

***Aspergillus fumigatus* conidia inhibit tumour necrosis factor- or staurosporine-induced apoptosis in epithelial cells**

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Abstract

A major innate immune response to inhaled conidia of the opportunistic pathogen *Aspergillus fumigatus* (Af) is the synthesis of pro-inflammatory cytokines, which include tumour necrosis factor (TNF)- α , a known inducer of apoptosis. Modulation of host cell apoptosis has been reported to be one of the mechanisms whereby pathogens overcome host cell defences. Our study was designed to investigate whether or not Af conidia could modulate apoptosis induced by TNF- α or staurosporine (STS). Exposure of epithelial cells treated by these inducers and exposed to Af conidia decreased the number of apoptotic cells detected by Annexin V staining, analysis of nuclear morphology, terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end-labelling reaction and immunoblotting. Inhibition of apoptosis by Af conidia was seen in cells of the A549 pneumocyte II line, human tracheal epithelial 16HBE and primary human respiratory cells. Inhibition of apoptosis by Af conidia was also observed when apoptosis was induced by co-cultivating A549 cells with activated human alveolar macrophages. Unlike Af conidia, conidia of *Cladosporium cladosporioides* as well as latex beads or killed Af conidia have no inhibitory effect on TNF- α or STS-induced apoptosis. For TNF-induced apoptosis, the observed anti-apoptotic effect of Af conidia was found to be associated with a significant reduction of caspase-3.

Introduction

Aspergillus fumigatus (Af) is a saprophytic mould, which is responsible for the majority of invasive mycosis in patients undergoing chemotherapy or organ transplantation (1). It propagates through airborne conidia (spores), which are inhaled into the small airways where they may germinate and initiate an infection. The invasion of airway epithelial cells is therefore a key step in the etiology of aspergillosis, especially since it has been shown that these cells are able to phagocytose Af conidia which can survive at least for some time in these cells (2–4).

Pathogenic micro-organisms have evolved different mechanisms to survive in the host environment: modulation of host

cell apoptosis is one of these mechanisms. *Escherichia coli* (5) and *Candida albicans* (6) have been found to induce apoptosis in neutrophils, and *Cryptococcus neoformans* has been shown to induce apoptosis in inflammatory cells in granulomas of rats with cryptococcal meningitis (7, 8). In contrast, inhibition of host cell apoptosis by the yeast form of *Histoplasma capsulatum* and the protozoa *Theileria parva* and *Toxoplasma* may provide an intracellular niche for the pathogens by extending the life span of infected host cells (9–11). Previous studies have shown that gliotoxin, the most abundant mycotoxin produced by Af, induces apoptosis in many types of cells (12). However, this secondary metabolite

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is only produced by hyphae and nothing is known about the influence of conidia of Af on host cell apoptosis.

Apoptotic cells show characteristic morphological changes, including cell shrinkage, nuclear/chromatin condensation, internucleosomal cleavage of DNA, membrane blebbing and formation of apoptotic bodies (13). Caspases, which are responsible for many biochemical and morphological changes associated with apoptosis, are synthesized as inactive pro-enzymes, most of which have to be activated by proteolytic cleavage (14). There are various alternative inducers of apoptosis such as the proteins of the tumour necrosis factor receptor (TNFR) family and stimuli such as cytotoxic agents, cell metabolites and other pathological insults (15, 16). The receptor-mediated (extrinsic) signalling pathway involves the formation of a death-inducing signalling complex (DISC), with subsequent activation of the initiator caspase, which either directly activates the effector caspases or cleaves cytosolic factors, leading to the release of cytochrome-c and activation of another initiator caspase, and subsequently of effector caspases (17–19). The chemically mediated (intrinsic) pathway, which is classically linked to mitochondrial disturbance, is characterized by the release of cytochrome-c from mitochondria, following the activation of the initiator caspases, which in turn activate effector caspases (14, 16). Caspase-3 is one of the effector caspases which is situated at nodal points in both apoptotic pathways. When activated by proteolytic cleavage, caspase-3 cleaves vital cellular proteins involved in the apoptotic process (19, 20). However, a caspase-independent apoptotic pathway has also been identified recently (21, 22).

The studies described in this report were designed to investigate whether Af conidia could induce or modify apoptosis and hence affect host cell survival. Exposure of alveolar macrophages to Af conidia results in the synthesis of TNF- α (23), which in turn may induce apoptosis in epithelial cells. Here, we describe *in vitro* experiments which have been mainly carried out with a transformed human type II pneumocyte cell line (A549) as a model for alveolar epithelial cells, using TNF- α and staurosporine (STS) as inducers of apoptosis. Cycloheximide (CXH), a translational inhibitor, has been used together with TNF, to favour DISC-dependent activation of initiator caspase (24–26). STS was selected as an alternative inducer of apoptosis for the investigation of the chemical-mediated pathway. The results of the morphological analysis of stained nuclei, flow cytometry, terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end-labelling (TUNEL) assay and western blot analysis demonstrated that Af conidia only slightly induce apoptosis in untreated A549 cells. In contrast, Af conidia drastically inhibit apoptosis initiated by either TNF- α or STS. The mechanism of inhibition of apoptosis following STS treatment remains unknown, while conidia-mediated inhibition of cytokine-induced death appears to be caspase-3 dependent. The inhibition of apoptosis by Af conidia was also observed in the human tracheal epithelial 16HBE cell line, in airway epithelial primary culture cells as well as in A549 cells co-cultivated with activated human alveolar macrophages (HAM) cells in the absence of the addition of TNF or STS: this points to the biological significance of our findings.

Methods

Fungal strains and growth conditions

Af CBS 144.89 and *Cladosporium cladosporioides* IP1232 (Institut Pasteur, Paris, France) were used throughout this study. Conidia of Af and *C. cladosporioides* were obtained from cultures grown on YM agar (0.3% yeast extract, 2% malt extract, 0.5% peptone and 0.5% agar) for 3 days at 37°C and 25°C, respectively. Conidia were harvested by flooding the plates with sterile distilled water and then suspending the conidia in 0.15 M PBS. For some experiments, Af conidia were killed by incubation in a 3% PFA solution overnight followed by washings with 0.2 M glycine and PBS.

Human cells and growth conditions

Type II pneumocyte cell line A549 derived from a human lung carcinoma was obtained from American Type Culture Collection [ATCC CCL 185 (27)] and maintained in Kaighn's modification of HAM's F12 medium supplemented with 10% of FCS (Invitrogen), pen/strep (16 mg ml⁻¹ penicillin and 100 mg l⁻¹ streptomycin), 2 mM L-glutamine and 1.5 g l⁻¹ sodium bicarbonate. The cells were grown until confluent. Unless specified otherwise, all incubations were at 37°C in an incubator with a humidified atmosphere of 5% CO₂. Trypsin/EDTA (Invitrogen) was used to release adherent cells for subculturing when this was required.

Human tracheal epithelial SV40-transformed cells (16HBE) were provided by Boisivieu-Ulrich (Cytophysiology and Toxicology laboratory, University of Paris 7, France). 16HBE cells were maintained in Eagle MEM medium (Invitrogen) with 2% UltrosorG (Invitrogen), pen/strep, 2 mM L-glutamine (Sigma) and 1.5 g l⁻¹ sodium bicarbonate (Sigma) and were grown until confluent (28).

