Inhibition of the alveolar macrophage oxidative burst by a diffusible component from the surface of the spores of the fungus *Aspergillus fumigatus*

Joan Slight, Wendy J Nicholson, Colin G Mitchell, Nathalie Pouilly, Paul H Beswick, Anthony Seaton, Kenneth Donaldson

Abstract

Background **– *Aspergillus fumigatus* is a fungus that grows on dead and decaying organic matter in the environment and whose spores are present ubiquitously in the air. The fungus causes a range of diseases in the human lung. A study was undertaken to demonstrate and partially characterise an inhibitor of the macrophage respiratory burst from the surface of *A fumigatus* spores that could be an important factor in allowing the fungus to colonise the lung.**

Methods **– The spore-derived inhibitor of the respiratory burst of rat alveolar macrophages, as measured by generation of superoxide anion, was demonstrated in Hank's balanced salt solution extracts of four clinical isolates and an environmental isolate of *A fumigatus*. The time course of the release of the inhibitor into aqueous solution was assessed and the cytotoxic potential of the spore-derived inhibitor towards macrophages was tested using the propidium iodide method. An oxygen electrode was used to confirm the superoxide anion measurements. Molecular weight cutoff filters were used to determine the size of the inhibitor as assessed in the respiratory burst assay and also by its ability to inhibit macrophage spreading on glass. The crude diffusate from the spore surface was fractionated by reversed phase high pressure liquid chromatography (HPLC) and the fractions analysed for inhibitory activity, protein, and carbohydrate content.**

Results **– A small molecular weight (<10 kD) heat stable toxin was released from the spores of clinical and environmental isolates of *A fumigatus* within minutes of deposition in aqueous solution. The key effect of the toxin demonstrated here was its ability to inhibit the oxidative burst of macrophages as measured by superoxide anion release. The inhibition was not due to cell death or detectable loss of membrane integrity as measured by permeability to propidium iodide. The toxin was not a scavenger of superoxide anion. Oxygen electrode studies suggested indirectly that the inhibitor acted to inhibit the assembly of the macrophage NADPH-oxidase complex. Fractions of <10 kD also inhibited the spreading of alveolar macrophages, confirming that the toxin had an additional effect on macrophages that leads to loss of adherence or impairment of cytoskeletal function. In reversed phase HPLC fractions the inhibitory activity eluted with an associated carbohydrate, although the exact chemical nature of the toxin remains to be elucidated.**

Conclusions **– This spore toxin may, through its ability to diffuse rapidly into lung lining fluid, diminish the macrophage respiratory burst and play a part in allowing *A fumigatus* to persist in the lung and manifest its well known pathogenic effects. Future research will be focused on further molecular characterisation of the toxin and elaboration of the effect of the toxin on intracellular signalling pathways involved in the activation of alveolar macrophages.**

(*Thorax* 1996;**51**:389–396)

Keywords: *Aspergillus fumigatus*, macrophage, superoxide anion.

Biomedicine and Biotechnology Research Group, Department of Biological Sciences, Napier University, Edinburgh EH10 5DT, UK
J Slight
W J Nicholson
C G Mitchell
N Pouilly
P H Beswick
K Donaldson

Department of Environmental and Occupational Medicine, University of Aberdeen, Medical School, Forresterhill, Aberdeen, UK
A Seaton

Correspondence to:
Dr K Donaldson.

Received 28 June 1995
Returned to authors
23 October 1995
Revised version received
9 November 1995
Accepted for publication
14 November 1995

Aspergillus fumigatus is a fungus that grows on dead and decaying organic matter in the environment and whose spores are present ubiquitously in the air. The fungus causes a range of diseases in the human lung.[1 2] The size of the spores, around 3 μm in diameter and therefore highly respirable, is an important factor in determining the pathogenicity of the fungus. Mullins[3] described the presence of the spores of *A fumigatus* in greater numbers in the lungs at necropsy than would be anticipated from their presence in the air, suggesting that, over and above their respirability, they may have some survival advantage in the human lung over other spores.

Spores of *A fumigatus* survive longer in rabbit lungs following instillation than does a control spore,[4] and macrophages from animals immunosuppressed by cortisone treatment were impaired in their ability to inhibit spore germination in vitro, so mimicking the situation seen in immunosuppressed patients.

The spores and hyphae of *A fumigatus* show several activities that could contribute to its pathogenicity. A diffusible substance from the spores has been found to have the following effects on macrophages: inhibition of phagocytosis,[5] inhibition of the respiratory burst,[6]

and inhibition of chemotaxis and spreading.[7] A soluble extract from both hyphae and spores of *A fumigatus* has been found to detach epithelial cells in culture.[8] In addition, Reichard[9] extensively characterised a protease from the spores and hyphae of *A fumigatus*, a 32 kD chymotrypsin-like enzyme which detached cells in culture and also degraded elastin. Although the authors suggested that this enzyme might be involved in hyphal invasiveness, it is important to note that an antibody raised against the protease stained the spores as well as the hyphae. Protease-mediated detachment of cells in culture is preceded by a reduction in cell spreading which is a characteristic described for the spore product.[7]

When spores deposit in the lung they will make contact first with the epithelial cells that line the airspaces and they should then be phagocytosed by macrophages. Since *A fumigatus* enters the lung as a spore and spores are normally removed efficiently by the macrophage system in the alveolar region, we have based our hypothesis of the pathogenicity of *A fumigatus* on the release of a toxin from spores that prevents efficient phagocytosis and killing.[1] We report here on the ability of alveolar macrophages to mount an oxidative burst in the presence of diffusible material from the surface of the spores. Following confirmation of the release of an inhibitor of the oxidative burst by *A fumigatus* spores, we have characterised the molecular size of this substance by differential centrifugation and have used high pressure liquid chromatography (HPLC) in partial purification.

