Aspergillus fumigatus germ tube growth and not conidia ingestion induces expression of inflammatory mediator genes in the human lung epithelial cell line A549

Anne-Pauline Bellanger,¹ Laurence Millon,¹ Khaled Khoufache,² Danièle Rivollet,² Ivan Bièche,³ Ingrid Laurendeau,³ Michel Vidaud,³ Françoise Botterel² and Stéphane Bretagne²

Correspondence

Stéphane Bretagne
bretagne@univ-paris12.fr

¹UMR 6249 CNRS, Laboratoire de Parasitologie-Mycologie, Hôpital Jean Minjoz, Besançon, France

²UMR BIPAR 956, Laboratoire de Parasitologie-Mycologie, Hôpital Henri Mondor and Université Paris-Est, Créteil, France

³UMR INSERM 745, Université Paris Descartes, Faculté des Sciences Pharmaceutiques et Biologique, Paris, France

Inhalation of conidia is the main cause of invasive pulmonary aspergillosis (IPA) and the respiratory epithelium is the first line of defence. To explore the triggering factor for the inflammatory response to *Aspergillus fumigatus*, the species mainly responsible for IPA, this study analysed the differential expression of three inflammatory genes in A549 cells after challenge with live and killed conidia. The influence of steroids, one of the main risk factors for developing IPA, was also investigated. Quantification of mRNAs of the inflammatory mediator genes encoding interleukin (IL)-8, tumour necrosis factor (TNF)- α and granulocyte-monocyte colony-stimulating factor (GM-CSF) was carried out using real-time PCR. Ingestion rates were studied for the conidia of *A. fumigatus* and *Penicillium chrysogenum* using a fluorescence brightener. Similar results were obtained for both species, with ingestion rates ranging from 35 to 40 %. Exposure of A549 cells to live *A. fumigatus* conidia only induced a four- to fivefold increase in the mRNA levels of the three genes, starting 8 h after the initial contact. Both inactivation of live *A. fumigatus* conidia and treatment by dexamethasone (10^{-7} M) prevented the overexpression of TNF- α , IL-8 and GM-CSF. Fungal growth, rather than conidia ingestion, appears to be the main stimulus for the production of inflammatory mediators by epithelial cells, and this production is inhibited by steroid therapy. These results underline the role that the epithelium plays in the innate response against IPA.

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INTRODUCTION

Invasive pulmonary aspergillosis (IPA) is an opportunistic infection whose incidence is increasing at the same rate as the number of severely immunocompromised patients (Lin *et al.*, 2001). Neutropenia and high-dose steroid treatments are the main risk factors for IPA, and these underline the importance of an appropriate inflammatory response if fungal invasion is to be avoided. Most authors stress the important roles that alveolar macrophages and neutrophils play in controlling infection (Romani, 2004; Walsh *et al.*, 2005). The release of innate immune-related molecules from professional phagocytic cells in the course of

Aspergillus fumigatus infection has been widely studied (Meier *et al.*, 2003; Pylkkanen *et al.*, 2004; Cortez *et al.*, 2006). However, the respiratory epithelium is the first tissue that inhaled conidia encounter, and it probably participates in the efficient coordinated response against IPA. In animal models of IPA, most inhaled conidia are trapped in the upper respiratory tract, and the respiratory epithelium coordinates with alveolar macrophages (Stephens-Romero *et al.*, 2005).

A few studies have investigated the production of inflammatory mediators by studying the human lung epithelial cell line A549 after stimulation with moulds or mould extracts (Borger *et al.*, 1999; Kauffman *et al.*, 2000; Huttunen *et al.*, 2003; Zhang *et al.*, 2005; Tai *et al.*, 2006). These studies have focused on fungi as the source of allergens, and on species other than *A. fumigatus*, which are

Abbreviations: ANOVA, analysis of variance; C_t, cycle threshold; GM-CSF, granulocyte-monocyte colony-stimulating factor; IL, interleukin; IPA, invasive pulmonary aspergillosis; TNF, tumour necrosis factor.

often more allergenic (Kauffman *et al.*, 2000). To our knowledge, there are no reports on the response of A549 cells to germinating *A. fumigatus* or on comparisons with other mould species. Spores of other mould species usually outnumber *A. fumigatus* conidia in inhaled air, although *A. fumigatus* is the main aetiological agent of IPA. Therefore, differences observed between *A. fumigatus* conidia and the spores of other species after the challenge of respiratory epithelium could help explain why *A. fumigatus* is a frequently encountered aetiological agent of invasive infection.