Primary epithelial cells were obtained from human nasal turbinates (HNT) of patients undergoing turbinectomy (P. Herman, CHU Lariboisière, Paris, France) as described previously (29). Briefly, HNT were washed in Dulbecco's modified Eagle medium DMEM/F12 (Invitrogen) and incubated with 2 mg ml⁻¹ pronase (Protease XIV; Sigma) in DMEM/F12 supplemented with pen/strep, at 4°C for 16–20 h under slow rotary agitation (80 r.p.m.). After washing, aggregates were discarded and dissociated cells were filtered using a 30- μ m pore filter. The cell suspension was then plated for 2 h at 37°C on plastic dishes (Falcon) to eliminate contaminating fibroblasts. After centrifugation, the supernatant containing the epithelial cells was plated on collagen type I-coated wells and cultivated in DMEM/F12 supplemented with 10% FCS and pen/strep.

HAM were obtained from a bronchoalveolar lavage of a patient who had received a lung transplant (B. Philippe, Foch Hospital, Suresnes, France). Cells were harvested by centrifugation (400 $\times$ g for 10 min at 4°C), re-suspended in RPMI complete medium containing 10% FCS and seeded on cell culture plates at 10⁶ per well. Non-adherent cells were removed by washing after 2 h incubation.

Induction of apoptosis

Either A549 or 16HBE cells were seeded at 5 $\times$ 10⁵ cells per well in 1 ml of DMEM/F12 on 18-mm-diameter cover slips

(Marienfeld, Germany) in 12-well plates (Nunc, Nuclon™ Surface) in triplicate and grown for 16 h at 37°C. Primary epithelial cells were seeded at 5×10^6 cells per well and grown for 48 h. After washing the cover slips with PBS-BSA (PBS-5% BSA, Fraction V, Sigma), the inducers of apoptosis were added to the wells in 1 ml of medium. To induce apoptosis, two different types of inducers were used: (i) 1 μ M STS (Sigma) and (ii) 20 ng ml⁻¹ of TNF- α (Sigma) with 2 μ g of CHX (Sigma). After 7 h of incubation conidia germinated, however, the apoptotic features of cells, incubated with TNF- α in the absence of CHX, appeared much later (data not shown). To efficiently induce apoptosis and decrease the time of incubation, TNF- α was used together with CHX, since CHX has been shown to suppress survival signals induced by an engagement of TNFR (23). The presence of CHX is therefore only required to allow us to study the role of conidia on induced apoptosis in the absence of conidial germination.

To investigate early stages of apoptosis by flow cytometry, the induction of apoptosis was performed for 1 h, a period when maximal numbers of apoptotic cells and minimal number of necrotic cells were detected (data not shown). The control cells were incubated either with CHX or medium alone. We did not detect any difference between CHX-treated cells (data not shown) and cells incubated with medium alone. None of the treatments inducing apoptosis affected the viability of conidia (data not shown).

To study the late stages of apoptosis, A549 cells were incubated with STS or TNF- α for 6 h: this duration of treatment was defined by results reported by others (25, 30) and on our own unpublished observation showing the absence of the 17-kDa active form of caspase-3 after 4 h of incubation.

HAM cells have also been used as a source of TNF for apoptosis induction. In order to estimate the optimal incubation time, preliminary assay of apoptosis induction in A549 with 20 ng ml⁻¹ of TNF- α during different periods (6 h, 24 h and 48 h) was performed. The highest number of apoptotic cells was found after 24 h incubation (data not shown). Therefore, for a 24-h incubation, different doses of TNF- α were tested by morphological criteria for apoptosis induction in A549 cells. Apoptotic features of cells were observed at a concentration of TNF- α as low as 5 ng ml⁻¹ of TNF- α (data not shown). A549 cells were seeded at 5×10^5 cells per well on 18-mm diameter cover slips in 12-well plates and grown for 16 h. After washing with PBS-5% BSA, the cover slips bearing A549 cells were transferred onto the surface of an 18-h-old culture of alveolar macrophages stimulated with 1 μ g ml⁻¹ of LPS for the same time of culture. Under these culture conditions, HAM produced 10 ng ml⁻¹ of TNF- α , as measured in culture supernatant using a commercial ELISA kit (BD Biosciences).

Exposure to conidia

Following washing of A549, 16HBE or primary culture cells with PBS, Af conidia ranging from 5×10^6 to 10^8 conidia per millilitre of medium were added to the cells simultaneously with the inducer of apoptosis. In experiments when activated HAM have been used as a source of TNF, 10^7 conidia per millilitre have been added to the cells. The lower concentration of conidia was chosen on the basis of the results of Wasylnka and Moore (31); the higher concentrations were chosen to

investigate dose dependence. After incubation, unbound conidia were removed by washing wells with PBS-5% BSA. The resulting number of apoptotic cells was compared with cultures incubated either with the inducers or the medium (control). To test for the specificity of the effect of live Af conidia, some wells received instead 5×10^6 to 10^8 *C. cladosporioides* conidia, latex beads or PFA-killed Af conidia.

Flow cytometry analysis

A549 cells were labelled with an FITC-Annexin V and stained with propidium iodide (PI) using an apoptosis detection kit (Sigma). The procedure consists of (i) the binding of FITC-Annexin V to phosphatidylserine which translocates from the interior to the exterior of the cell membranes at the start of the apoptotic process and (b) the binding of PI to DNA in cells where the membrane has been totally compromised (32). A549 cells were prepared as described above. Induction of apoptosis was performed for 1 h. Harvested cells were centrifuged for 5 min at $250 \times g$ and after washing with PBS were re-suspended in binding buffer (10 mM HEPES/NaOH, pH 7.5 containing 140 mM NaCl and 2.5 mM CaCl₂) at a concentration of 1×10^6 cells per millilitre. Five microlitres of 1:10 dilution of FITC-Annexin V conjugate (50 μ g ml⁻¹ in 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl) and 10 μ l of PI solution (100 μ g ml⁻¹ in 10 mM potassium phosphate buffer, pH 7.4, containing 150 mM NaCl) were added to 500 μ l of cell suspension and incubated in the dark at room temperature for 15 min. A suitable dilution of FITC-Annexin V conjugate was determined in preliminary experiments. The relative level of apoptotic cells was detected using a FACScan system (BD Biosciences) using CellQuest 3.3 software (33).

Detection of apoptotic body formation and chromatin condensation

Nuclei of the control cells, of cells treated with the inducers of apoptosis for 6 h and of cells exposed to Af conidia in the presence or absence of inducers, together with untreated control cells, were stained with DNA binding dye Hoechst 33342 and then analysed using a fluorescence microscope. The cover slips were washed with PBS-5% BSA and the cells fixed with PBS-4% (wt/vol) PFA (pH 7.4) for 1 h. Chromatin condensation and apoptotic body formation was assessed by staining with Hoechst 33342 (1.5 μ M; Sigma) for 30 min at room temperature. After washing, the cover slips were mounted on slides with ProLong antifade Vectashield (Vector Laboratory). Samples were viewed with a Zeiss fluorescence microscope using $\times 400$ magnifications. For each sample, cells from five random fields were counted and the percentage of apoptotic cells was calculated as the number of apoptotic cells divided by the total number counted multiplied by 100.

TUNEL assay of DNA fragmentation

TUNEL technology, based on labelling of DNA strand breaks, was performed using an In Situ Cell Death Detection kit (Roche Molecular Biologicals) (34). After induction of apoptosis for 6 h at 37°C, cover slips were washed with PBS-5% BSA, the cells were fixed with PBS-4% (wt/vol) PFA (pH 7.4) for 1 h and then permeabilized in a solution containing 0.1% Triton X-100/0.5%

sodium citrate for 30 min at room temperature. Cover slips were washed again and 50 μ l of TUNEL reaction mixture was added for 1 h and incubated in a humidified dark chamber at 37°C. After detection of incorporated FITC by anti-FITC antibody, conjugated with alkaline phosphatase, substrate was added and stained cells were analysed by light microscopy.