Methods

Inhouse-bred adult Wistar rats were used throughout.

BRONCHOALVEOLAR LAVAGE

Rats were killed by intraperitoneal injection of Euthatal (Rhône Mérieux Ltd). The lungs were dissected free of the thoracic cavity and lavaged with 4×8 ml sequential volumes of saline at 37°C to obtain the alveolar macrophages which were >95% pure, the remainder being lymphocytes.

FUNGAL SPORES AND *A FUMIGATUS* DIFFUSATE

A single strain of *A fumigatus* was isolated from the sputum of a patient with allergic bronchopulmonary aspergillosis. Spores were obtained from cultures that had been grown for 10 days at 30°C on malt agar. Spore suspensions were prepared in Hank's balanced salt solution (HBSS, Gibco) by gentle homogenisation. Spore counts were performed using an improved Neubauer chamber. To obtain *A fumigatus* diffusate the spores were incubated at a concentration of 10^8/ml in HBSS at 37°C for one hour in an orbital incubator. The soluble *A fumigatus* diffusate was passed firstly through a Whatman number 1 filter paper followed by a 0·22 μm sterile filter and stored at −80°C.

PRESENCE OF *A FUMIGATUS* DIFFUSATE INHIBITOR ON SPORES FROM OTHER STRAINS OF *A FUMIGATUS*

All of the results described in the present paper were obtained with the single clinical isolate referred to above. However, in order to determine whether the inhibitor was present on *A fumigatus* spores in general three further clinical isolates were obtained from Dr Les Milne, Western General Hospital, Edinburgh, plus a standard environmental isolate type STD 2140 from the Mycology Reference Laboratory, Bristol. *A fumigatus* diffusate was made from these as described above and their inhibitory activity in the superoxide assay was determined.

SUPEROXIDE ANION ASSAY

Superoxide anion was measured according to the method of Johnson.[10] The reaction mixture contained 1·2 ml HBSS or *A fumigatus* diffusate, 150 μl dextrose (Sigma) and 150 μl of cytochrome C (Sigma) to yield final concentrations of 2 mg/ml and 1 mg/ml, respectively. Bronchoalveolar cells were suspended at a concentration of 5×10^6/ml in HBSS and 50 μl were added to the reaction mixture. Either phorbol myristate acetate (PMA) (final concentration 1 μg/ml; Sigma) or zymosan (final concentration 100 μg/ml; Sigma) were added as triggers of superoxide anion release. Superoxide dismutase (SOD) controls (final concentration 38 μg/ml; Sigma) were included in each assay. Only the portion of the reduced cytochrome C that could be inhibited by superoxide dismutase was used to estimate the amount of superoxide anion released. All assays were performed in triplicate and incubated at 37°C for two hours. The supernatants were then read at 550 nm and 468 nm, measuring reduced and oxidised cytochrome C, respectively. The absorbance obtained by subtracting the reading at 468 nm from that at 550 nm was used to calculate the SOD inhibitable cytochrome C reduction. This was converted to superoxide anion concentration[10] and expressed as nmol of superoxide anion produced/10^6 cells.

To obtain kinetic measurements of superoxide release, samples were set up as above in the absence and presence of *A fumigatus* diffusate at dilutions of 1/4, 1/8, and 1/16. The cuvette compartment was maintained at 37°C and the absorbance read every five minutes for 30 minutes.

RECOVERY OF OXIDATIVE BURST FOLLOWING *A FUMIGATUS* DIFFUSATE TREATMENT

To determine whether macrophages could recover from the inhibitory effect of diffusate alveolar macrophages were pretreated at 2×10^6/ml with HBSS (control) or a twofold concentrated sample of diffusate. This was obtained by freeze drying the original diffusate and was used in order to keep the ratio of cell number:dilution of diffusate the same as in previous experiments. The preincubation of 90 minutes was carried out in 24-well tissue culture plates coated with poly(2-hydroxyethyl

methylmethacrylate) (Sigma 12 mg/ml) to prevent macrophage attachment. After preincubation the cells were centrifuged at 1000 rpm for five minutes, washed, and counted. Cells pretreated in *A fumigatus* diffusate were assayed in both *A fumigatus* diffusate and HBSS to determine the inhibitory effect of *A fumigatus* diffusate and the ability of cells to recover from this inhibition respectively. A SOD control was included throughout the experiment. The samples were assayed for 90 minutes at 37°C and read at 550 nm and 468 nm as previously described.

