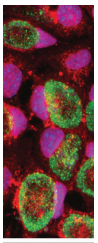

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RESEARCH ARTICLE

Increased production of gliotoxin is related to the formation of biofilm by *Aspergillus fumigatus*: an immunological approach

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Aspergillus fumigatus can form a major health problem in immunocompromised patients. Accurate detection can strongly impact on the outcome of such an infection. In this paper, an antibody against gliotoxin, produced by *A. fumigatus*, was obtained, and it was shown that it holds promise for the diagnosis of *A. fumigatus* infection in the human host.

Keywords
Aspergillus fumigatus; biofilm; gliotoxin; immunofluorescent assay; fungal biofilm-associated infection.

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Abstract

Gliotoxin (GT) belongs to the epipolythiodioxopiperazine class of toxins secreted from certain fungi including *Aspergillus fumigatus*, which is the most prolific producer of this secondary metabolite. Recently, enhanced amounts of GT were found in *in vitro* biofilm-grown *A. fumigatus* mycelium. To further correlate the *A. fumigatus* biofilm growth phenotype with the enhanced secretion of GT, a polyclonal antibody (pAb) was produced by immunizing mice against GT. By an indirect immunofluorescent assay, pAb was then able to recognize specifically GT onto *A. fumigatus* Af293 biofilm formed on human pulmonary epithelial cells. Then, treating Af293 biofilms with a compound which reduces the GT disulfide bonds provoked shutdown of the GT-specific immunofluorescence (IF) signals along the hyphae. To explore the potential of GT for diagnostic use, pAb was shown to react with GT on hyphae into *Aspergillus* culture-positive respiratory tract specimens from patients with probable invasive aspergillosis (IA) and into tissue specimens from the lungs of patients with proven IA. As the presence of fungal hyphae in clinical specimens strongly indicates the *in vivo A. fumigatus* growth as a biofilm, anti-GT antibodies could be a specific and sensitive diagnostic tool for detecting *A. fumigatus* biofilm-associated clinical infections.

Introduction

Aspergillus fumigatus is an ubiquitous, saprotrophic mold that is considered the most important air-borne fungal pathogen of humans (Dagenais & Keller, 2009), as a result of its abundant production of small conidia which are continuously inhaled but normally eliminated by the innate immune response (Brakhage *et al.*, 2011). Depending on the host immune status, *A. fumigatus*-related diseases range from allergic reactions and chronic colonization with scarce invasiveness (e.g., aspergilloma) to systemic infections (e.g., invasive pulmonary aspergillosis) with high mortality rates (Thompson & Patterson, 2011).

Both localized and invasive forms of aspergillosis are characterized by the mycelial development of *A. fumigatus* from inhaled conidia (Loussert *et al.*, 2010; Müller *et al.*, 2011), that parallels the well-established behavior of the fungus to form biofilm *in vitro* (Mowat *et al.*, 2007, 2008; Seidler *et al.*, 2008). The *in vivo* biofilm growth, consisting of an intricate hyphal network surrounded by an extracellular

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matrix (ECM; Beauvais *et al.*, 2007), may account for the *A. fumigatus* resistance against phagocytic and antimicrobial attacks (Ramage *et al.*, 2012). The ECM, that makes biofilms the most successful forms of microbial life on earth (Flemming & Wingender, 2010), is in *A. fumigatus* composed of galactomannan, α-1,3-glucans, monosaccharydes, polyols, melanin, and proteins, including major antigens and hydrophobins (Beauvais *et al.*, 2007). Of note, gliotoxin (GT), the major and the most potent toxin produced by *A. fumigatus* (Kwon-Chung & Sugui, 2009), was seen to increase in *A. fumigatus* cultures grown as biofilm, thus implying a potentially significant role for this metabolite under *in vivo* conditions (Bruns *et al.*, 2010).

Clinical biofilm-associated infections are difficult to diagnose (and treat), and this has prompted the search for new diagnostic targets, which exploit the existing differences between planktonic and sessile microbial cells (Hall-Stoodley *et al.*, 2012), as well as for new antifungal therapies (Bugli *et al.*, 2013). While advanced molecular *in situ* or imaging techniques may be effective to demonstrate biofilms *in vivo*, older immunohistochemical or immunofluorescent techniques using specific polyclonal or monoclonal sera could still represent a useful targeted tool to identifying pathogens in host tissues. However, use of these antibodies is often limited by that they are thought to bind to the ECM nonspecifically and by almost overall lack of commercially available antibodies specific for fungal pathogens (Loussert *et al.*, 2010; Hall-Stoodley *et al.*, 2012).

In this study, we generated an anti-GT mouse polyclonal antibody (pAb) to develop an immunofluorescent assay that allowed to elucidate the relationship between *A. fumigatus* biofilm growth phenotype and GT production. This assay proved to detect GT not only on hyphae cultured onto the human lung epithelial cell line A549, but also, directly, on hyphae into respiratory tract specimens or lung tissues from patients with invasive aspergillosis (IA). As the presence of fungal hyphae in clinical specimens strongly indicates the *in vivo A. fumigatus* growth as a biofilm, anti-GT antibodies were envisaged as a specific and sensitive diagnostic tool for detecting *A. fumigatus* biofilm-associated clinical infections.

Materials and methods

Reagents and cells

GT (purity: ≥ 98.0%) was purchased from Sigma–Aldrich (Milan, Italy), and a stock solution was freshly prepared as appropriate. The human lung adenocarcinoma epithelial cell line A549 was from European Collection of Cell Cultures (Wiltshire, UK) and was cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Milan, Italy), supplemented with 10% fetal calf serum (FCS; Invitrogen).

Human samples

Sputum and bronchoalveolar lavage (BAL) fluid samples were obtained as part of the routine microbiological and/or histological examination at the Services of Microbiology and Pathology of the Università Cattolica del Sacro Cuore, Rome, Italy. All samples were from patients with hematological malignancies, who were diagnosed with probable or proven IA, according to the revised European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) case definitions (De Pauw *et al.*, 2008). Part of the samples underwent routine microbiological cultures, and fungal isolates were identified at the species level by morphological and molecular methods (De Hoog *et al.*, 2000; Balajee *et al.*, 2009). For microscopic examination, a smear from each sample was made and subjected to a Gomori–Grocott silver stain to detect hyphal elements. Septated hyphae exhibiting angular dichotomous branching were regarded as suggestive for *Aspergillus* species. Additionally, histological samples were taken at the autopsy from the lungs of patients who died for IA. The tissue samples were fixed in phosphate-buffered saline (PBS) with 4% paraformaldehyde, then embedded in paraffin, sectioned (4 μm), and mounted on glass slides, and subsequently investigated by the periodic acid-Schiff (PAS) and the Gomori–Grocott silver staining. Control tissue samples were also obtained from patients with fatal cases that showed no histopathological signs of IA on autopsy; one of them was diagnosed with pulmonary fusariosis, and the other one with a disseminated form of zygomycosis involving lungs and other organs.