Caspase analysis

In order to monitor the presence of both pro-caspase-3 and the active form of caspase-3, western blot analysis of the cell lysates was performed. Following the induction of apoptosis, cells exposed (or not) to *Af* conidia and untreated controls without conidia were simultaneously harvested by trypsinisation. After centrifugation at $200 \times g$, the cell pellets were re-suspended in 30 μ l of buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.1% triton, 0.1% SDS and a cocktail of protease inhibitors: 2.5 mM orthovanadate, 10 mM para-nitrophenylphosphate, 10 μ g ml⁻¹ leupeptine and 10 μ g ml⁻¹ aprotinin. The resulting lysates were centrifuged for 10 min at $15\,000 \times g$ at 4°C. Protein concentration was determined using a bicinchoninic acid-based protein assay (Interchim) and equal amounts of protein were mixed with 2 $\times$ Laemmli sample buffer (62.5 mM Tris-HCl pH 6.8; 8.4% SDS, 5% mercaptoethanol, 8.5% glycerol, 0.25% bromophenol blue) and maintained at 100°C for 7 min before loading 20 μ g of cell extract per lane onto 15% SDS-polyacrylamide gels (35). After electrophoresis, the separated proteins and molecular weight standards were electrophoretically transferred onto 0.2 μ m Immuno-Blot PVDF membranes. The membranes were blocked with 10% skimmed milk in 0.1 M Tris buffer, 0.9% NaCl, 0.05% Tween 20, pH 7.2, and 3% normal goat serum (Sigma), incubated for 3 h with polyclonal rabbit antibody specific for human caspase-3 (Abcam, UK) diluted 1:250 in the blocking solution and after washing were incubated with goat anti-rabbit antibody (1:1000) coupled with peroxidase (Sigma) for 1 h. The membranes were then washed in PBS and incubated with enhanced chemoluminescence reagents (Amersham). The optical density of the bands, with the relative molecular weights 32 and 17 kDa corresponding to pro-caspase-3 and the active form of caspase-3, was estimated using the ImageQuant system. To compare the intensities of the bands in the different lines, the values of the bands were divided by the value of the band corresponding to pro-caspase-3 in the control untreated cells without conidia.

To confirm the involvement of caspase, different concentrations of the caspase inhibitor Z-VAD.FMK or FMK negative control ranging from 0.125 to 0.5 mM were added simultaneously with the inducer of apoptosis to the cells exposed to conidia. The percentage of apoptosis was assessed according to the nuclear morphology of the cells stained with 1.5 μ M Hoechst 33342.

Statistical analysis

At least three different assays were performed per experiment. The differences in the number of apoptotic cells for TNF- and STS-induced apoptosis exposed or not to conidia were assessed by analysis of variance. *P*-values <0.05 were considered to be significant. Tukey's honestly significant difference test was applied for comparison of means between

groups. Means followed by the same letter are not significantly different. The values are expressed as mean $\pm$ SEM.

Results

*Inhibition of apoptosis in A549 cells exposed to *Af* conidia*

Flow cytometry analysis. Flow cytometry of FITC-Annexin V-labelled cells stained with PI allowed an analysis of the early stages of apoptosis of the host cells. At the highest concentration, *Af* conidia inhibited TNF- and STS-induced apoptosis of A549 cells. The level of apoptotic cells, after 1 h incubation with TNF or STS was $14 \pm 3\%$ and $20 \pm 1.5\%$, respectively: an example of one replicate is shown in Fig. 1(A). TNF treatment of cells and simultaneous exposure to *Af* conidia leads to a statistically significant ($P < 0.05$) dose-dependent decrease of the number of apoptotic cells from $14 \pm 3\%$ in the controls to $1.5 \pm 2\%$ with 10^8 , to $6.7 \pm 2\%$ with 10^7 and to $9.8 \pm 1\%$ with 5×10^6 conidia (Fig. 1B). The equivalent effect in STS-treated cultures was from $20 \pm 1.5\%$ in controls to $7 \pm 1.5\%$, $13 \pm 1\%$ and $17 \pm 1.2\%$, respectively ($P < 0.05$). In an untreated control, $2.5 \pm 0.5\%$ apoptotic cells were found. No apoptosis was induced in the cultures following addition of 10^7 or 5×10^6 *Af* conidia. Spontaneous apoptosis was weakly increased to $4.5 \pm 1\%$ when cells were exposed to 10^8 conidia ($P < 0.05$) (Fig. 1B).

Detection of apoptotic body formation and chromatin condensation

The anti-apoptotic role of *Af* conidia was verified on advanced stages of apoptosis, when TNF- or STS-treated cells were simultaneously exposed to conidia for 6 h (see Fig. 2(A) as a representative example of the inhibition of late stages of apoptosis by conidia). Quantification of the differences in the number of apoptotic cells for TNF- and STS-induced apoptosis showed that $45 \pm 5\%$ of TNF-treated and $23 \pm 4\%$ of STS-treated cells displayed features of apoptosis, while CHX-treated cells (data not shown) or untreated control cells were unaffected by apoptosis (Fig. 2B). Exposure of A549 cells to *Af* conidia inhibited the apoptotic process: the number of apoptotic cells induced by both inducers was significantly decreased ($P < 0.05$) in the samples of cells treated with the inducers and exposed to *Af* conidia (Fig. 2B). Figure 2 shows that the anti-apoptotic effect of *Af* conidia was dose dependent. Exposure to 5×10^6 , 10^7 and 10^8 conidia decreased the number of apoptotic cells from $45 \pm 5\%$ in control to $33 \pm 3\%$, $25 \pm 2\%$ and $20 \pm 2\%$ for TNF-induced apoptosis and from $23 \pm 4\%$ in control to $16 \pm 2\%$, $10 \pm 2\%$ and $7 \pm 1.5\%$ ($P < 0.05$) for STS-induced apoptosis, respectively. In cell cultures containing 5×10^8 conidia without inducers of apoptosis, 1.5% of the cells were apoptotic.

Detection of nuclear DNA nicks using TUNEL technology

The inhibitory effect of *Af* conidia on advanced stage of apoptosis was confirmed by TUNEL. After 6 h incubation, the cells treated either with TNF- α or STS have shown intense positive TUNEL staining in comparison with control cells or cells exposed only to *Af* conidia (Fig. 3). The number