TIME COURSE OF *A FUMIGATUS* DIFFUSATE TOXIN RELEASE

To determine at what stage the *A fumigatus* diffusate was released into the supernatant, spores (10^8/ml) were added to HBSS and incubated as previously described for 2, 15, 30, and 60 minutes. At each time point a sample was taken and centrifuged at 3000 rpm for 10 minutes to pellet the spores. The supernatant was removed and filtered through a 0·22 μm filter. The spore pellet was resuspended in fresh HBSS and incubated for a further 60 minutes, then centrifuged and filtered as before. All the supernatants were then tested using the superoxide anion assay.

TESTING FOR SCAVENGING ACTIVITY OF *A FUMIGATUS* DIFFUSATE

To test for a possible scavenging effect of *A fumigatus* diffusate on superoxide anion we used a cell-free superoxide generating system. Superoxide anion was generated by incubating 200 μl xanthine oxidase (20 μg/ml; Sigma) with 200 μl of 400 mM acetaldehyde for 10 minutes at 37°C. This resulted in the generation of approximately 15 nmol superoxide anion. To this superoxide anion anion, 800 μl of the following were added: (1) HBSS (control), (2) SOD (38 μg/ml) which catalyses the conversion of superoxide anion to hydrogen peroxide, (3) *A fumigatus* diffusate (1/4 dilution). Cytochrome C and dextrose were added as before and the mixture incubated for a further 10 minutes at 37°C. The absorbance was read as previously described.

EFFECT OF *A FUMIGATUS* DIFFUSATE ON CYTOCHROME C

In order to ascertain that *A fumigatus* diffusate itself was not reducing cytochrome C or interfering with its reduction, we studied the direct effect of *A fumigatus* diffusate on cytochrome C and on the reduction of cytochrome C by ascorbic acid in the absence of cells.

OXYGEN ELECTRODE STUDIES

To enable us to confirm the oxidative burst in alveolar macrophages, 4×10^6 cells were suspended in 1·5 ml HBSS and added to the oxygen electrode. Potassium cyanide, an inhibitor of mitochondrial oxygen uptake, was added to a final concentration of 0·5 mM. In the first series of experiments, PMA (1 μg/ml) was added prior to *A fumigatus* diffusate (1/4 dilution) while in the second series *A fumigatus* diffusate was added before PMA stimulation. The results were expressed as nmol oxygen consumed/10^6 cells/minute.

VIABILITY

To determine whether the *A fumigatus* diffusate was lethal to cells, 4×10^5 alveolar macrophages were incubated in a range of dilutions of *A fumigatus* diffusate for two hours at 37°C. Following this incubation the percentage viability was determined by the propidium iodide (Sigma) exclusion method.

STABILITY OF THE *A FUMIGATUS* DIFFUSATE

To determine whether *A fumigatus* diffusate was heat stable, samples were heated to 60°C, 80°C, and 100°C for 15 minutes. *A fumigatus* diffusate was also assessed for its ability to withstand three cycles of freezing at −80°C and thawing. The treated *A fumigatus* diffusate was then tested for its ability to inhibit macrophage superoxide anion production.

SIZE CHARACTERISATION OF *A FUMIGATUS* DIFFUSATE TOXIN USING FILTER CENTRIFUGATION

To concentrate and separate a range of molecular weight fractions of *A fumigatus* diffusate samples were subjected to centrifugation using molecular weight cutoff microconcentrators <50 kD, <30 kD, <10 kD, and <3 kD (Macrosep and Microsep, Flowgen). The tubes were centrifuged according to the manufacturer's instructions for varying lengths of time depending on the fraction required. The resulting filtrates were assayed for their ability to inhibit the oxidative burst.

HPLC

A fumigatus diffusate (500 ml) was prepared in 0·9% (w/v) NaCl as described earlier, freeze dried, and resuspended in 5 ml of a buffer containing 50 mM sodium phosphate pH 7·5 (phosphate buffer). The sample (up to 0·1 ml) was loaded onto an LKB Ultropac TSK-ODS 120T reversed phase HPLC column (5 μm pore size, 4·6 × 250 mm) equilibrated with the same buffer. Material adhering to the column was eluted with a linear gradient of 0–100% (v/v) acetonitrile, containing phosphate buffer. Fractions (0·5 ml) were collected and prepared for assay by freeze drying to remove solvents. Fractions, reconstituted in water, were tested for their effect on the macrophage oxidative burst as described above.

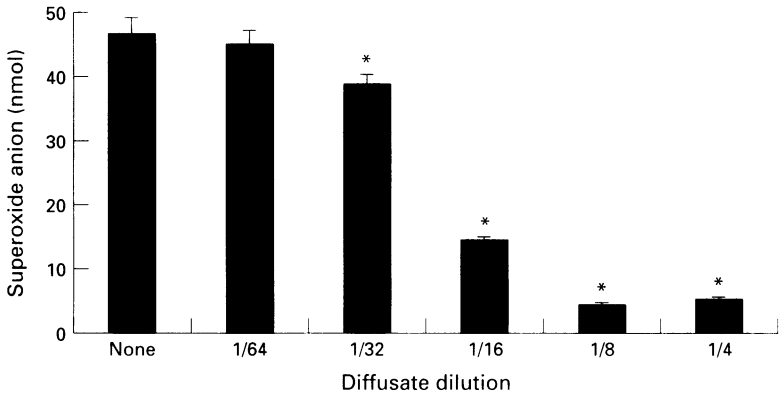

*Figure 1 Effect of increasing dilution of A fumigatus diffusate on the release of superoxide anion by phorbol myristate acetate (PMA) stimulated macrophages. *p<0·05 compared with untreated control.*