The aim of our study was to investigate the early inflammatory signals in airway epithelial cells after exposure to *A. fumigatus* conidia using real-time quantitative PCR for reliable quantification of expression of selected inflammatory mediator genes (Stordeur *et al.*, 2002; Asselah *et al.*, 2005). We used in our study *Penicillium chrysogenum* as a control for conidia ingestion (Botterel *et al.*, 2008). *P. chrysogenum* is a mould that is highly predominant in the environment, with a conidial size (3–4 µm diameter) similar to that of *A. fumigatus*. Although *P. chrysogenum* can germinate at 37 °C (de Hoog *et al.*, 2000), this fungus is rarely responsible for invasive disease. We also aimed to explore the effect of steroids on the development of the inflammatory response of the A549 cell line after challenge with live *A. fumigatus* conidia.

METHODS

Fungal strains. *A. fumigatus* strain IP 2279.94 and *P. chrysogenum* strain IP 1652.86 (both from the Pasteur Institute) were grown in 2% malt agar tubes (Bio-Rad) for 7 days at their optimal growth temperature: 37 and 27 °C for *A. fumigatus* and *P. chrysogenum*, respectively. The conidia were harvested with PBS/0.01% Tween 20 and 1 ml of conidia suspension (10^6 ml⁻¹) was used to seed the wells containing A549 cells. For some experiments, conidia were inactivated either chemically with 10% (v/v) formaldehyde or by heating at 100 °C in water for 1 h, and were resuspended in culture medium at 10^6 conidia ml⁻¹.

Cell lines. The alveolar epithelial cell line A549 was purchased from the German Collection of Micro-organisms and Cell Cultures (DSMZ) and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen), with 10% heat-inactivated fetal bovine serum (Biowest) and 25 µg gentamicin ml⁻¹ (Sigma-Aldrich). The medium was changed every 2 days and cells were confluent after 5 days. Cell cultures were checked for the presence of *Mycoplasma* spp. every month using the Venor GeM Mycoplasma Detection PCR kit (BioValley). A549 cells were cultivated in 24-well microtitre plates (VWR). Confluent A549 cells ($\sim 5 \times 10^6$ cells per well) were seeded with conidial suspensions. Plates were centrifuged for 5 min at 1500 r.p.m. to optimize and standardize contact between the cells and conidia. The supernatants were removed and the conidia were counted. Wells were refilled with fresh, pre-warmed culture medium. Cell exposure was terminated at 2, 4, 8 and 24 h. These experiments were performed at least three times under all conditions.

Quantification of conidia ingestion. The internalized conidia were quantified using the fluorescence brightener Blankophor-P-fluessig [4,4'-bis[(4-anilino-6-substituted-1,3,5-triazine-2-yl) amino stilbene-2,2'-disulfonic acid], kindly provided by Bayer (Botterel *et al.*, 2008).

The brightener stained only the external conidia. Ten microlitres of the dye was diluted in 1 ml culture medium and 300 µl of this dilution was added to the wells and incubated for 10 min at 37 °C to stain non-internalized conidia. The wells were washed twice to collect any remaining unattached conidia and to remove the dye. The A549 cells were then lysed with 300 µl distilled water to allow counting of all conidia associated with or ingested by the cells. An Axioskop 40 microscope (Zeiss) with a filter combination including a barrier filter at 420 nm was used to classify the conidia. When UV light was stimulated below 400 nm, the brightener emitted a very intense bluish, yellowish or white light (Ruchel & Margraf, 1993). The blue conidia were classified as non-internalized and the non-fluorescent conidia as internalized.