Fungal strain and sessile growth conditions

The *A. fumigatus* Af293 (ATCC MYA-4609, CBS 101355)-type strain was used throughout this study, after it was retrieved from a frozen glycerol stock, plated on Sabouraud's agar (SDA: Kima, Padua, Italy), and incubated at 37 °C in the presence of 5% CO_2 until sporulation. Conidia were harvested, suspended in 0.025% Tween-20 (Sigma–Aldrich) solution, washed twice in PBS (Sigma–Aldrich), and finally resuspended in PBS. The conidial inoculum (1×10^5 cells mL^{-1}) was dispensed into polystyrene, flat-bottom, 24-well microtiter plates (Thermo Scientific, Milan, Italy), and grown statically for 48 h at 37 °C in DMEM-containing FCS. Serum was added to the culture medium because it is present *in vivo* and promotes the growth and the formation of biofilm by *A. fumigatus* (Seidler *et al.*, 2008; Toyotome *et al.*, 2012). For comparison, conidia were inoculated to another microtiter plate in DMEM without FCS and were used as a control. After biofilm formation, the medium was aspirated, and the plates were washed in PBS by repeated pipetting to remove planktonic and/or nonadherent cells. Biofilm architectures on the wells were observed by optical microscopy at magnifications of 10 × and 40 × (data not shown). For absolute quantification, biofilm biomass was assessed as described elsewhere (Mowat *et al.*, 2007). Briefly, biofilms were stained with 0.5% crystal violet solution for 5 min and were then rinsed with distilled water. The dye bound was extracted with 200 μL of 95% ethanol and was quantified by measuring the $A_{490\ nm}$ with a microtiter plate reader (Bio–Rad Laboratories, Hercules, CA) to determine the level of staining which is proportional to the amount of biological material produced

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(Mowat *et al.*, 2007; data not shown). Biofilm formation was confirmed by immunoassay analysis using the anti-galactomannan monoclonal antibody EBA2 (Platelia™ *Aspergillus* Galactomannan EIA: BioRad, Marnes La Coquette, France) that showed a positive labeling of the hyphal surface (data not shown).

Biofilm formation on human pulmonary epithelial cells

Af293 biofilm was formed on A549 cells using an *in vitro* coculture model as described elsewhere (Seidler *et al.*, 2008). Briefly, the cells were grown to confluence on 13-mm-diameter glass coverslips (Bioscience Tools, San Diego, CA) placed into a standard 24-well cell culture plate (Thermo Scientific) in two different media, DMEM with 10% FCS (the medium for biofilm formation) and DMEM with 10% PBS (the control medium for nonbiofilm growth). The Af293 conidial suspension prepared as described above was added to the cells and incubated at 37 °C under 5% CO_2 for 2 h. After removal of nonadherent conidia, fresh DMEM (containing FCS or PBS) was added and cell monolayers were incubated for up to 48 h to allow the fungus to produce biofilm. Then, the culture medium was aspirated, collected in sterile Eppendorf tubes, and stored until to use for high-performance liquid chromatography (HPLC) analysis, whereas coverslips were removed and used for indirect immunofluorescence (IF) assays (see below).

Measurement of GT by HPLC analysis

To measure GT produced by biofilm-growing Af293 cocultivated with A549 cells, supernatants of cell cultures were subjected to solid-phase extraction, by loading 1 mL of each spiked sample solution onto an Oasis® HLB cartridge (Waters Corporation, Milford, MA) which had previously been conditioned with 2 mL of methanol–water mixture (1 : 1), according to the manufacturer's instructions. The cartridge was then washed with 1 mL of 5% methanol in water, and the analyte was eluted with 1 mL absolute methanol. For HPLC measurements, the extract was evaporated to dryness under a flow of N_2 at 50 °C, the residue was redissolved in 0.2 mL of methanol and used for the GT analysis essentially as described elsewhere (Boudra & Morgavi, 2005). Briefly, samples were measured on a Jasco HPLC system equipped with a UV-Visible detector ($\lambda = 270$ nm) using a ReproSil-Pur C18-AQ column (100 × 4.6 mm, 5 μm: Dr. Maish HPLC GmbH, Ammerbuch-Entringen, Germany). Twenty microliter of sample were manually injected, using the eluents A: H_2O, 0.1% trifluoroacetic acid and B: acetonitrile (ACN) and a flow rate of 1 mL min^{-1}, at the start of a gradient solvent program: 30 min 10–50% ACN, 2 min to 90% ACN, 4 min at 90% ACN, 1 min to 10% ACN, and held at 10% ACN. GT eluted from the HPLC column after 20 min as revealed by the injection of pure GT powder (see above). GT concentrations were determined by interpolation from a calibration curve (25–1000 ng mL^{-1}) prepared using the GT powder.