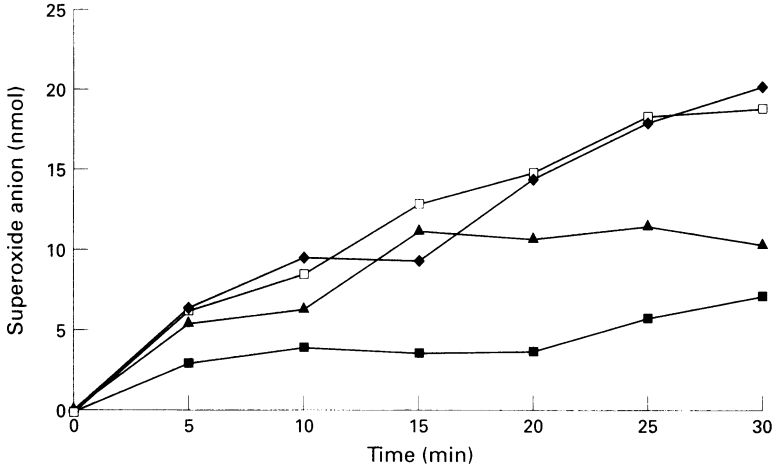

Figure 2 Time course of superoxide anion production by stimulated macrophages alone (♦) or in the presence of A fumigatus diffusate at dilutions of 1/4 (■), 1/16 (▲), or 1/64 (□).

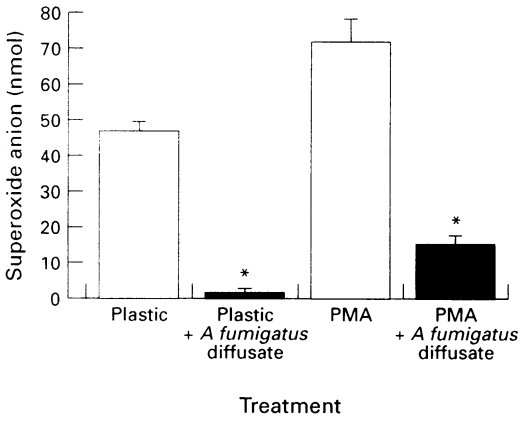

*Figure 3 Inhibition of superoxide anion release by A fumigatus diffusate in unstimulated and phorbol myristate acetate (PMA) stimulated macrophages. *p<0·05 compared with the respective control.*

CARBOHYDRATE AND PROTEIN ANALYSIS

Total sugar was measured by the phenol-sulphuric acid method.[11] Protein was measured using BioRad reagent.

MACROPHAGE SPREADING ASSAY

This assay is based on the ability of macrophages to spread over a period of one hour on glass.[12] Alveolar macrophages were suspended at 2×10^5/ml in Minimal Essential Medium (Gibco) supplemented with 10% (v/v) fetal calf serum. A volume of 0·9 ml of this suspension was added to each chamber of a four chamber slide (Lab-Tek, Gibco) and the slides were incubated at 37°C for two hours to allow the cells to adhere. The cells were then washed in phosphate buffered saline to remove non-adherent cells and either 0·9 ml of whole or fractionated *A fumigatus* diffusate added. A cell control containing medium alone was included in each experiment. Following a further incubation for one hour at 37°C the preparations were fixed and stained with Diff-Quik (Baxter Diagnostics). The area of 400 cells from each treatment was measured using an image analyser (Kontron M14).

DATA ANALYSIS

Unless stated otherwise, experiments were carried out in triplicate and performed on two or three separate occasions. The raw data were subjected to analysis of variance with the Tukey multiple comparison test for significance of differences between treatments with $p<0·05$ taken as the level of significance. In the experiments addressing recovery of macrophage oxidative burst after *A fumigatus* diffusate treatment two way analysis of variance (with experiments and treatments as the two factors) was used because of the unusual variability between experiments; "recovery" and control treatments were then compared by the *t* test using a pooled estimate of the standard error.

Results

DEMONSTRATION OF INHIBITION OF THE MACROPHAGE RESPIRATORY BURST BY *A FUMIGATUS* DIFFUSATE IN THE ABSENCE OF LETHALITY

The *A fumigatus* diffusate derived from 10^8 spores/ml was taken as the initial concentration of spore diffusate. Dilution of the *A fumigatus* diffusate showed that there was activity at 1/32 dilution but this had been diluted out at 1/64 (fig 1). A time course of superoxide anion production (fig 2) confirmed that the activity was effectively diluted out at 1/64 but was present in a clearly dose-related manner at 1/16 and 1/4 dilutions. When the 1/4 dilution of *A fumigatus* diffusate was added to macrophages that were stimulated by adherence to plastic and by PMA there was significant inhibition of the oxidative burst as measured by the generation of superoxide anion in both cases (fig 3). This inhibition of the oxidative burst caused by the *A fumigatus* diffusate was 96% for plastic but was only 80% for PMA. When the viability of macrophages treated with various dilutions of the *A fumigatus* diffusate was tested, there was no evidence of lethality or loss of membrane integrity at any dose compared with the untreated macrophages (data not shown), as shown by propidium iodide staining.