Dexamethasone experiments. A549 cells were also challenged with *A. fumigatus* conidia in the presence of 10^{-6} and 10^{-7} M dexamethasone (Sigma). Dexamethasone was dissolved in 90% ethanol, diluted in culture medium and added to the A549 cells just after centrifugation of the plates and removal of the supernatant. The growth of *A. fumigatus* in DMEM at 37 °C in the presence of 10^{-3} – 10^{-7} M dexamethasone was also monitored for effects on fungal growth at 37 °C. Fungal germination was analysed at 4, 8 and 24 h.

mRNA quantification. RNA was extracted using an RNable kit (Eurobio). Determination of the concentration of nucleic acids was performed using a spectrophotometer (Nanodrop ND 1000) and the concentrations were adjusted to 250 ng µl⁻¹. Reverse transcription was carried out in a final volume of 20 µl as follows: 1 µg (4 µl) RNA extract was added to 2 µl 0.1 M dithiothreitol (Invitrogen), 0.5 µl Superscript II reverse transcriptase (200 U µl⁻¹; Invitrogen), 4 µl 5 × first-strand buffer [250 mM Tris/HCl (pH 8.3), 375 mM KCl, 15 mM MgCl₂; Invitrogen], 2 µl 5 mM dNTP mix (Amersham Pharmacia), 6 µl pdN₆ random hexamers (0.5 µg µl⁻¹; Amersham Pharmacia) and 0.5 µl RNasin (40 U µl⁻¹; Promega). Sterile water was used to dilute the cDNAs (1:20) before storing at –80 °C until amplification. For each reaction, negative controls without RNA and without reverse transcriptase were carried out.

The primers used are listed in Table 1. Real-time PCR was carried out in a LightCycler 1.5 (Roche) in a 20 µl final volume containing 2 µl $10 \times$ Fast Start SYBR Green Buffer (Fast Start SYBR Green kit; Roche Diagnostics), 3 mM MgCl₂ for granulocyte-monocyte colony-stimulating factor (GM-CSF; *CSF2* gene) and interleukin (IL)-8 (*IL-8* gene) and 5 mM MgCl₂ for tumour necrosis factor (TNF)-α (*TNF* gene), 50 µM sense and antisense primers, 8 µl cDNA (diluted 1:20) and sterile water. The thermal cycling conditions were: initial denaturation at 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 65 °C for 1 min. Quantitative values were obtained from the cycle threshold (C_t) number. Samples from three independent experiments were analysed in duplicate. The melting curves of each PCR were checked. The presence of contaminating DNA was checked by amplifying an intron of the human albumin gene. Each sample was normalized on the basis of its content compared with two reference mRNAs that were not co-regulated (Bieche *et al.*, 2005). The first gene, P0, also known as 36B4 (GenBank accession no. NM_001002), encodes acid ribosomal phosphoproteins. The second, the TBP gene (GenBank accession no. NM_003194), encodes a component of the DNA-binding protein complex TFIID. The results, expressed as the N -fold difference in target gene expression relative to the P0 or TBP gene (termed N_{target}), were determined by the formula $N_{\text{target}} = 2^{\Delta C_t \text{ sample}}$. The ΔC_t sample was determined by subtracting the mean C_t value of the target gene from the mean C_t value of the TBP or the P0 gene. The N_{target} values of the samples were subsequently normalized so that the median N_{target} value of the non-fungus control samples was 1.

Table 1. Primers sequences used in the study

Gene target	Sense primer (5'→3')	Antisense primer (5'→3')
IL-8 (IL-8 gene)	CACCGGAAGGAACCATCTCACTGT	TCCTTGGCAAACTGCACCTTCA
TNF- α (TNF gene)	GCCAGGCAGTCAGATCATCTT	CCTCAGCTTGAGGGTTGCTACA
c-Fos (FOS)	ACCACTCACCCGAGACTCCT	CCAGGTCCGTGCAGAAAGTCCT
GM-CSF (CSF2)	GAGACACTGCTGCTGAGATGAATG	CAGGTCGGCTCCTGGAGGT
P0	GGCGACCTGGAAGTCCAACT	CCATCAGCACCACAGCCTTC
TBP	TGCACAGGAGCCAAGAGTGAA	CACATCACAGCTCCCCACCA
Albumin (intron)	GCTGTCATCTCTTGTGGGCTGT	ACTCATGGGAGCTGCTGGTTC

Statistical analysis. Data are presented as means $\pm$ SEM of three independent experiments. Statistical analysis was performed using XLSTAT 2007. Differences were considered statistically significant for $P < 0.05$. A one-way analysis of variance (ANOVA) test was used to detect significant variations in mRNA levels for each target gene during exposure. A t -test was used to detect significant differential mRNA levels with or without dexamethasone.