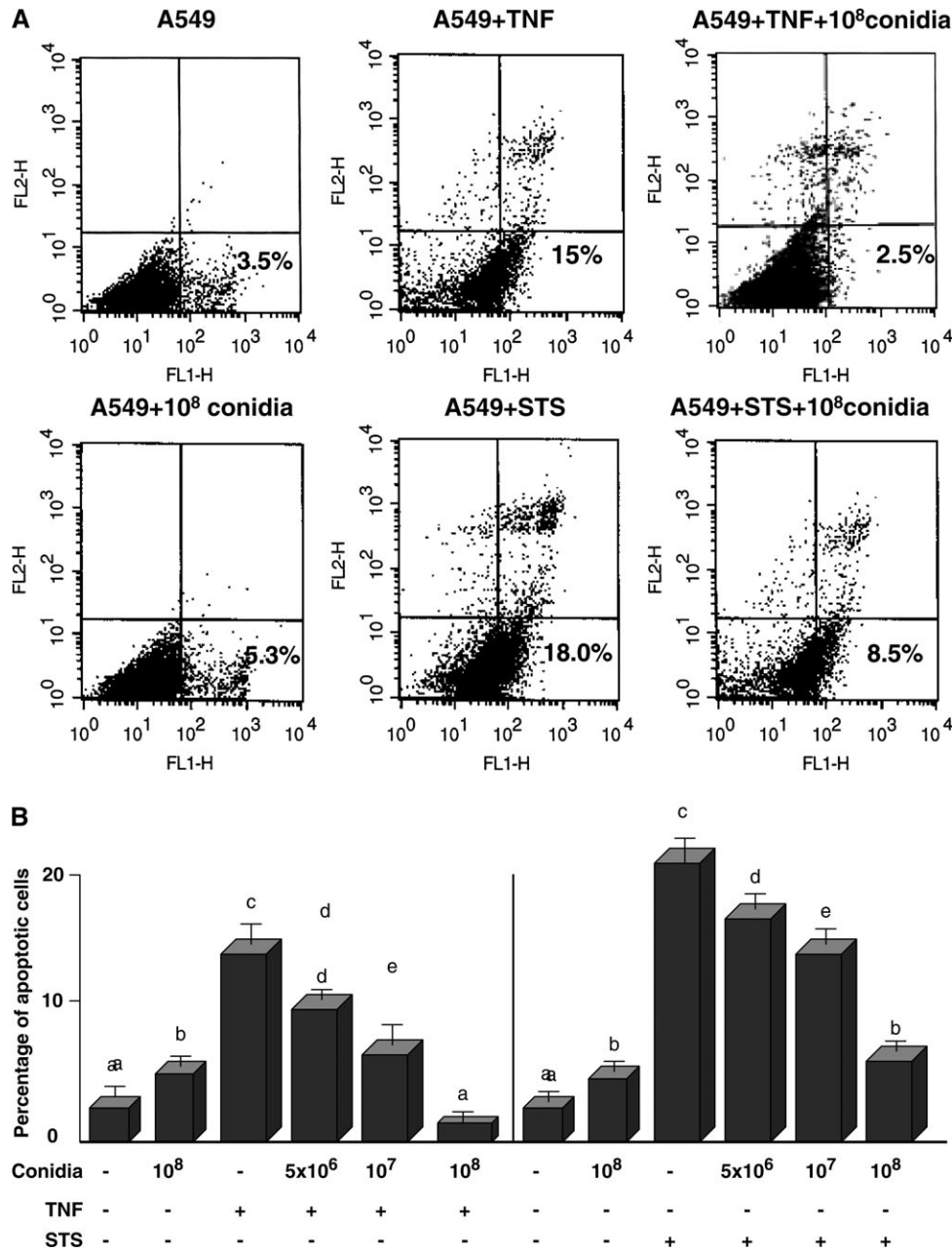


Fig. 1. (A) An example of contour diagram of FITC–Annexin V/PI obtained by flow cytometry of A549 cells exposed to Af conidia and treated with the inducers of apoptosis is shown. FITC–Annexin V fluorescence is presented on the horizontal axis and PI fluorescence on the vertical axis. The lower left quadrant of the cytograms represents the viable cells (FITC[−]/PI[−]) and the upper right quadrant represents the necrotic cells (FITC⁺/PI⁺), while apoptotic cells were recorded in the lower right quadrant (FITC⁺/PI[−]) and dead cells in the upper left quadrant (FITC[−]/PI⁺). In each case, the fluorescence of 10 000 cells was assessed. Percentage of the early-stage apoptotic cells is marked in the right lower quadrant. Abbreviations: (A549), control untreated A549 cells; (A549 + STS), A549 cells treated with 1 μ m of STS; (A549 + STS + 10⁸ conidia), A549 cells treated with 1 μ m of STS and exposed to 10⁸ of conidia; (A549 + 10⁸ conidia), A549 cells exposed to 10⁸ of conidia; (A549 + TNF), A549 cells treated with 20 ng ml^{−1} of TNF- α and 2 μ g of CXH; (A549 + TNF + 10⁸ conidia), A549 cells treated with 20 ng ml^{−1} of TNF- α , 2 μ g of CXH and exposed to 10⁸ of conidia. (B) Inhibition of apoptosis is depending on the dose of the conidia. The percentage of apoptotic cells was calculated with data from triplicates of four experiments. Means followed by the same letter are not significantly different. +, presence; −, absence of TNF, STS or conidia.

of apoptotic cells after TNF and STS treatment reached 53 $\pm$ 6% and 20 $\pm$ 4%, respectively. In contrast, control cells remained TUNEL negative, while only 1% of cells exposed to 10⁷ conidia per millilitre were TUNEL positive. The TUNEL assay has also confirmed that exposure to conidia in-

hibited TNF- and STS-induced apoptosis in A549 cells. Exposure to 10⁷ conidia per millilitre decreased the number of TUNEL-stained cells in TNF-treated culture from 53 $\pm$ 6% to 22 $\pm$ 4% (P < 0.05) and in STS-treated culture from 20 $\pm$ 4% to 5 $\pm$ 2% (P < 0.05).