Production and characterization of GT-specific mouse pAb

Prior to immunization, GT (2 mg mL^{-1} in dimethyl sulfoxide) was reacted with bovine serum albumin (BSA) and thyroglobulin (TG), respectively, to obtain GT/BSA and GT/TG conjugates, by a procedure described elsewhere (Fox *et al.*, 2004). Female BALB/c mice (6–8 weeks old; 25–30 g: Harlan Italy, San Pietro al Natisone, Italy) were housed at the Unit for Laboratory Animal Medicine of the Università Cattolica del Sacro Cuore and submitted to an experimental protocol under approval by the Institutional Animal Use Committee. A total of six female BALB/c mice (three per group) were first immunized subcutaneously (200 μL per dose) with 10 μg each of GT/BSA or GT/TG conjugate emulsified in an equal volume of complete Freund's adjuvant. Mice were boosted three times at three-week intervals, and finally were challenged intraperitoneally with 5-μg injections of both conjugates (one per group) mixed 1 : 1 with incomplete Freund's adjuvant. Three weeks after the last immunization, sera were prepared from blood taken from the animals and were tested using indirect enzyme-linked immunosorbent assay (ELISA) as described below. After visualization on a SDS-8% polyacrylamide gel, conjugates were electro-transferred, then subjected to immunoblot analysis using 1 : 2500 diluted anti-GT antiserum in PBST (PBS containing 0.05% Tween-20) with 0.5% skim milk at 37 °C for 2 h. The blots were washed with PBST, and an alkaline phosphatase-conjugated goat anti-mouse (FC-specific) IgG (Sigma–Aldrich) was used as a secondary antibody at room temperature for 1 h. After washing, bands were visualized using an BCIP/NBT liquid substrate (Sigma–Aldrich) according to the manufacturer's instructions. For ELISA, flat-bottom 96-well microtiter plates (Thermo Scientific) were coated with 10 μg mL^{-1} of GT/BSA or GT/TG in PBS overnight at 4 °C. Following removal of the conjugate, 100 μL of BSA (10 mg mL^{-1}) in PBS were added and plates were incubated for 1 h at room temperature before washing two times with PBST. Serial dilutions (from 1 : 200 to 1 : 64 000) of sera obtained from mice immunized with GT/BSA or GT/TG were added (100 μL in PBST) to wells and incubated in a humidified atmosphere for 2 h at 37 °C. After washing, bound antibody was detected using a horseradish peroxidase-conjugated goat anti-mouse IgG antibodies in conjunction with the water-soluble enzyme substrate o-phenylenediamine (Millipore, Milan, Italy). The reaction was stopped by the addition of 1 N H_2SO_4. Absorbance was read at 450 nm using the microtiter plate reader. In the case of competitive ELISA, GT conjugates and free GT were individually diluted (0–60 μg mL^{-1}) and were added separately to the aforementioned microtiter plates with appropriate dilutions of anti-GT pAbs (Fox *et al.*, 2004). The percent inhibition was calculated using the following formula: percent inhibition = 100 × [(OD with inhibitor − OD without inhibitor)/OD without inhibitor]. Inhibition vs. concentration curves were plotted for each inhibitor and used to calculate the IC_{50} (50% inhibitory concentration) values. All results are representative of two separate experiments.

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Detection of GT by indirect IF microscopy

A549 cells, grown on glass coverslips in 24-well plates and cocultivated with *A. fumigatus* Af293 as described above, were washed with PBS, fixed with 4% paraformaldehyde for 5 min, and blocked for 30 min with 1% BSA in PBS. Monolayers were stained using the anti-GT pAb (which was generated against GT/BSA) diluted 1 : 500 in PBS-0.5% BSA for 2 h at 37 °C. After washing with PBS, bound antibodies were detected by incubation for 1 h with fluorescein isothiocyanate (FITC)-conjugated secondary anti--mouse IgG (Sigma–Aldrich) diluted 1 : 200 in PBS-0.5% BSA. After the cells were washed with PBS, coverslips were mounted on glass slides and observed on a Zeiss Axiophot (Jena, Germany) fluorescence microscope under 100 × magnification. Similarly, glass coverslips with Af293 biofilms, allowed to form on as described above, were microscopically analyzed following incubation for 2 h in RPMI with or without 200 μM dithiothreitol (DTT: Sigma–Aldrich), which reduces the internal disulfide bond of GT (Speth *et al.*, 2011). Indirect IF studies were also performed on the aforementioned human clinical samples. Before microscopy examination, fluid samples (i.e., sputum and BAL) were directly spotted on glass slides and heat-fixed, whereas tissue samples were deparaffinized using 2 × 3 min xylene, 1 × 3 min xylene-absolute ethanol mixture (1 : 1), 1 × 3 min 95% ethanol, 1 × 3 min 70% ethanol, 1 × 3 min 50% ethanol, and 3 × 3 min washes in sterile water. The specificity of the anti-GT pAb was verified by comparing staining intensities of tissue samples from patients with IA to those from patients without *A. fumigatus* infection.

Results

GT-specific pAbs generated in mice

GT has been detected and quantified in human serum and other clinical specimens from patients at risk of IA (Lewis *et al.*, 2005a; Domingo *et al.*, 2012), as well as in culture filtrates of clinical *A. fumigatus* isolates (Lewis *et al.*, 2005b; Kupfahl *et al.*, 2008). However, under static conditions, the mycelial growth is greater than in shaken, submerged conditions (Beauvais *et al.*, 2007), and this seems to affect the ECM composition with respect to proteins like surface hydrophobins or metabolites (Bruns *et al.*, 2010). To investigate the possibility of detecting GT directly on *A. fumigatus* mycelium grown as a biofilm, an anti-GT mouse pAb was generated using a previously developed strategy (Fox *et al.*, 2004). By this, GT was not utilized as an immunogen *per se*, but two GT/carrier protein conjugates, as obtained by coupling GT with BSA or TG, respectively, were synthesized (Fig. 1a) to enhance the immune response against the GT moieties and then to facilitate toxin immobilization on ELISA microtiter plates (Fox *et al.*, 2004). As it was expected, both pAb preparations reacted specifically with the corresponding GT conjugates by immunoblot analysis, resulting in respective bands of ~67 and ~170 kDa, whereas no reactive bands were observed with unconjugated BSA and TG (Fig. 1b).

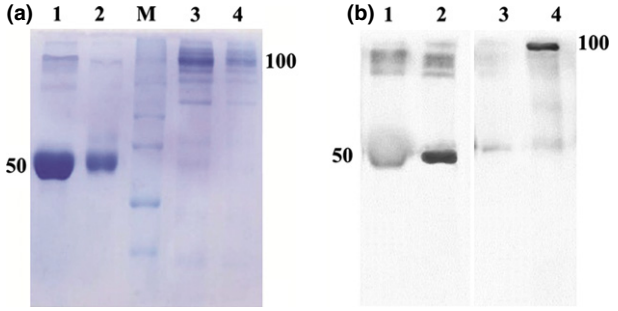

Fig. 1 SDS-polyacrylamide gel electrophoresis and immunoblot analyses of conjugates (2 μg per lane), as obtained by coupling GT with carrier proteins, such as BSA and TG. (a) GT/BSA (lane 2) and GT/TG (lane 4) conjugates were run with the unconjugated BSA (lane 1) and TG (lane 3), and their sizes were approximately determined using a molecular weight marker (lane M). (b) After electro-transferring, GT/BSA (lane 2) and GT/TG (lane 4) conjugates were visualized by immunodetection analysis using a 1 : 2500 diluted GT-specific mouse antiserum as a primary antibody.