Table 1 Presence of inhibitor on spores derived from three clinical isolates and on an environmental sample of A fumigatus

Isolate	*% inhibition of the respiratory burst*
Clinical 1	18·34 (3·49)*
Clinical 2	56·84 (4·50)*
Clinical 3	67·68 (2·01)*
Environmental (STD 2140)	75·15 (7·09)**

Data are mean (SE) of 3* or 2** separate experiments.

Table 2 Effect of phorbol myristate acetate (PMA) and A fumigatus diffusate (AfD) on oxygen consumption by cyanide treated macrophages

	Treatment 1	*Oxygen consumption (%)*	*Treatment 2*	*Oxygen consumption (%)*
Experiment 1	PMA	91·4	AfD	111·2
Experiment 2	PMA	53·0	AfD	95·5
Experiment 1	AfD	20·5	PMA	10·3
Experiment 2	AfD	31·3	PMA	7·7

Data are percentage oxygen consumption above control levels.

PRESENCE OF *A FUMIGATUS* DIFFUSATE INHIBITOR ON OTHER STRAINS OF *A FUMIGATUS*

The experiments using isolates of *A fumigatus* other than that used throughout this paper are shown in table 1. All of the isolates caused a significant degree of inhibition of the respiratory burst, although there was some variability.

OXYGEN ELECTRODE STUDIES

We confirmed the inhibition of the oxidative burst of macrophages treated with the *A fumigatus* diffusate by the use of an oxygen electrode. Cells were treated with cyanide to inhibit the mitochondrial respiration related uptake of oxygen and data were normalised, with 100% representing the amount of oxygen uptake by cyanide treated cells. The uptake of oxygen was followed in cyanide treated cells further exposed to PMA then *A fumigatus* diffusate, and *A fumigatus* diffusate then PMA (table 2).

From the data it is clear that *A fumigatus* diffusate inhibits the uptake of oxygen caused by PMA stimulation; however, if PMA is given first then there is no real effect of *A fumigatus* diffusate and, in fact, *A fumigatus* diffusate causes a slight increase in the uptake of oxygen in both cases. These data were confirmed using the superoxide anion assay. In these experiments PMA added concomitantly with *A fumigatus* diffusate caused a 44% inhibition of the oxidative burst ($p<0·05$). When cells were pretreated with PMA for 15 minutes and the *A fumigatus* diffusate then added, the inhibition was lowered to 23% (not significantly different).

Table 3 Demonstration that A fumigatus diffusate does not act by scavenging superoxide

Acetaldehyde + xanthine oxidase	*A fumigatus diffusate*	*Superoxide dismutase*	*Mean (SE) superoxide anion (nmol)*
+	−	−	15·3 (1·5)
+	+	−	17·7 (1·4)
+	−	+	0 (0)

RECOVERY OF THE OXIDATIVE BURST AFTER TREATMENT OF MACROPHAGES WITH *A FUMIGATUS* DIFFUSATE

In view of the non-lethal nature of the *A fumigatus* diffusate we examined whether macrophages treated with *A fumigatus* diffusate recovered their ability to mount an oxidative burst if they were further incubated in the absence of the *A fumigatus* diffusate. There was substantial inhibition of the oxidative burst in the presence of *A fumigatus* diffusate, whilst subsequent incubation in medium without *A fumigatus* diffusate showed recovery of the oxidative burst (all data as mean (SE) nmol superoxide in three separate experiments): control 104·7 (11·1); *A fumigatus* diffusate treated and not allowed to recover 28·86 (7·7); *A fumigatus* diffusate treated and allowed to recover 58·8 (11·0). The approximate doubling of the mean release of superoxide anion shown by the recovered macrophages compared with those remaining in *A fumigatus* diffusate attained a significant increase over the *A fumigatus* diffusate treated release ($p<0·02$) in two way analysis of variance. This analysis was used to take into account the exceptional interexperiment variability arising in these experiments because of the problems of variable adherence between experiments.

TIME COURSE OF RELEASE OF *A FUMIGATUS* DIFFUSATE INHIBITOR FROM THE SPORE SURFACE

In these experiments the rate at which the *A fumigatus* diffusate toxin was released from the surface of spores was studied. The amount of toxin produced by spores after two minutes was no different from that present after 60 minutes (results are mean (SE) superoxide anion release in three repeated experiments): control 61·20(1·09); *A fumigatus* diffusate prepared at two minutes 21·65 (4·09); *A fumigatus* diffusate prepared at 60 minutes 19·05 (2·26). This suggests that the *A fumigatus* diffusate diffuses from the spores into aqueous solution in two minutes.

EXCLUSION OF SCAVENGING AS A MODE OF ACTION OF THE *A FUMIGATUS* DIFFUSATE

To preclude direct scavenging of superoxide produced by the macrophages as an explanation of the preceding results, superoxide was generated in a cell-free system and the effect of *A fumigatus* diffusate studied; SOD was used as a positive control. The results (table 3) show that *A fumigatus* diffusate at a 1/4 dilution, which had dramatic inhibitory activity on the oxidative burst, showed no scavenging activity against superoxide anion.