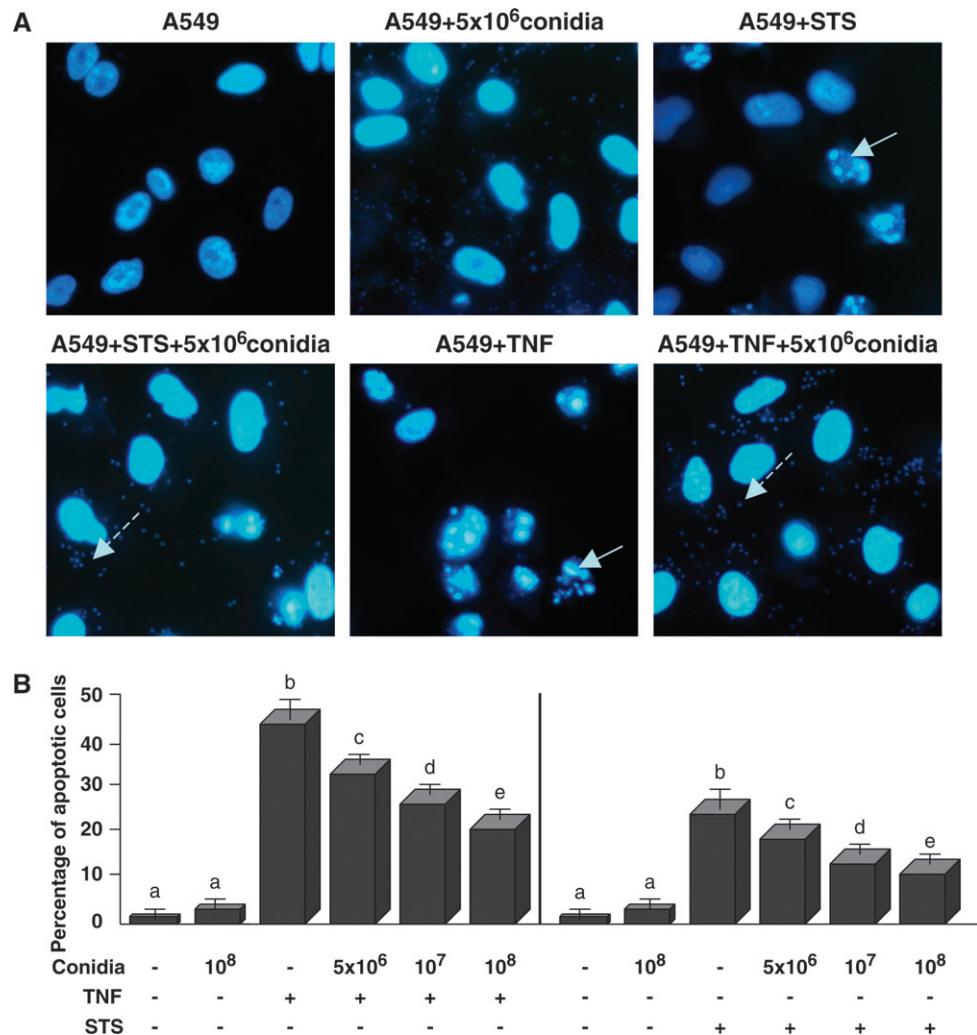


Fig. 2. (A) Conidial inhibition of apoptotic body formation/chromatin condensation induced by 6 h of STS or TNF treatment. After 6 h of treatment with 5×10^6 conidia (the late stage of apoptosis), in the presence or absence of the inducers of apoptosis ($1 \mu\text{M}$ STS or 20 ng ml^{-1} of TNF and $2 \mu\text{g}$ of CXH per millilitre), cells were fixed with PBS-4% PFA for 1 h. Chromatin condensation and apoptotic bodies' formation were assessed by staining with Hoechst 33342. Solid arrows indicate apoptotic bodies. The broken line arrows indicate Af conidia. One representative experiment is shown. (B) Inhibition of late stage of apoptosis also depends on the dose of the conidia. The percentage of cells with apoptotic bodies was computed from triplicates of four experiments. Means followed by the same letter are not significantly different. +, presence; -, absence of TNF, STS or conidia. Abbreviations are as in Fig. 1.

Implication of caspase-3 in the inhibition of TNF-induced apoptosis by Af conidia

Since caspase-3 has been implicated as a general effector of apoptosis (36), we used western blot analysis to test whether or not caspase-3 activation took place in cells treated with the inducers of apoptosis and Af conidia. Anti-caspase-3 antibody detected a 32-kDa band corresponding to pro-caspase-3 in the control cell lysate (Fig. 4). After 6 h of TNF treatment, the 32-kDa band disappeared and a 17-kDa band corresponding to the active form of caspase-3 appeared. In TNF-treated cells exposed to Af conidia, the intensity of the 17-kDa band decreased, while the 32-kDa pro-caspase-3 was not detected. The effect was dose dependent: the ratio of the intensity of the 17-kDa band in control to that in TNF-treated cells was 0.80, which dropped to 0.75 and 0.30 for cultures treated with 10^7 and 10^8 conidia, respectively. The intensity of the 17-kDa band

in lysate of the cells exposed to 5×10^6 conidia was equal to those in TNF-treated cells without conidia (data not shown). These results suggest that conidial inhibition of apoptosis in TNF-treated A549 cells was due to the degradation of either caspase-3 or pro-caspase-3. In contrast, in the lysates of cells treated with STS or cells treated with STS and 10^7 or 10^8 conidia, the 32-kDa band only was observed. The results showed that under our experimental conditions, STS-triggered apoptosis of A549 cells was caspase-3 independent.

Involvement of caspase during TNF-induced apoptosis was confirmed by the use of the caspase inhibitor Z-VAD.FMK. Exposure to Z-VAD.FMK did not affect STS-induced apoptosis (Fig. 5). Supplementation of conidia with 0.125 to 0.5 mM Z-VAD.FMK did not increase the inhibition of apoptosis induced by the conidia (Fig. 5). In contrast, Z-VAD.FMK inhibited TNF-induced apoptosis in a dose-dependent manner, while

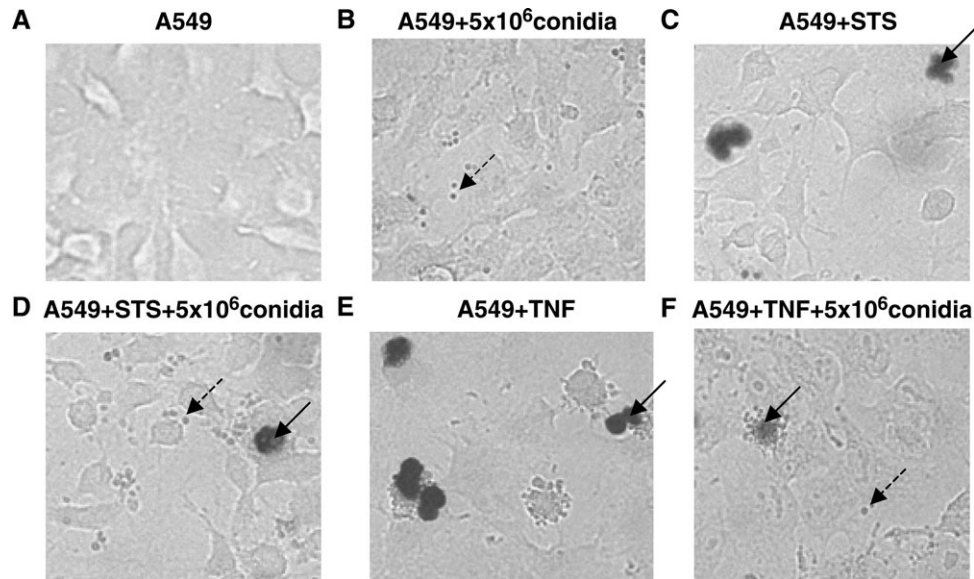


Fig. 3. Example of the inhibition of apoptotic cell death by Af conidia using TUNEL. The arrows indicate positive TUNEL staining. One representative experiment is shown. Same treatment as cells of Fig. 2; legend and abbreviations as in Fig. 1 caption.

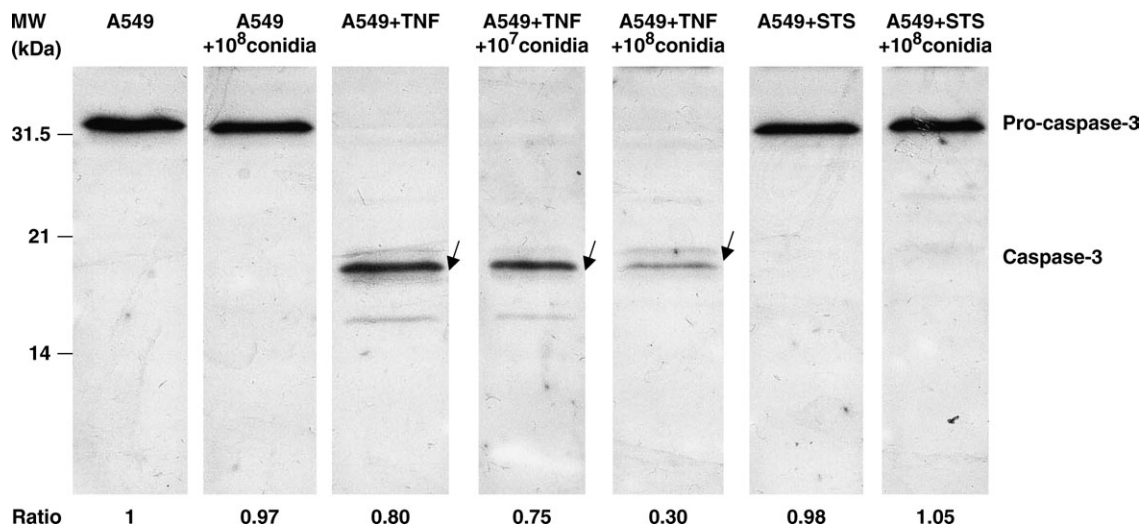


Fig. 4. Western blot analysis of pro-caspase-3 processing in TNF- and STS-treated A549 cells. Control A549 cells, cells exposed to Af conidia in the presence or absence of inducers of apoptosis ($1 \mu\text{M}$ STS or 20 ng ml^{-1} of TNF and $2 \mu\text{g}$ of CXH per millilitre) and cells treated only with the inducers of apoptosis for 6 h (the late stage of apoptosis) were harvested and cell extracts were used for western blot analysis. Pro-caspase-3 and caspase-3 were detected by anti-caspase-3 antibody. At the left side, the molecular weight standards are shown. The arrows indicate active 17-kDa caspase-3. The values of the band intensities were divided by the value of the band corresponding to pro-caspase-3 in the control cells. The ratio is shown at the bottom of the figure. Abbreviations are as in Fig. 1.

control cultures without the reagent showed no change in the number of apoptotic cells (Fig. 5). Furthermore, it was shown that the inhibitory effect of Z-VAD.FMK was additive to the effect induced by conidia, suggesting that the inhibitions by Z-VAD.FMK and conidia might both target caspase (Fig. 5).