The reactivity of both antisera was compared by ELISAs showing that the pAb raised against GT/BSA exhibited slightly higher titers rather than pAb raised against GT/TG ($OD_{450\ nm}$ s at the 1 : 16 000 dilution was 0.75 and 0.60, respectively; Fig. 2a and b). Notably, a great specificity was shown by both pAbs, as documented by the absence of binding to both unconjugated BSA and TG when the protein carriers were immobilized at concentrations as the same as used for the GT/conjugates. Thus, specificity of the pAb against GT/BSA was further assessed by competitive ELISAs. While free carrier did not interfere with antibody binding to the immobilized toxin conjugate, the addition of GT/BSA to an 1 : 16 000 dilution of anti-GT/BSA pAb fully inhibited antibody binding to the immobilized GT/BSA (10 μg mL^{-1}) with an IC_{50} value of 10 μg mL^{-1} (Fig. 2c). Also, to evaluate the inhibition of toxin conjugate antiserum by free toxin, GT was added to microtiter wells precoated with GT/BSA (10 μg mL^{-1}) after the toxin was pre-incubated with the antiserum for 12 h. This was because of the low avidity displayed by IgG for free GT as indicated by other authors (Fox *et al.*, 2004). As depicted in Fig. 2d, GT was capable of interfering with the antiserum (1 : 16 000) binding to immobilized GT/BSA in a concentration-dependent manner, by which 50% binding inhibition (IC_{50}) was achieved at 50 μg mL^{-1} of toxin. In both immunoblot and ELISAs, pre-immune mouse antisera did produce neither reactive bands nor binding against the immobilized GT/BSA or GT/TG conjugates. Consistent with previous studies (Fox *et al.*, 2004), these results show that a sensitive and specific antibody was raised in mice against the *A. fumigatus* mycotoxin.

pAb binds to GT on *A. fumigatus* hyphae cocultured with human lung epithelial cells

Preliminarily, IF microscopy was employed to demonstrate the capability of our pAb to specifically recognizing GT on the

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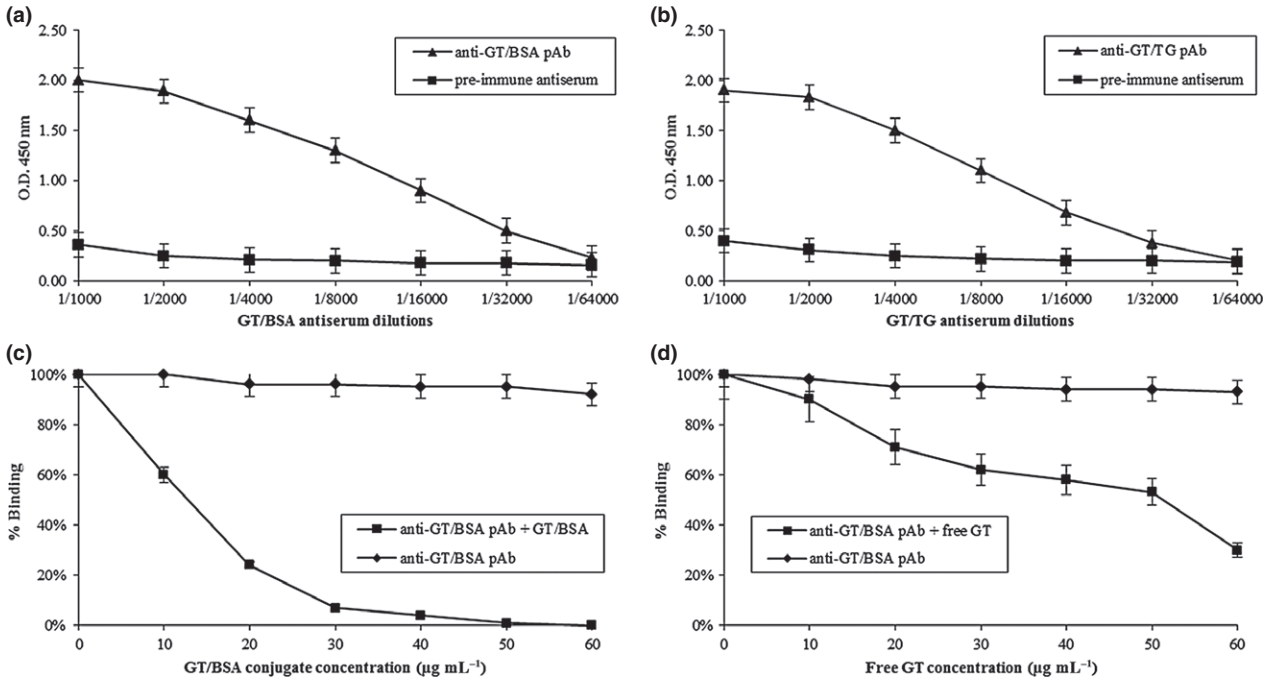

Fig. 2 Binding of mouse polyclonal antibodies (pAbs) to GT by indirect and competitive ELISA. (a) The reactivity of anti-GT/BSA pAb was determined against the immobilized GT/TG conjugate (5 μg mL^{-1}). (b) The reactivity of anti-GT/TG pAb was determined against the immobilized GT/BSA conjugate (5 μg mL^{-1}). The antibody titers in (a) and (b) are expressed as the highest reciprocal dilution of serum at which $OD_{450\ nm}$ was ≥ 0.2. (c) The inhibition profile for binding of anti-GT/BSA pAb (1 : 16 000) to immobilized GT/BSA (10 μg mL^{-1}) was obtained in the presence of GT/BSA conjugate by adding reactants (pAb and inhibitor) directly to coated microtiter wells. (d) The inhibition profile for binding of anti-GT/BSA pAb (1 : 16 000) to immobilized GT/BSA (10 μg mL^{-1}) was obtained in the presence of free GT by adding reactants (pAb and inhibitor) to coated microtiter wells after they were incubated together for 12 h. The inhibition vs. concentration curves in (c) and (d) were plotted for each inhibitor, and were used to calculate the IC_{50} values.

