
- Harvey RJ, and Lund VJ. Biofilms and chronic rhinosinusitis: Systematic review of evidence, current concepts and directions for research. *Rhinology* 45:3–13, 2007.
- Palmer J. Bacterial biofilms in chronic rhinosinusitis. *Ann Otolaryngol Rhinol Laryngol Suppl* 196:35–39, 2006.
- Ramadan HH. Chronic rhinosinusitis and bacterial biofilms. *Curr Opin Otolaryngol Head Neck Surg* 14:183–186, 2006.
- Vlastarakos PV, Nikolopoulos TP, Maragoudakis P, et al. Biofilms in ear, nose, and throat infections: How important are they? *Laryngoscope* 117:668–673, 2007.
- Healy DY, Leid JG, Sanderson AR, and Hunsaker DH. Biofilms with fungi in chronic rhinosinusitis. *Otolaryngol Head Neck Surg* 138:641–647, 2008.
- Foreman A, Psaltis AJ, Tan LW, and Wormald PJ. Characterization of bacterial and fungal biofilms in chronic rhinosinusitis. *Am J Rhinol Allergy* 23:556–561, 2009.
- Ramage G, Mowat E, Jones B, et al. Our current understanding of fungal biofilms. *Crit Rev Microbiol* 35:340–355, 2009.
- Chandra J, Zhou G, and Ghannoum MA. Fungal biofilms and antimicrobials. *Curr Drug Targets* 6:887–894, 2005.
- Mukherjee PK, and Chandra J. *Candida* biofilm resistance. *Drug Resist Updat* 7:301–309, 2004.
- Seneviratne CJ, Jin L, and Samaranayake LP. Biofilm lifestyle of *Candida*: A mini review. *Oral Dis* 14:582–590, 2008.
- Villena GK, and Gutiérrez-Correa M. Morphological patterns of *Aspergillus niger* biofilms and pellets related to lignocellulolytic enzyme productivities. *Lett Appl Microbiol* 45:231–237, 2007.
- Mowat E, Butcher J, Lang S, et al. Development of a simple model for studying the effects of antifungal agents on multicellular communities of *Aspergillus fumigatus*. *J Med Microbiol* 56:1205–1212, 2007.
- Seidler MJ, Salvenmoser S, and Müller FMC. *Aspergillus fumigatus* forms biofilms with reduced antifungal drug susceptibility on bronchial epithelial cells. *Antimicrob Agents Chemother* 52:4130–4136, 2008.
- Meltzer EO, Hamilos DL, Hadley JA, et al. Rhinosinusitis: Establishing definitions for clinical research and patient care. *Otolaryngol Head Neck Surg* 131(suppl):S1–S62, 2004.
- Heydorn A, Nielsen AT, Hentzer M, et al. Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* 146:2395–2407, 2000.
- Heydorn A, Ersbll BK, Hentzer M, et al. Experimental reproducibility in flow-chamber biofilms. *Microbiology* 146:2409–2415, 2000.
- COMSTAT Software. Available online at [www.comstat.dk](http://www.comstat.dk); last accessed May 2011.
- Deshazo RD, O'Brien M, and Chapin K. A new classification and diagnostic criteria for invasive fungal sinusitis. *Arch Otolaryngol Head Neck Surg* 123:1181–1188, 1997.
- Ebbens FA, Georgalas C, and Fokkens WJ. The mold conundrum in chronic hyperplastic sinusitis. *Curr Allergy Asthma Rep* 9:114–120, 2009.
- Ebbens FA, Georgalas C, and Fokkens WJ. Fungus as the cause of chronic rhinosinusitis: The case remains unproven. *Curr Opin Otolaryngol Head Neck Surg* 17:43–49, 2009.
- Fokkens WJ, Ebbens F, and van Drunen CM. Fungus: A role in pathophysiology of chronic rhinosinusitis, disease modifier, a treatment target, or a no role at all? *Immunol Allergy Clin North Am* 29:677–688, 2009.
- Boase S, Valentine R, Singhal D, et al. A sheep model to investigate the role of fungal biofilms in sinusitis: Fungal & bacterial synergy. In press.
- Loussert C, Schmitt C, Prevost MC, et al. In vivo biofilm composition of *Aspergillus fumigatus*. *Cell Microbiol* 12:405–410, 2010.
- Woodworth BA, Tamashiro E, Bhargava G, et al. An in vitro model of *Pseudomonas aeruginosa* biofilms on viable airway epithelial cell monolayers. *Am J Rhinol* 22:235–238, 2008.
- Uppuluri P, Chaturvedi AK, and Lopez-Ribot JL. Design of a simple model of *Candida albicans* biofilms formed under conditions of flow: Development, architecture, and drug resistance. *Mycopathologia* 168:101–109, 2009.
- Seneviratne G, Zavahir JS, Bandara WMM, et al. Fungal-bacterial biofilms: Their development for novel biotechnological applications. *World J Microbiotechnol* 24:739–743, 2008.
- Wargo MJ, and Hogan DA. Fungal-bacterial interactions: A mixed bag of mingling microbes. *Curr Opin Microbiol* 9:359–364, 2006.
- Amitani R, et al. *Aspergillus* culture filtrates and sputum sols from patients with pulmonary aspergillosis cause damage to human respiratory ciliated epithelium in vitro. *Eur Respir J* 8:1681–1687, 1995.
- Chaudhary N, Staab JF, and Marr KA. Healthy human T-cell responses to *Aspergillus fumigatus* antigens. *PLoS One* 5(2):e9036. doi: 10.1371/journal.pone.0009036.
- Jain N, and Fries BC. Antigenic and phenotypic variations in fungi. *Cell Microbiol* 11:1716–1723, 2009.
- Thomas DP, Bachmann SP, and Lopez-Ribot JL. Proteomics for the analysis of the *Candida albicans* biofilm lifestyle. *Proteomics* 6:5795–5804, 2006. □

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.