STABILITY OF THE INHIBITORY ACTIVITY IN *A FUMIGATUS* DIFFUSATE

A fumigatus diffusate retained its inhibitory activity compared with unheated *A fumigatus* diffusate following treatment at 60°C, 80°C, and

Table 4 Heat and freeze stability of the A fumigatus diffusate toxin

Treatment	Mean (SE) superoxide anion (nmol)
Pooled control	61·2 (1·1)
A fumigatus diffusate untreated	17·6 (2·8)
60°C	23·8 (4·4)
80°C	22·7 (3·4)
100°C	36·6 (7·6)
Pooled control	59·7 (2·6)
A fumigatus diffusate freeze thaw once	8·5 (1·4)
A fumigatus diffusate freeze thaw twice	6·2 (0·1)
A fumigatus diffusate freeze thaw thrice	0 (0)

Table 5 Presence of A fumigatus diffusate in fractions produced by centrifugation through various sizes of molecular weight filter

Treatment	Superoxide anion production (nmol)
None	52·8 (3·0)
Whole *A fumigatus* diffusate	26·4 (2·1)*
<100 kD	24·0 (2·2)*
<50 kD	26·1 (2·1)*
<30 kD	27·1 (1·5)*
<10 kD	25·7 (1·2)*
<3 kD	41·3 (1·7)
<1 kD	46·3 (2·1)

* $p<0.05$ compared with no treatment.

100°C (table 4). There was a suggestion that the 100°C treatment was less inhibitory than the unheated, but there was no significant difference between unheated and 100°C treatment. Likewise, three cycles of freeze thawing had no effect on the inhibitory activity of *A fumigatus* diffusate (table 4).

SIZE CHARACTERISATION OF THE *A FUMIGATUS* DIFFUSATE TOXIN

When the *A fumigatus* diffusate was centrifuged through various molecular weight cutoff filters it was clear the inhibitory activity was less than 10 kD but not less than 3 kD in size (table 5). The ability of the *A fumigatus* diffusate to inhibit the spreading of alveolar macrophages was also assessed in the <30 kD and <10 kD size fractions. The results, shown in fig 4, reveal that the whole *A fumigatus* diffusate caused a marked

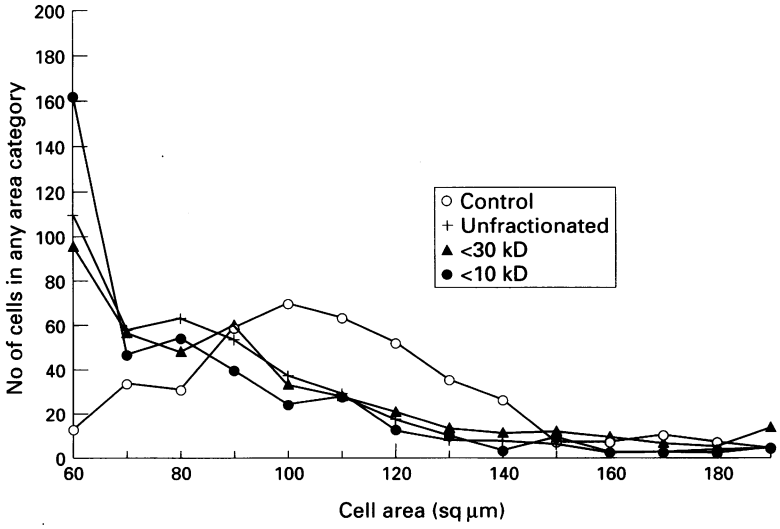

Figure 4 Effect of different molecular weight fractions of A fumigatus diffusate on macrophage spreading.

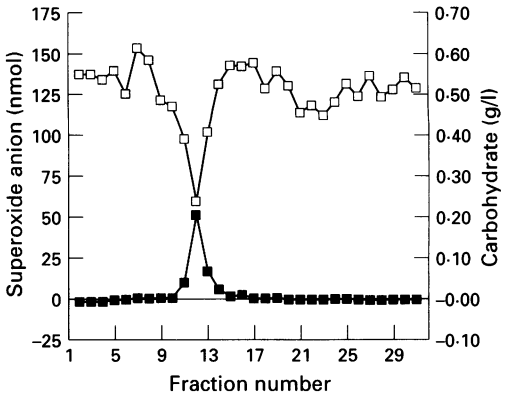

Figure 5 Effect of HPLC fractions on superoxide anion production by macrophages (□) and their carbohydrate content (■).

reduction in the spreading of macrophages and that this activity was present in <30 kD and <10 kD size fractions.

HPLC, PROTEIN, AND CARBOHYDRATE ANALYSIS

When HPLC fractions of *A fumigatus* diffusate were tested for their ability to inhibit the oxidative burst of macrophages there was inhibitory activity in fractions 10, 11, and 12. This corresponded to material which did not adhere to the reversed phase column and also to a peak of carbohydrate (fig 5). There was 10 μg/ml protein in the whole *A fumigatus* diffusate but no detectable protein in any of the active fractions. Material which adhered to the column was eluted with acetonitrile revealing several peaks of absorbance at 206 nm. None of these fractions showed activity (data not shown).