hyphae from *A. fumigatus* Af293 grown as a biofilm on polystyrene plates in the presence of FCS, as the binding was inhibited by the adding of free GT (data not shown). The immunolabeling intensity strongly diminished as the Af293 strain was cultured without FCS, supporting the concept that serum accelerates the *A. fumigatus* growth leading to development of a fungal community (Toyotome *et al.*, 2012). As the quantity of GT is related to the formation of a mature biofilm (Bruns *et al.*, 2010), it was expected that the absence of serum provoked shutdown of the GT-specific IF signal. To further evaluate the surface accessibility of GT, IF microscopy analyses were carried out with Af293 biofilms adherent to human lung epithelial cell monolayers. This *in vitro* coculture model has been established previously (Seidler *et al.*, 2008) to mimic the *in vivo* situation in *A. fumigatus* infections of the respiratory tract (Müller *et al.*, 2011), which generally are characterized by a spherical mass of hyphae or by bronchial casts containing mucus and mycelia (Ramage *et al.*, 2012). The mouse pAb, which bonds to GT-expressing hyphae, was thus added to the wells containing Af293 biofilm-coated slides after 2 days of coculturing in the presence or absence of FCS. After FITC-based immunofluorescent staining, Af293 cocultured in the FCS-containing medium produced tightly intertwined hyphae displaying an intense bright-green fluorescence (Fig. 3a), in contrast to Af293 cocultured in the medium without FCS where the hyphae displayed a low fluorescence level (Fig. 3b). Interestingly, no green-stained hyphae were observed after Af293 biofilm cocultures were incubated with the nonimmunized mouse serum (Fig. 3c) or the anti-GT pAb pre-adsorbed with GT (Fig. 3d) used both as a primary antibody, or directly with the FITC-conjugated secondary anti-mouse antibody (Fig. 3e). To confirm these findings, GT from the supernatant of cells cocultured for 48 h with biofilm-forming or non-biofilm-forming *A. fumigatus* was extracted and quantified by HPLC. In a representative experiment, GT concentration was estimated to be 9.66 μg mL^{-1} in the case of Af293 mycelium grown as a biofilm, while it was as far as 0.04 μg mL^{-1} when Af293 grew planktonically. Moreover, as shown in the Fig. 4, we were able to identify a peak that migrates the same distance as the pure GT, in addition to an unidentified peak that was supposed to be the inactive derivative bis(methylthio) GT (bmGT) of GT (Domingo *et al.*, 2012). Taken together, these data show that the production of GT by biofilm-growing *A. fumigatus* increased compared to the non-biofilm-growing fungus and that the extent of produced GT allowed to detect the *A. fumigatus* mycotoxin easily and reliably.

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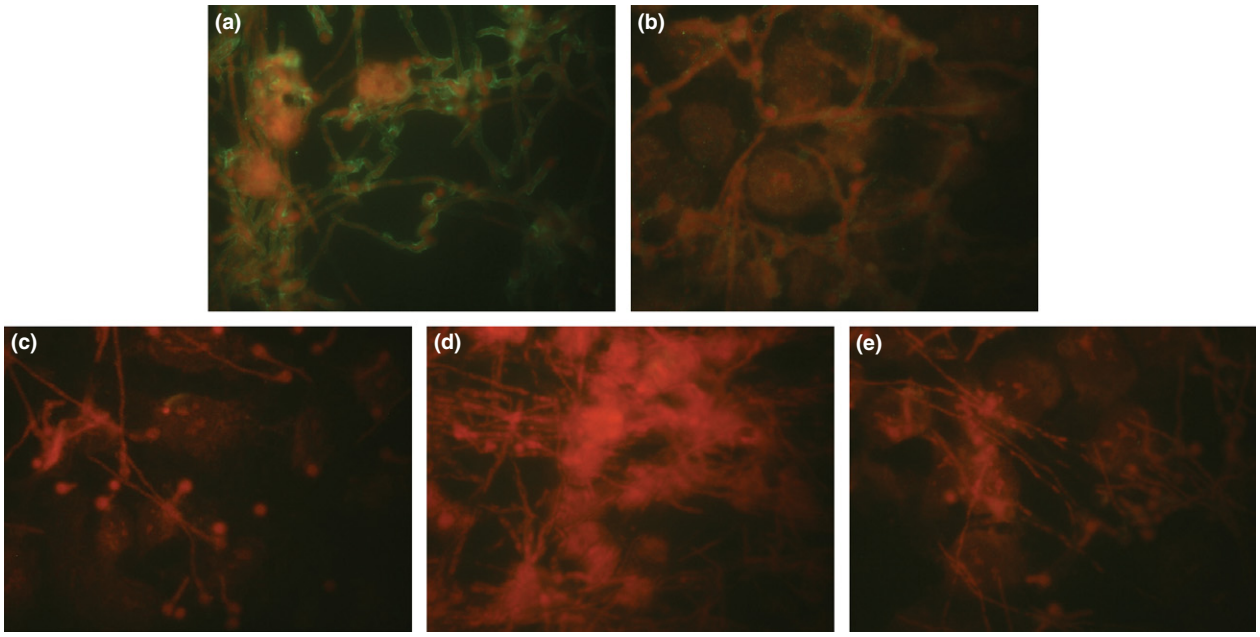

Fig. 3 Microscopic images of *Aspergillus fumigatus* Af293 biofilm-growing hyphae cocultured with human lung epithelial A549 cells, after immunofluorescent staining with the mouse GT-specific pAb as a primary antibody. (a) Intense fluorescence is visible over the surface of intertwined hyphae grown on the cell monolayer in FCS-containing culture medium. (b) Low fluorescence is visualized over the surface of hyphae grown for comparison in a non-FCS-containing culture medium (the control medium for nonbiofilm growth). The images in (a) and (b) are compared with fluorescent-negative controls, as obtained by incubation of the aforementioned cocultures with the nonimmunized mouse serum (c) and the mouse pAb pre-adsorbed with GT (d) as primary antibodies, or directly with the FITC-conjugated secondary anti-mouse antibody (e). The green color is due to the FITC-labeled antibody, whereas the dull red color is due to the Evans blue counterstain.

GT is localized on the hyphae outer surfaces

Mycotoxins, including GT, are originated and released by the fungal growth. To determine whether GT might be transiently bound to the external fungal cell wall before being released into the extracellular environment, we used the reducing compound DTT to treat biofilms of *A. fumigatus* Af293. DTT reduces GT disulfide bonds that are essential for its toxic effects (Speth *et al.*, 2011). The mouse pAb, which bonds to GT-expressing hyphae, was therefore added to the wells containing Af293 biofilm-coated slides after they were kept in the presence or absence of DTT. After FITC-based immunofluorescent staining, the DTT-treated biofilm displayed a low fluorescence level (Fig. 5a), in contrast to the intense fluorescence signal that was detectable along the hyphae from the untreated biofilm (Fig. 5b). These findings demonstrate that pAb was hampered to react against the reduced form of GT and confirm the accessibility of GT onto the hyphae outer surfaces.

pAb binds to GT on hyphal-positive infected specimens from patients with aspergillosis