Inhibition of apoptosis in TNF- and STS-treated cells is induced specifically by Af conidia

As shown in Fig. 6, 5×10^6 Af conidia strongly inhibited TNF-induced apoptosis from $45 \pm 5\%$ to $27 \pm 2\%$ for STS-induced

apoptosis and from $19 \pm 3\%$ to $11 \pm 2\%$ for STS-induced apoptosis. *Cladosporium cladosporioides* conidia, even at doses as high as 10^8 , had no inhibitory effect. Percentage of apoptotic cells exposed to *C. cladosporioides* was $44 \pm 2\%$ versus $45 \pm 5\%$ in control cells for TNF-induced apoptosis and $18 \pm 2\%$ versus $19 \pm 3\%$ in control cells for STS-induced apoptosis, respectively ($P > 0.05$). Moreover, exposure to $2\text{-}\mu\text{m}$ latex beads or PFA-fixed conidia did not induce a significant reduction of apoptosis induced by STS or TNF (Fig. 6). These results support the conclusion that the inhibition of apoptosis is specific and depends on live conidia of Af.

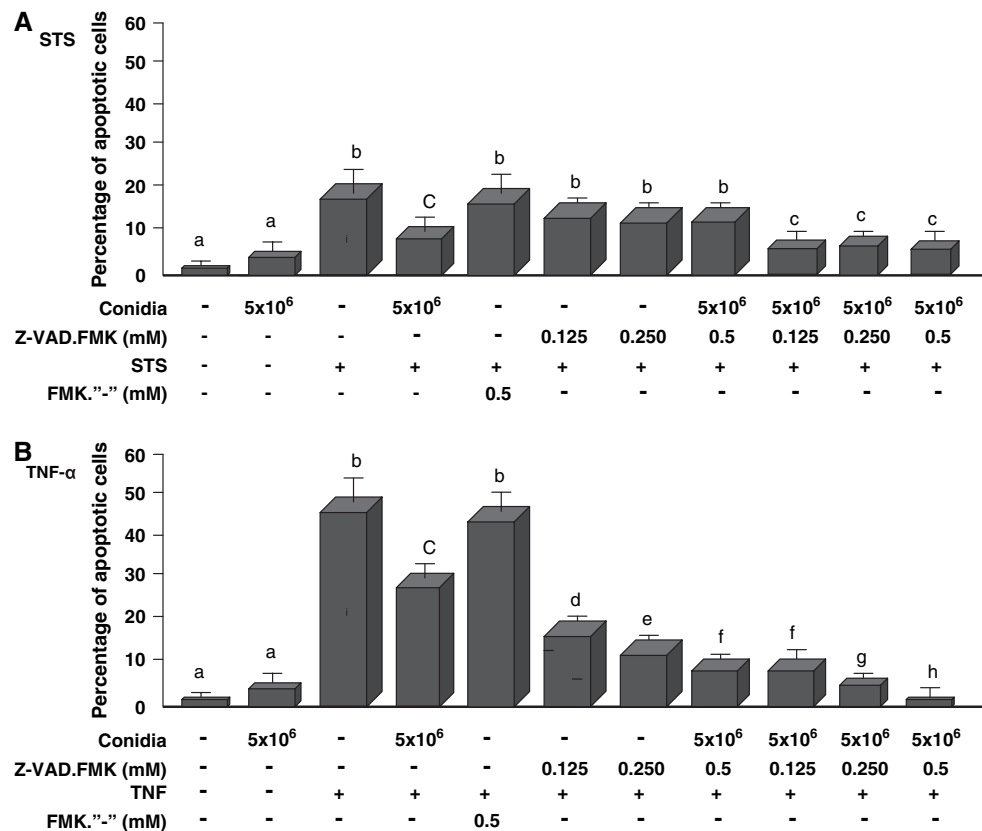


Fig. 5. Caspase inhibitor Z-VAD.FMK does not modulate STS-induced apoptosis (A) but increases conidial inhibition of TNF-induced apoptosis (B). A549 cells were seeded at 5×10^5 cells per well on cover slips and grown for 16 h at 37°C. Caspase inhibitor Z-VAD.FMK or FMK negative control ranging from 0.125 to 0.5 mM was added simultaneously with the inducer of apoptosis to the cells exposed or not to conidia. Nuclear morphology was assessed by staining with the nuclear dye Hoechst 33342. The percentage of the late-stage apoptotic cells was calculated from four experiments. Results are presented as means $\pm$ SEM. Means followed by the same letter are not significantly different. +, presence; -, absence of TNF, STS, conidia or inhibitor.

Af conidia inhibit apoptosis in cells other than A549

Inhibition of apoptosis following exposure to (10^7) conidia also occurred in human tracheal epithelial 16HBE cells treated with TNF and STS, (Fig. 7) although the 16HBE cells were less sensitive to the apoptotic inducers than A549 cells: for example, for TNF and STS, 16HBE cells showed that 16% and 18% of cells, respectively, were apoptotic, the relative numbers were 4% and 6% after exposure to conidia. There were no signs of apoptosis in cultures exposed only to 10^7 conidia or in control cultures receiving no conidia.

After 6 h of incubation with TNF or STS, epithelial primary cells obtained from HNT displayed apoptotic features in $15 \pm 3\%$ of TNF-treated cells and $14 \pm 4\%$ of STS-treated cells while untreated control cells were unaffected by apoptosis (Fig. 8). The exposure of HNT cells to *Af* conidia inhibited TNF- or STS-induced apoptosis. Exposure to 10^7 conidia decreased the number of apoptotic cells from $15 \pm 3\%$ to $4 \pm 3\%$ ($P < 0.05$) for TNF-induced apoptosis and from $14 \pm 4\%$ to $3 \pm 1.5\%$ ($P < 0.05$) for STS-induced apoptosis (Fig. 8). There were no signs of apoptosis in cell cultures containing 10^7 conidia without inducers of apoptosis or in control cultures receiving no conidia. Exposure to PFA-fixed conidia did not reduce the number of apoptotic cells significantly.

Conidia also inhibited the apoptosis of A549 cells induced by their co-culture with LPS-activated HAM (as the source of TNF for apoptosis induction). In the co-culture growth conditions, the amount of apoptotic cells was low ($6 \pm 1\%$). After exposure to *Af* conidia, the number of apoptotic cells was significantly reduced (to $2 \pm 1.5\%$) ($P < 0.05$) (Fig. 9).