RESULTS AND DISCUSSION

Using quantitative PCR, we observed a significant increase in mRNA levels in A549 epithelial cells for all three selected inflammatory mediators (IL-8, TNF- α and GM-CSF) when they were exposed to live *A. fumigatus* conidia. This increase was observed at 8 h and continued up to 24 h. This overexpression of mRNA was not observed when the cells were exposed to inactivated *A. fumigatus* conidia, or to live or inactivated *P. chrysogenum* conidia. The presence of 10^{-7} M dexamethasone prevented the increase in mRNA levels of IL-8, TNF- α and GM-CSF.

Standardization of techniques

After centrifugation of cell cultures with conidial suspensions, approximately 10 % did not adhere to the A549 cells and were removed. There was no statistical difference between the ingestion rates of conidia for *A. fumigatus* (35.3 ± 3.3 %) and *P. chrysogenum* (37.7 ± 2.0 %) at 4 h post-exposure.

After RNA extraction, we first checked for contaminating genomic DNA by amplifying an intron of the human albumin gene. The C_t values were always >37 and were similar for all of the cDNAs, confirming the absence of DNA contamination. To normalize mRNA expression from different wells, a relative quantification of the target genes and of the endogenous reference P0 and TBP genes using $\Delta\Delta C_t$ was performed. Because similar results were obtained for both reference genes, only the results standardized with the P0 gene are reported here.

mRNA quantification

We focused our study on three mediators whose role has been well established in the inflammatory response. The absence of this response is one of the well-known risk factors for IPA, i.e. prolonged neutropenia and steroid therapy. IL-8 is a chemokine involved in both the recruitment of

neutrophils and the neutrophil phagocytosis of *A. fumigatus* conidia (Richardson & Patel, 1995), TNF- α is a cytokine that plays a role in chemokine production and neutrophil activity (Mehrad *et al.*, 1999; Phadke & Mehrad, 2005) and GM-CSF is a growth factor known to be crucial in the maturation process of granulocytes and macrophages (Walsh *et al.*, 2005). These three mediators are released in response to activation of the NF- κ B transcriptional complex.

Exposure to live *A. fumigatus* conidia induced a progressive increase in IL-8, TNF- α and GM-CSF mRNA levels (Fig. 1). A significant overexpression of the mRNA of all three was observed as early as 8 h post-exposure ($P \leq 0.005$, $P \leq 0.002$ and $P \leq 0.003$, respectively). This increase was not observed when the cells were exposed to inactivated *A. fumigatus* conidia. No significant variation for any of the three inflammatory mediators was observed when the epithelial cells were exposed to live or inactivated *P. chrysogenum* conidia. Germinating *A. fumigatus* conidia were observed in A549 cells as early as 8 h post-exposure. In contrast, with live *P. chrysogenum* conidia and with the inactivated conidia of both species, no germination was observed during the entire 24 h of the experiment.

We hypothesized that ingestion of conidia by A549 cells could be the triggering factor for the inflammatory response. Overexpression of chemokines and of chemokine receptor genes (IL-8 gene, *CCL4*, *CXCL20* and *CXCL2*) has been described as early as 2 h in human monocyte models using cDNA microarray analysis (Cortez *et al.*, 2006). In our study, we were unable to detect any significant increase before 8 h. If conidia ingestion were the triggering event, an inflammatory response would have occurred for both fungal species because the rate of internalized conidia was similar for both species: around 30–40 %, as reported previously (Wasylnka & Moore, 2002).