Finally, we tested the capability of our pAb-based immunofluorescent assay to detect GT directly in fluid and tissue samples (for a total of 14 examined) from immunocompromised patients suffering from hematological malignancy or undergoing hematopoietic stem cell transplant. As specified above, they were classified as probable or proven IA according to EORTC/MSG guidelines (De Pauw *et al.*, 2008). While light-microscopy examination of a Gomori–Grocott silver stained smear from all 10 fluid (six BAL and four sputum) samples revealed hyphae compatible with *Aspergillus* species (data not shown), cultures from the same samples yielded *A. fumigatus* as a uniquely isolated fungal species. Therefore, the samples were investigated by IF microscopy for GT, and an example of IF assays is shown in Fig. 6. As for all the fluid samples analyzed, fluorescently brilliant-green hyphae were noticed (Fig. 6a), and this sharply contrasted with the lack of fluorescent signals in samples from control patients with no evidence of aspergillosis (Fig. 6b), that indeed tested culture negative for *Aspergillus* species and light-microscopy negative for fungal hyphae. Furthermore, an IF investigation of preserved lung tissues was carried out, using histological samples obtained postmortem from four patients who had deceased or had negative autopsy findings for IA. In the former two cases, patients had histopathological evidence of tissue invasion by septated, acutely branching hyphae, together with an *Aspergillus* positive culture result (De Pauw *et al.*, 2008). In the latter two cases, hyphae compatible with fungal species were seen in the tissues together with the isolation of *Fusarium* or *Mucor* species from commonly sterile body sites (De Pauw *et al.*, 2008). Thus, as exemplified in Fig. 6c, the IF microscopy for GT showed hyphae localized in all the pathological tissues examined that appeared brilliant-green

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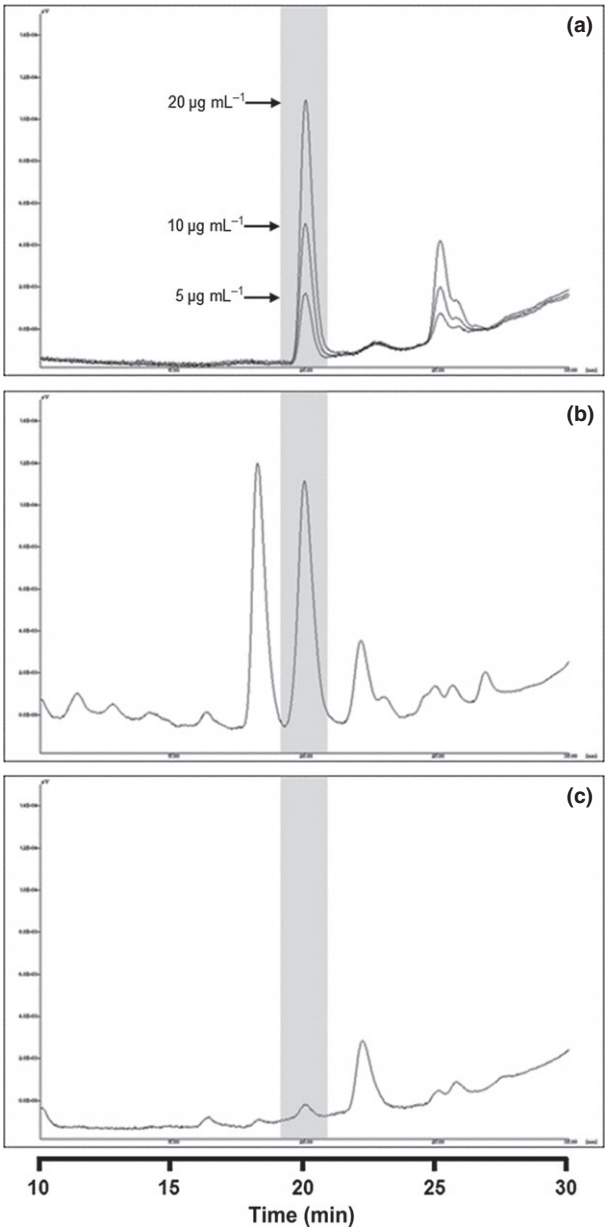

Fig. 4 Detection and quantification of GT by HPLC analysis. (a) Chromatograms of different concentrations of pure GT used as a standard, showing overlapping peaks (outer: 20 μg mL^{-1}; middle: 10 μg mL^{-1}; and inner: 5 μg mL^{-1}) with a retention time of 20 min (gray zone). (b) Chromatograms of GT extracted from supernatants of A549 cells cocultured for 48 h with biofilm-forming *Aspergillus fumigatus* Af293, showing a peak that migrates as the GT standard (gray zone). (c) Chromatograms of GT extracted from supernatants of A549 cells cocultured for 48 h with non-biofilm-forming *A. fumigatus* Af293, showing no apparent peak migrating as the GT standard (gray zone).

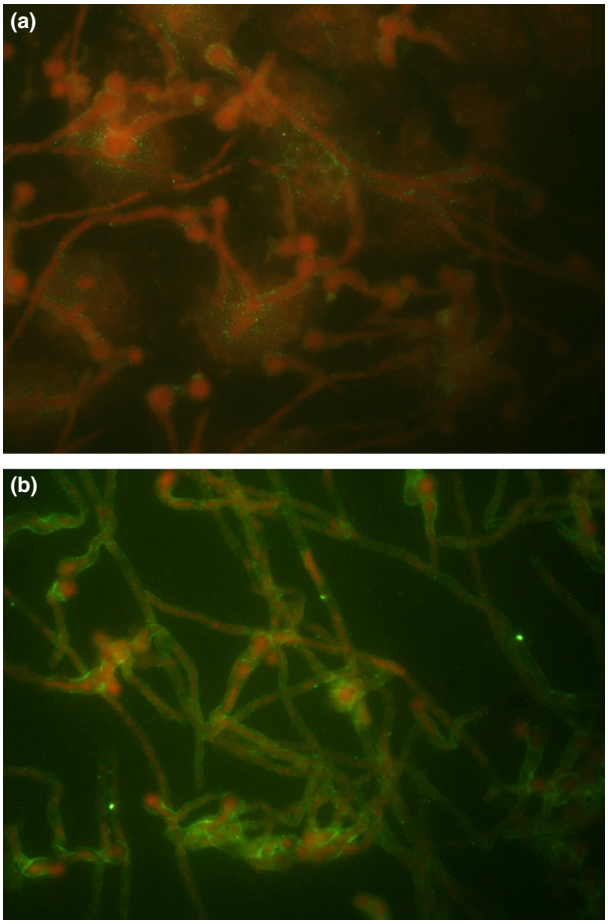

Fig. 5 Microscopic images of *Aspergillus fumigatus* Af293 biofilm-growing hyphae following incubation with (a) and without (b) 200 μM DTT, which reduces GT disulfide bonds that are essential for its toxic effects. After immunofluorescent staining with the mouse GT-specific pAb as a primary antibody, a very low-level fluorescence along the fungal hyphae was seen in (a), as opposed to the intense fluorescence seen in (b), indicating that the loss of GT activity consequent to the DTT-reducing action may hamper the specific binding of pAb to the *A. fumigatus* mycotoxin. The green color is due to the FITC-conjugated secondary anti-mouse antibody, whereas the dull red color is due to the Evans blue counterstain.

over a fully negative background, consistently with Gomori–Grocott silver positive hyphal elements observed at the light-microscopy evaluation in the same tissue samples (Fig. 7). Expectedly, negative controls consisting of tissue samples derived from the patients with fungal disease other than IA (i.e., fusariosis), an example of which is shown in Fig. 6d, revealed no fluorescent signals, in opposition to the strong positivity seen with traditional or fungus-specific (i.e., Gomori–Grocott silver) staining techniques for the same samples (data not shown). In summary, we demonstrated that the antibody-based GT detection assay may be a sensitive and specific targeted approach for *A. fumigatus* infections, including those related to fungal biofilms.