Discussion

The influence of *Af* conidia on host cell apoptosis has been investigated *in vitro* using epithelial cells since they are the likely primary target of inhaled *Af* conidia. We examined the effect of *Af* conidia on apoptosis of A549 cells treated with TNF- α . TNF- α was selected as an inducer of apoptosis since its role during *Af* infection has been well established: the release of TNF- α has been demonstrated *ex vivo*, using whole blood from healthy volunteers, stimulated by non-viable conidia and hyphal fragments from *Af* (23). Furthermore, it has been shown that TNF- α is produced in the lungs of normal and immunocompromised mice (2 and 6 ng ml⁻¹, respectively) in animals responding to the intra-tracheal administration of *Af* conidia at concentrations that are compatible with those used in this study (37, 38). Resident alveolar

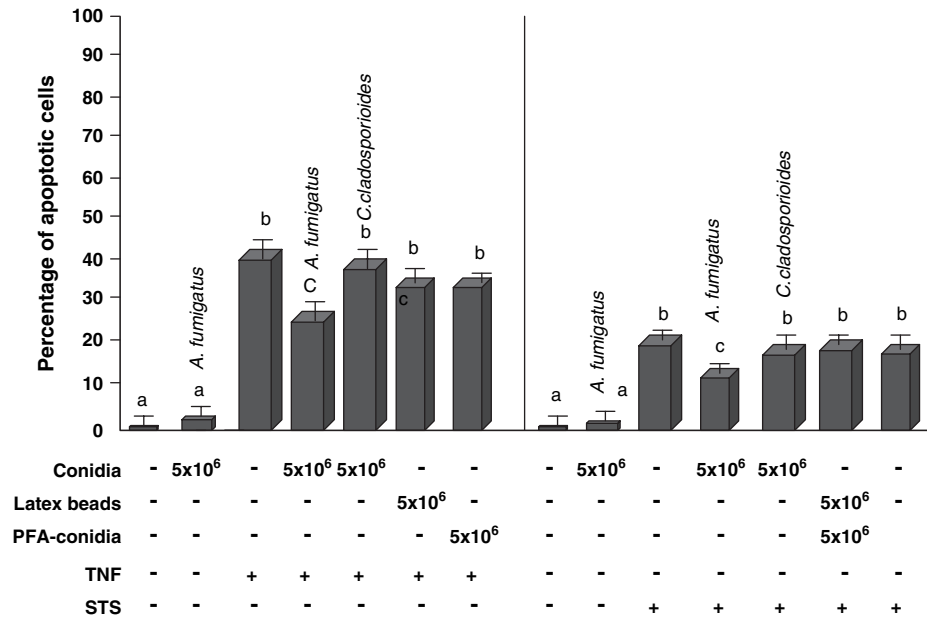


Fig. 6. *Af* conidia specifically inhibit TNF- and STS-induced apoptosis of A549 cells. A549 cells were treated with the inducers of apoptosis (1 μ M STS or 20 ng ml⁻¹ of TNF and 2 μ g of CXH per millilitre) for 6 h in the presence or absence of 5×10^6 conidia of *Af*, *Cladosporium cladosporioides*, PFA-fixed conidia or latex beads. Apoptotic cells were assessed by staining with nuclear dye Hoechst 33342. The percentage of apoptotic cells was calculated from triplicates of four experiments. Results are presented as means $\pm$ SEM. Means followed by the same letter are not significantly different. +, presence; -, absence of TNF, STS or conidia.

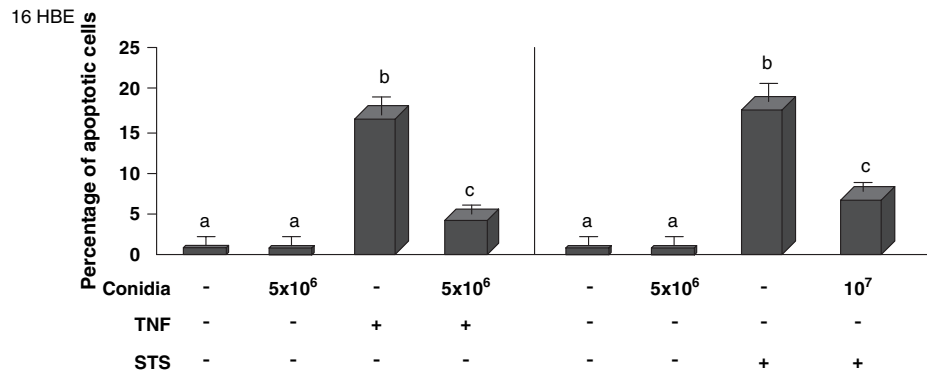


Fig. 7. *Af* conidia decreased the number of apoptotic cells in 16HBE cells. HBE cells were treated for 6 h with 5×10^6 conidia, in the presence or absence of the inducers of apoptosis. Chromatin condensation and apoptotic body formation were assessed by staining with Hoechst 33342. The number of apoptotic cells was compared with control and with cultures incubated either with the inducers or with conidia. The percentage of apoptotic cells was calculated on the base of the data from four experiments. Means followed by the same letter are not significantly different. +, presence; -, absence of TNF, STS or conidia.

macrophages are responsible for the early release of TNF- α , whereas the later release and higher levels of this cytokine are produced by recruited neutrophils (37). Our study has shown for the first time that *Af* conidia do not promote apoptosis of epithelial cells but in contrast are able to inhibit early, as well as late stages of drug-induced apoptosis. The biological significance of this finding is emphasized by the inhibition of apoptosis of A549 cells co-incubated with human-activated macrophages or human respiratory epithelial primary culture cells. Alteration of apoptosis could play a pivotal role in the first step of the interaction between *Af* and the host since it would allow intracellular conidium survival with subsequent germination and host invasion.

It has been reported that many pathogenic agents interact at check(nodal)-points in the cascade of events leading to apoptosis (39). For example, baculovirus contains an anti-apoptotic factor that inhibits caspases (40); EBV (41) and herpes virus (42) encode proteins that are homologues of the cellular anti-apoptotic protein Bcl-2. Thus, in the case of *Af* conidia a strong inhibition of induced apoptosis yet also show a weak augmentation of spontaneous apoptosis.

The complexity of the apoptotic process is illustrated in our study where *Af* conidia are shown to have an inhibitory effect and the response to *Af* is morphotype specific. Such findings were repeatedly observed in immunological reactions to conidia and hyphae of *Af* (43, 44). In the case of apoptosis,