Discussion

This study has confirmed the presence of an inhibitor of the macrophage oxidative burst that is released from the surface of the spores of *Aspergillus fumigatus*.[6] Although the study was carried out using a single clinical isolate of *A fumigatus*, limited experiments were carried out with three more clinical and a single standard environmental isolate. The presence of the inhibitor on the spores of all of these isolates suggests that this inhibitor is universally present on *A fumigatus* spores.

The ability of *A fumigatus* diffusate toxin to inhibit the levels of superoxide produced by macrophages was not a consequence of scavenging of the superoxide anion as has been reported for other organisms,[13] but was a genuine effect on the generation of the oxidative burst. Neither was the inhibition a result of killing of the macrophages since no lethality could be detected by a standard assay at dilutions of *A fumigatus* diffusate that caused inhibition of the oxidative burst. The *A fumigatus* diffusate toxin was able to inhibit the oxidative burst of macrophages responding to plastic adherence and PMA stimulation. This indicates that the *A fumigatus* diffusate toxin acts at a point in the common pathway on which both of these stimuli converge.

The rapid release of the toxin, most of the activity being released in the first two minutes, suggests that the activity is present in a freely available form on the surface of the spore where it can enter solution with ease; it is therefore unlikely to be a spore metabolite. This suggests that the *A fumigatus* diffusate could easily enter solution in lung lining fluid following deposition where it could exert effects on macrophages and epithelial cells in vivo. The *A fumigatus* diffusate toxin was also stable to refreezing and to heat such that, even after raising the temperature to 100°C, inhibitory activity was still present. This supports the contention that the activity is not a protein, as does the experiment of centrifugation through molecular weight cutoff filters which revealed the activity to be less than 10 kD. However, this does not exclude the possibility that the inhibitor is a small peptide. *A fumigatus* diffusate activity causing inhibition of macrophage spreading was also <10 kD, suggesting that there may be more than one inhibitor or that the inhibitor is pleiotropic, affecting more than one macrophage activity.

HPLC fractionation of the *A fumigatus* diffusate showed the activity to co-elute with a carbohydrate peak; there was no protein present in any of these fractions and, indeed, in the unfractionated *A fumigatus* diffusate there was less than 10 μg/ml protein and in the active HPLC fraction there was no detectable protein. The data therefore suggest that the active component of *A fumigatus* diffusate is of small molecular weight (<10 kD) and may have a carbohydrate component. It is not therefore the 32 kD chymotrypsin-like enzyme derived from *A fumigatus* spores in a previous study.[9]

The hyphae of *A fumigatus* have been found to release a number of other low molecular weight toxins including gliotoxin, helvolic acid, fumigatin, and fumagillin.[14] In addition, the hyphae of *A fumigatus* release an inhibitor of the complement system, the other key defence system of the alveolar region of the lung.[15] Hyphal gliotoxin[16] is believed to be an important factor in allowing the hyphae to grow in tissue. Gliotoxin is able to modulate lung cell functions such as attachment of epithelial cells and fibroblasts dramatically, as well as to inhibit phagocytosis by macrophages; other important functions of the host immune defence are also impaired by gliotoxin, including induction of cytotoxic and alloreactive T cells.[17]

At a molecular weight of 326, gliotoxin is smaller than the size range that we have demonstrated for the *A fumigatus* diffusate toxin, but the binding of gliotoxin to a larger carrier molecule cannot be ruled out. Gliotoxin is reported not to be present in cultures of *A fumigatus* until three days,[18,19] which suggests that it is a hyphal product rather than a spore product. However, the spores and hyphae of *A fumigatus* have a number of antigens in common, as has been demonstrated by the use of monoclonal antibodies.[20] Provisional experiments with commercially available gliotoxin (data not shown) have shown no inhibitory activity against the macrophage oxidative burst, suggesting that the *A fumigatus* diffusate toxin is not gliotoxin.

Many agents cause inhibition of the leucocyte superoxide anion generating system[21] and these can be broadly divided into two groups – those which inhibit the activation of the oxidase in whole cells but do not inhibit the isolated enzyme and those which exert a direct inhibitory effect on the enzyme. Preliminary studies using cell-free extracts of macrophages strongly suggest that the *A fumigatus* diffusate inhibitor is the former (data not shown). Oxygen electrode studies described in this paper revealed that, once activated by PMA, *A fumigatus* diffusate had no effect on oxygen uptake whereas, given prior to PMA, it inhibited oxygen uptake. This again suggests that *A fumigatus* diffusate operates at the level of the activation of the oxidase and, once the complex is assembled, the toxin has no effect. As an inhibitor of activation of the NADPH assembly and activation, *A fumigatus* diffusate could operate at a large number of different points.[22] However, other studies that we have carried out with the *A fumigatus* diffusate have demonstrated its ability to cause rapid and dramatic inhibition of release of the cytokine tumour necrosis factor (TNF) and the level of TNF mRNA. Profound inhibition of the oxidative burst and cytokine secretion – two important macrophage responses that are quite different in final outcomes – suggest that some fundamental process in the intracellular signalling pathways may be inhibited by *A fumigatus* diffusate. Phosphorylation is one obvious candidate since phosphorylation of the p47 component is obligatory for activation of the phagocyte oxidase[22] and phosphorylation of IκB-α is an essential prerequisite for activation of the cytokine transcription factor NF-κB.[23] However, inhibition could be at any of a number of points and the *A fumigatus* diffusate may contain more than one inhibitor and so more than one pathway could be inhibited.