Discussion

Among the vast array of secondary metabolites produced by filamentous fungi (Palmer & Keller, 2010), GT is the best-known example of the epipolythiodioxopiperazine class

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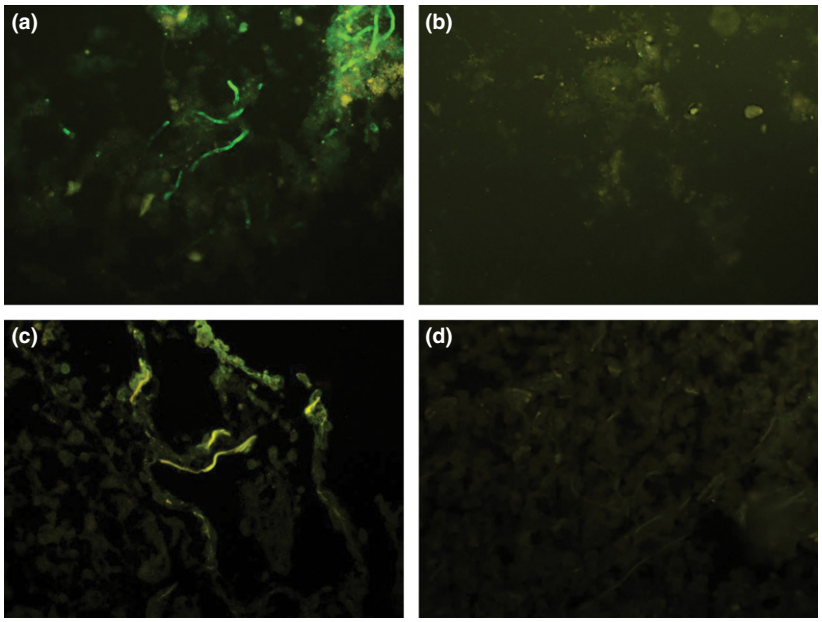

Fig. 6 (a) Microscopic image of an *Aspergillus* culture-positive BAL sample from a patient with IA, showing *Aspergillus fumigatus* hyphae after immunofluorescent staining with the mouse GT-specific pAb as a primary antibody. Intertwined hyphae appear brilliant green because of binding by an FITC-conjugated antibody against the pAb specific for GT. (b) Microscopic image of an *Aspergillus* culture-negative BAL sample from a patient with no IA, showing the complete absence of hyphae-associated fluorescent signals. (c) Microscopic image of a lung tissue from a patient with histologically documented IA, showing single hyphae that appear brilliant green because of GT-specific binding by the FITC-conjugated antibody. (d) Microscopic image of a lung tissue from a patient with histologically documented fusariosis, showing the complete absence of hyphae-associated fluorescent signals.

of toxins (Gardiner *et al.*, 2005) secreted from certain fungi, including *Aspergillus* species of which *A. fumigatus* is the most prolific maker (Lewis *et al.*, 2005b). The toxicity relies on the unusual intramolecular disulfide bridge, which can inactivate proteins via reaction with thiol groups (Scharf *et al.*, 2012), so that blockage of the disulfide bond by methylation leads to the formation of an inactive derivative known as bmGT (Domingo *et al.*, 2012). As a consequence of its negative immunomodulatory properties, GT has been the focus of intense scientific interest (Scharf *et al.*, 2012), but contradictory results were obtained from studies assessing the pathobiological role of the *A. fumigatus* toxin (Kwon-Chung & Sugui, 2009), likely related to the type of immunosuppressive regimen used to induce *A. fumigatus* infection in mice (Sugui *et al.*, 2007; Spikes *et al.*, 2008).

Nevertheless, there is evidence that GT is produced in the infected organs of patients with aspergillosis at a significant level (Kamei & Watanabe, 2005), as GT was detected in the lungs and sera of mice with experimental IA and in the sera of patients with cancer with IA (Lewis *et al.*, 2005a). Interestingly, GT concentrations in serum were substantially lower than the lung tissue concentrations (Lewis *et al.*, 2005a), according to the evidences that GT as well as bmGT are synthesized by *A. fumigatus* only in the hyphal stage (Dagenais & Keller, 2009) and that GT, in contrast to bmGT, is highly reactive and rapidly becomes cell associated (Waring *et al.*, 1994), thus decreasing its detectability in biological fluids (Domingo *et al.*, 2012). This may weaken the potential value of circulating GT as a diagnostic marker for IA, although clinically relevant specimens other than serum or plasma (Lewis *et al.*, 2005a; Domingo *et al.*, 2012) such as BAL fluid would need to be extensively investigated.

As already mentioned, genes encoding proteins involved in the biosynthesis of secondary metabolites were significantly upregulated in *A. fumigatus* grown *in vitro* at a maturation biofilm phase (Bruns *et al.*, 2010). *In vivo*, GT was recently seen to increase in *A. fumigatus*-positive BAL fluid of patients with cystic fibrosis (Coughlan *et al.*, 2012), of which lungs provide a natural environment favoring *A. fumigatus* colonization and biofilm formation (Ramage *et al.*, 2011). Consistently, BAL in some patients reveals the presence of numerous hyphae resembling a fungus ball (aspergilloma; Jayshree *et al.*, 2006), a localized infection that has typical biofilm characteristics (Loussert *et al.*, 2010), although intricate hyphal networks are also important in the pathogenesis of IA, which is normally typified by tissue invasion and angioinvasion (Thompson & Patterson, 2011), as in other forms of aspergillosis including the allergic bronchopulmonary aspergillosis (Mowat *et al.*, 2007). Thus, regardless from the clinical presentation, almost all these diseases share similar morphological features as a result of the *in vivo* development of *A. fumigatus* multicellular communities (Loussert *et al.*, 2010).