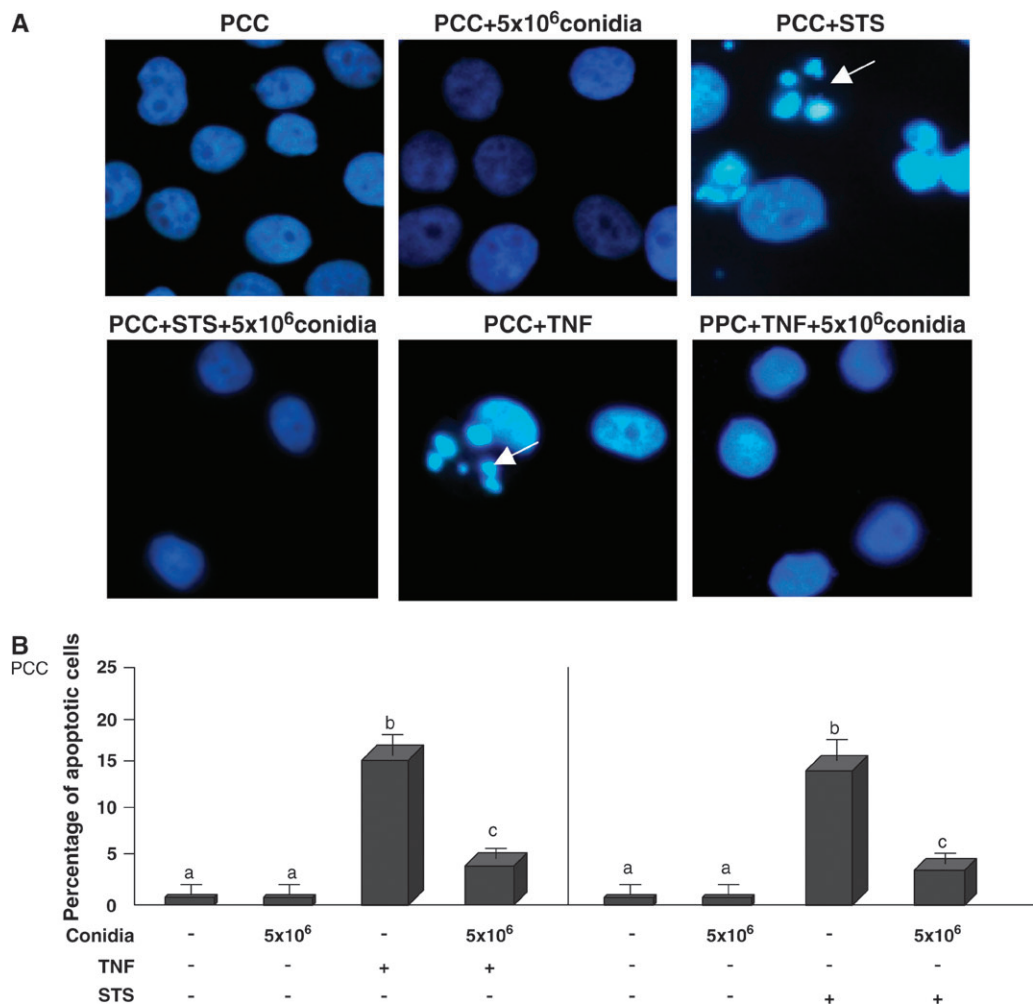


Fig. 8. Inhibition of apoptosis in airway primary culture cells exposed to *Af* conidia. Primary epithelial cells obtained from the HNT were seeded at 5×10^6 cells per well on cover slips and grown for 48 h at 37°C. Induction of apoptosis with STS and TNF and exposure to 5×10^6 conidia have been performed as described above. (A) Analysis of nuclear morphology. The arrows indicated apoptotic bodies. (B) Quantification of the apoptotic inhibition. Means followed by the same letter are not significantly different. +, presence; –, absence of either TNF, STS or conidia.

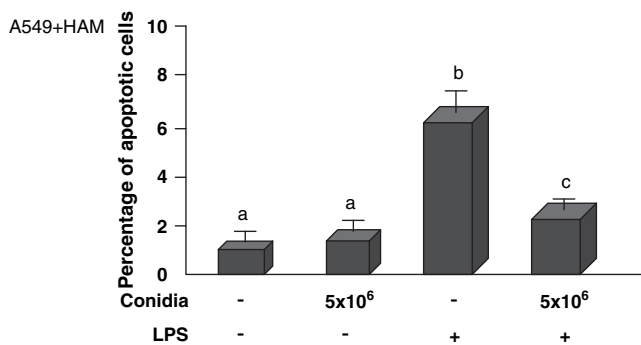


Fig. 9. *Af* conidia inhibit apoptosis induced by co-culture of HAM with A549 cells. HAM were stimulated with $1 \mu\text{g ml}^{-1}$ of LPS and grown for 18 h at 37°C. Subsequently the cover slips containing A549 cells were transferred into the wells with HAM and exposed or not to 5×10^6 *Af* conidia. The number of A549 apoptotic cells assessed by staining with dye Hoechst 33342 was compared in co-culture containing either control HAM or non-stimulated HAM incubated with conidia. Means followed by the same letter are not significantly different. +: presence; -: absence of either LPS or conidia.

the various fungal forms elicit a different response: conidia inhibit apoptosis whereas hyphae secrete gliotoxin, a well known inducer of apoptosis in many cell types, including macrophages and monocytes (12, 45–47).

Processing of pro-caspase-3 was observed in TNF-induced apoptotic cells, resulting in the complete loss of an inactive 32-kDa pro-caspase-3 and the appearance of an active 17-kDa form. We have shown that the level of active caspase-3 as well as pro-caspase-3 was inhibited by the exposure of TNF-treated cells to *Af* conidia. We do not know whether the inhibition of TNF-induced apoptosis by *Af* conidia takes place upstream or downstream of caspase-3 activation. A variety of pathogens can inhibit caspase-3, for example, the anti-apoptotic effect of cytomegalovirus infection was shown to be associated with a significant reduction of caspase-3 activity (48), and the intracellular pathogen *Leishmania major* was found to inhibit the spontaneous apoptosis of neutrophils via a mechanism involving the inhibition of caspase-3 activation (49). Another intracellular pathogen, *Chlamydomonas pneumoniae*, inhibits apoptosis induced by treatment with

drugs or by death receptor ligation. The observed inhibition was associated with a lack of caspase-3 activation in infected cells: the protective effect was confined to the cells inhabited by *Chlamydomophila* (25). It has been reported that herpes simplex virus (HSV-1) interferes with apoptotic processes in infected cells: HSV-1 was able to prevent apoptosis which was induced by various stimuli, including treatment with TNF- α and STS. A further parallel with the situation seen after Af conidia treatment is that wild-type HSV-1 infection itself could induce low-level apoptosis in cells (50, 29). It was concluded that HSV-1 acted at multiple metabolic checkpoints.

The other apoptosis inducer used was STS. If conidia similarly inhibit STS- and TNF-induced apoptosis, the mechanisms of induction and inhibition are clearly different for these two drugs. TNF-dependent apoptosis is induced through the caspase pathway which is blocked by Z-VAD.FMK which in contrast has no effect on STS-induced apoptosis and pro-caspase-3 remains uncleaved. These observations indicate that STS-induced apoptosis in A549 cells does not require caspase activity. The lack of processing of pro-caspase-3 in cells exposed to STS was also reported for HeLa H21 cells (30). While HeLa H21 cells were efficiently killed after TNF or STS treatment, extensive processing of caspase-3 was only observed after TNF treatment. Recently, it was shown that STS-induced apoptosis of some cancer cells, including A549 cells, is caspase independent (51). These results support the view that various apoptotic stimuli may activate different steps of apoptosis. At this stage a role for other proteolytic caspases cannot be excluded.

Using three different convergent methods, we have shown that Af conidia inhibited apoptosis induced by two divergent pro-apoptotic stimuli, TNF and STS. The nature and mode of action of the conidial factors that block apoptosis are unknown: conidial molecules could be present in the airborne conidia and released upon contact with the cell or synthesized *de novo* in the presence of the epithelial cells; two or several molecules may inhibit the caspase-dependent and caspase-independent apoptotic pathways or both pathways could be inhibited by the same molecule: at this stage, we are unable to exclude dependence on the presence of conidia in early stages of germination, not distinguishable by standard light microscopy, releasing active products. Further experiments are required to fully understand the role of conidia in the inhibition of apoptosis.

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Abbreviations

Af	<i>Aspergillus fumigatus</i>
CHX	cycloheximide

DISC	death-inducing signalling complex
HAM	human alveolar macrophages
HNT	human nasal turbinates
HSV-1	herpes simplex virus
PI	propidium iodide
STS	staurosporine
TNF	tumour necrosis factor
TUNEL	terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labelling

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