However, in our study, a sharp increase appeared at the same time as the hyphal growth of *A. fumigatus*, which started after 6–8 h of culture in liquid medium (Manavathu *et al.*, 1999). In addition, exposure to inactivated *A. fumigatus* conidia did not induce any significant modifications of the mRNA levels of the factors studied. Similar results for cytokine production using ELISA were obtained using non-viable fragments of mycelium and inactivated *A. fumigatus* conidia (Zhang *et al.*, 2005). Recently, infection with hyphae, but not with

Fig. 1. Standardized mRNA expression of the genes encoding IL-8, TNF- α , GM-CSF and P0 after exposure of A549 cells to live *A. fumigatus* conidia calculated with the $\Delta\Delta C_t$ method, with P0 as the reference gene. Values were calculated from three independent experiments and gene expression was subsequently normalized against the median values of the uninfected controls. The data are presented as means $\pm$ SEM: significant overexpression at 8 h (#) for IL-8 ($P \leq 0.005$), TNF- α ($P \leq 0.002$) and GM-CSF ($P < 0.003$), and at 24 h (*) for the three factors ($P \leq 0.0001$), and unchanged expression of the control gene (P0), were analysed by ANOVA.

conidia, was shown to stimulate endothelial cells to synthesize IL-8 and TNF- α *in vitro* using ELISA (Chiang *et al.*, 2008). We cannot rule out the fact that heating or chemical inactivation may have destroyed epitope structures and heat-labile molecules such as proteases, which are potentially involved in inflammatory signalling. However, our results are in agreement with a recent report on the role of germinating conidia in the innate immune system *in vitro* and in animal models (Hohl *et al.*, 2005). Conidial

swelling is accompanied by an enrichment of β -glucans on the membrane surface, which triggers inflammatory responses via Dectin-1 recognition (Hohl *et al.*, 2005).

mRNA quantification in the presence of dexamethasone

Exposure of A549 cells to live conidia of *A. fumigatus* in the presence of 10^{-6} M (not shown) and 10^{-7} M dexametha-

Fig. 2. Standardized mRNA expression of the genes encoding IL-8, TNF- α , GM-CSF and P0 after exposure of A549 cells to live *A. fumigatus* conidia with (white columns) and without (black columns) 10^{-7} M dexamethasone using the $\Delta\Delta C_t$ method, with P0 as the reference gene. Values were calculated from three independent experiments and gene expression was subsequently normalized against the median values of the uninfected and non-steroid controls. The data are presented as means $\pm$ SEM: absence of mRNA overexpression at 24 h ($P \leq 0.0001$ for each) by *t*-test compared with wells exposed to live conidia of *A. fumigatus* without dexamethasone. The control gene (P0) remained unchanged.

sone resulted in the absence of overexpression at 8 h for IL-8 ($P \leq 0.005$), TNF- α ($P \leq 0.002$) and GM-CSF ($P < 0.003$) and at 24 h ($P \leq 0.01$ for each) (Fig. 2). In addition, dexamethasone at concentrations of 10^{-3} – 10^{-7} M did not modify the growth of *A. fumigatus* conidia (data not shown).

Given the significant role that germinating conidia play in IPA, the disappearance of overexpression when the conidia-exposed cells were treated with 10^{-7} M dexamethasone supports the role of steroids as a risk factor for IPA, not only at the macrophage level, but also at the respiratory epithelium level (Nissen & Yamamoto, 2000). Although the role that steroids play in the NF- κ B pathway of the cells is a probable explanation, we cannot completely rule out the fact that steroids could inhibit the synthesis of a specific fungal compound that could be responsible for the immune stimulation of A549 cells.

These initial results show that germinating *A. fumigatus* conidia induce overexpression of inflammatory mediators by epithelial cells in the first 24 h after exposure. Further studies are needed to define in more detail the chemokines, adhesion molecules and other possible pathways involved. However, these results suggest that the respiratory epithelium plays a role in initiating innate immunity to prevent the occurrence of IPA. Our results also confirm that steroids significantly modify the normal inflammatory response of airway cells exposed to *A. fumigatus*, and this could explain at least in part why steroids are a risk factor for IPA.

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