Given that GT is clearly associated with the filamentous growth of *A. fumigatus*, should its detection directly in clinical samples or *ex situ*, perhaps by means of a simple

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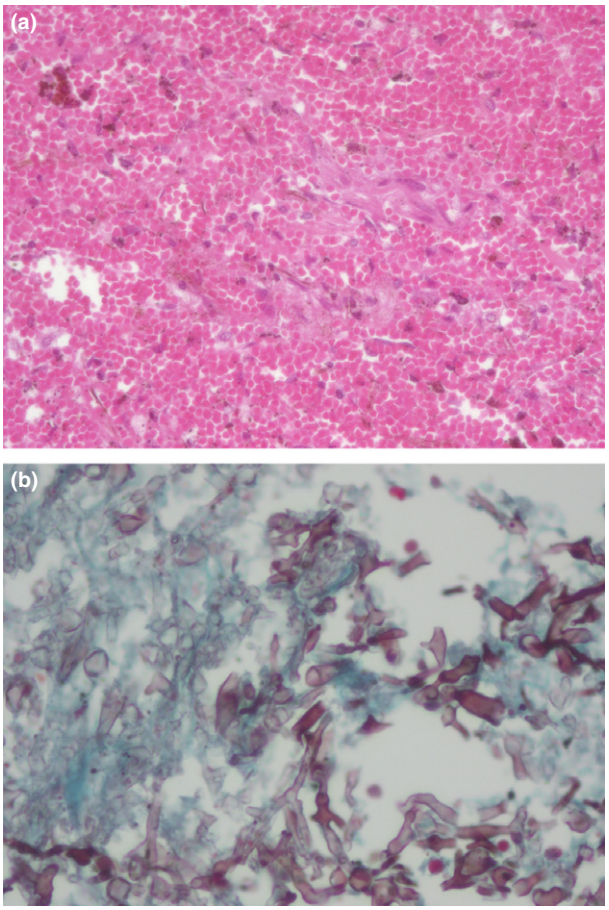

Fig. 7 Light-microscopy histological examination of sections of lung tissue samples obtained at the autopsy from a patient who had deceased for culture-proven invasive *Aspergillus fumigatus* infection. Septated, acutely branching hyphae were visible after the sections were stained with periodic acid-Schiff (a) and Gomori–Grocott silver (b). Images were examined at 40 × magnification.

immunofluorescent assay, be expected as a key indicator of *A. fumigatus* biofilm-related infection. First, a proof of principle was obtained by demonstrating the ability of a mouse pAb, here generated against GT, to recognize specifically the mycotoxin on biofilm-growing *A. fumigatus* hyphae onto human pulmonary A549 cells. Second, after demonstrating its accessibility toward the toxin on the outer hyphal surface, we found that our pAb was able to bind to hyphae present in respiratory tract (i.e., BAL and sputum) samples or in lung tissues of patients with probable or proven IA, respectively. The specificity of detection was analyzed by testing in parallel samples from patients without evidence of *A. fumigatus* infection; in this case, no IF staining occurred on the hyphae inside BAL (and sputum) samples, as well as inside tissue samples from patients with a culture and histologically documented diagnosis of fungal disease other than IA, such as fusariosis or zygomycosis. It is noticeable that acute-angle dichotomous branching hyphae of *Aspergillus* are histologically undistinguishable from those of *Fusarium* using a variety of traditional stains like PAS and Gomori–Grocott silver and that cultures should be subsequently used to confirm identification of the infecting organism. However, the culture that is regarded as the 'gold standard' of medical mycology (Azie *et al.*, 2012) cannot be an exclusive diagnostic criterion for biofilm-associated infection (Hall-Stoodley *et al.*, 2012). Therefore, unlike culture and light-microscopy, an antibody-based diagnostic assay that detects biofilm-specific fungal antigens in a biological fluid or aspirate would be desirable.

Previous studies have shown the essential role of galactomannan (and galactosaminogalactan) polysaccharides in structuring the *A. fumigatus* biofilm ECM produced *in vivo*, and α-1,3-glucans in the cohesion of hyphae, but, notably, no other ECM constituents such as proteins, metabolites, or monosaccharydes have been investigated by immunocytochemical techniques because of the lack of specific antibodies (Loussert *et al.*, 2010). Although the use of antigalactomannan monoclonal antibodies can be not disregarded to demonstrate *A. fumigatus* ECM *in vitro*, we focused our efforts on a nonpolysaccharidic (and antigenically weak) compound, namely GT, that could be exploited in a diagnostic setting due to its presence at the hyphal surface, as here shown. However, it sounds astonishing that a very small and soluble molecule as GT is bound to fungal biofilm components, as well as there are no any hints from our own and other authors' studies proving the binding of GT to the hyphal surface. In fungi, the composition of biofilm matrix varies with the species, so, unlike *A. fumigatus*, the ECM of *Candida albicans*, the major opportunistic pathogen other than *A. fumigatus* (Brakhage *et al.*, 2011), is rich in β-1,3 glucan (Taff *et al.*, 2012). By contrast, GT is also produced by *C. albicans* and significantly contributes to the platelet function inhibition via interaction with thiols (Bertling *et al.*, 2010). Thus, our present data seem to point toward expanding the biofilm diagnostic toolbox available nowadays (Hall-Stoodley *et al.*, 2012) to include fungus-specific assays, but further studies will be necessary before the GT immunological detection can be regarded as an invaluable tool for diagnostic use in clinical practice.

Some other limitations of the present work should be considered. Firstly, as the experiments were performed with a polyclonal mouse antiserum, only limited amounts of this antiserum are available, and it remains unclear whether a newly produced antiserum gives the same result. Secondly, no experiments were performed to demonstrate the advantage of using GT detection in lung biopsies over detection of other *A. fumigatus* surface proteins (e.g., glycosylhydrolases/glycosyltranferases Asp f9, Asp f16, and Crf1). Thirdly, while our pAb failed to detect hyphae inside tissue samples from patients infected by *Fusarium*, a well-known toxigenic fungus, we were unable to ascertain whether pAb did cross-react with mycotoxins other than GT. Fourthly, no information was given about the capability of pAb to detect GT in serum specimens from animals or patients with aspergillosis, that would strengthen the use of pAb for fungal diagnostics directly on primary clinical specimens.

In conclusion, fungal infections, particularly those biofilm-associated, are destined to become a serious threat

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in the next future, posing better diagnosis and effective treatment as key points to mitigate their human and healthcare costs (Tumbarello *et al.*, 2012). Continued research is needed to refine and improve the possibility for scientists and clinicians to distinguish and verify a biofilm infection as soon as possible, as well as to evaluate therapeutic regimens aimed at resolving this hard-to-treat infection.

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