In summary, we have confirmed the existence of a small molecular weight (<10 kD) toxin that is released from the spores of four clinical and one environmental isolate of *A fumigatus* within minutes of deposition in aqueous solution. A principal effect of the toxin is to inhibit the oxidative burst of macrophages without causing cell death. The *A fumigatus* diffusate toxin can be fractionated by reversed phase HPLC enabling us to demonstrate an associated carbohydrate. Further research will be focused on molecular characterisation of the *A fumigatus* diffusate toxin and elaboration of the effect of the *A fumigatus* diffusate toxin on intracellular signalling pathways in macrophages that leads to activation of the NADPH oxidase complex and cytokine gene expression.

1 Seaton A, Robertson MD. Aspergillus, asthma and amoebae. *Lancet* 1989;893–894.
2 Bateman ED. A new look at the natural history of aspergillus hypersensitivity in asthmatics. *Respir Med* 1994;**88**:325–7.
3 Mullins J, Harvey R, Seaton A. Sources and incidence of airborne *Aspergillus fumigatus*. *Clin Allergy* 1976;**6**:209–17.
4 Waldorf AR, Lentz SM, Diamond RD. In vivo bronchoalveolar macrophage defence against *Rhizopus oryzae* and *Aspergillus fumigatus*. *J Infect Dis* 1984;**150**:752–60.
5 Robertson MD, Seaton A, Milne LJR, Raeburn JA. Resistance of spores of *Aspergillus fumigatus* to ingestion by phagocytic cells. *Thorax* 1987;**42**:466–72.

6 Robertson MD, Seaton A, Milne LJR, Raeburn JA. Suppression of host defences by *Aspergillus fumigatus*. *Thorax* 1987;**42**:19–25.
7 Robertson MD, Seaton A, Raeburn JA, Milne LJR. Inhibition of phagocyte migration and spreading by spore diffusates of *Aspergillus fumigatus*. *J Med Vet Mycol* 1987; **25**:389–96.
8 Robinson BWS, Venaille TJ, Mende AHW, McAleer R. Allergens as proteases: *Aspergillus fumigatus* protease directly induces human epithelial cell detachment. *J Allergy Clin Immunol* 1990;**86**:726–31.
9 Reichard, V. Buthner S. Eiffert H. Ruchel R. Purification and characterisation of an extracellular serine protease from *Aspergillus fumigatus* and its detection in tissue. *J Med Microbiol* 1990;**33**:243–51.
10 Johnson RB, Godzik CA, Cohn ZA. Increased superoxide by immunologically activated and chemically elicited macrophages. *J Exp Med* 1978;**148**:115–27.
11 Dubois M, Gillies KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. *Anal Chem* 1956;**28**:350–6.
12 Donaldson K, Bolton RE, Brown DM, Douglas A. An improved macrophage spreading assay – a simple and effective measure of activation. *Immunol Commun* 1984; **13**:229–44.
13 Perry FE, Elson CJ, Mitchell TJ, Andrew PW, Catterall JR. Characterisation of an oxidative response inhibitor produced by *Streptococcus pneumoniae*. *Thorax* 1994;**49**: 676–83.
14 Arruda KL, Mann BJ, Chapman MD. Selective expression of a major allergen and cytotoxin, *Asp f* I in *Aspergillus fumigatus*: implications for the immunopathogenesis of aspergillus-related diseases. *J Immunol* 1992;**10**:3354–9.
15 Sturtevant JE, Latge J-P. Interactions between conidia of *Aspergillus fumigatus* and human complement component C3. *Infect Immun* 1992;**60**:1913–8.
16 Waring P, Eichner RD, Tiwari-Palni U, Mullbacher A. The isolation and identification of a new metabolite from *Aspergillus fumigatus* related to gliotoxin. *Tetrahedron Lett* 1986;**27**:735–8.
17 Müllbacher A, Eichner RD. Immunosuppression in vitro by a metabolite of a human pathogenic fungus. *PNAS* 1984;**81**:3835–7.
18 Müllbacher A, Waring P, Eichner RD. Identification of an agent in cultures of *Aspergillus fumigatus* displaying antiphagocytic and immunomodulating activity in vitro. *J Gen Microbiol* 1985;**13**:1251–8.
19 Eichner RD, AlSalami M, Wood PR, Müllbacher A. The effect of gliotoxin upon macrophage function. *Int J Immunopharmacol* 1986;**8**:789–97.
20 Reijula KE. Monoclonal antibodies bind identically to both spores and hyphae of *Aspergillus fumigatus*. *Clin Exp Allergy* 1992;**22**:547–53.
21 Cross A. Inhibitors of the leukocyte superoxide generating system. *Free Radical Biol Med* 1990;**8**:71–93.
22 Bastian NR, Hibbs JB. Assembly and regulation of NADPH oxidase and NO synthase. *Curr Opin Biol* 1994;**6**:131–3.
23 Brown K, Gerstberger S, Carlson L, Franzoso G, Siebenlist U. Control of IκB-alpha proteolysis by site-specific, signal induced phosphorylation. *Science* 1995;**267**:1485–9.