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Purification and Characterization of Factors Produced by *Aspergillus fumigatus* Which Affect Human Ciliated Respiratory Epithelium

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The mechanisms by which *Aspergillus fumigatus* colonizes the respiratory mucosa are unknown. Culture filtrates of eight of nine clinical isolates of *A. fumigatus* slowed ciliary beat frequency and damaged human respiratory epithelium in vitro. These changes appeared to occur concurrently. Culture filtrates of two clinical isolates of *Candida albicans* had no effect on ciliated epithelium. We have purified and characterized cilioinhibitory factors of a clinical isolate of *A. fumigatus*. The cilioinhibitory activity was heat labile, reduced by dialysis, and partially extractable into chloroform. The activity was associated with both high- and low-molecular-weight factors, as determined by gel filtration on Sephadex G-50. A low-molecular-weight cilioinhibitory factor was further purified by reverse-phase high-performance liquid chromatography and shown by mass spectrometry to be gliotoxin, a known metabolite of *A. fumigatus*. Gliotoxin significantly slowed ciliary beat frequency in association with epithelial damage at concentrations above 0.2 μg/ml; other *Aspergillus* toxins, i.e., fumagillin and helvolic acid, were also cilioinhibitory but at much higher concentrations. High-molecular-weight (≥35,000 and 25,000) cilioinhibitory materials had neither elastolytic nor proteolytic activity and remain to be identified. Thus, *A. fumigatus* produces a number of biologically active substances which slow ciliary beating and damage epithelium and which may influence colonization of the airways.

Aspergillus fumigatus is a widely distributed saprophytic fungus. *Aspergillus* species (most commonly, *A. fumigatus*), unlike many other fungal species, can colonize the respiratory mucosa. This can occur in the normal airway, and the individual may become sensitized, causing allergic bronchopulmonary aspergillosis (14, 16, 27). More commonly, the fungus colonizes damaged airways, such as in patients with healed tuberculosis, bronchiectasis, and cystic fibrosis (20, 21, 24, 25, 30, 39). Occasionally, *A. fumigatus* invades the mucosa, particularly if the immune defenses are compromised. Although the type 3 hypersensitivity reaction to *Aspergillus* antigens in the bronchial wall in allergic bronchopulmonary aspergillosis (14) and the importance of the neutrophil in the host defense against *Aspergillus* spp. (35) are well appreciated, the mechanisms of colonization of the respiratory mucosa are poorly understood.

We have previously shown that culture filtrates of most clinical isolates of *A. fumigatus* slow ciliary beat frequency (CBF) and damage human respiratory epithelium in vitro (1). The production of factors which impair ciliary function in vivo would contribute to colonization by perturbing the mucociliary system, a first-line defense of the airways against inhaled pathogens. Epithelial damage could permit invasion and also release nutrients for fungus growth.

Cilioinhibitory factors are produced by several kinds of bacteria which colonize the bronchial tree, including *Pseudomonas aeruginosa* (2, 15, 28, 42), *Streptococcus pneumoniae* (37), *Haemophilus influenzae* (29), and *Mycoplasma pneumoniae* (7). *A. fumigatus* produces a chymotrypsin-like proteinase which might cause epithelial damage (32). However, this study used amniotic rather than respiratory epithelium. In the present study, we purified a number of cilioinhibitory factors from a clinical isolate of *A. fumigatus* and fully characterized one of the factors.

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MATERIALS AND METHODS

Isolation of fungi. Nine clinical isolates of *A. fumigatus* (AF-1 to AF-9) were obtained from sputum of patients with pulmonary aspergillosis (chronic necrotizing pulmonary aspergillosis, four cases; aspergilloma, five cases) and underlying bronchopulmonary conditions (healed tuberculosis, six cases; emphysema, one case; bullae, one case; bronchiectasis, one case). All patients had normal systemic host defenses. AF-1 was derived from a 59-year-old male patient with chronic necrotizing pulmonary aspergillosis in association with pulmonary bullae. Two strains of *Candida albicans* (CA-1 and CA-2) were also isolated from sputum of two patients who had normal systemic host defenses and oral candidiasis.

Preparation of *A. fumigatus* culture filtrates. Conidia (5×10^7) were taken from potato dextrose agar (Difco Laboratories, Detroit, Mich.) slants previously inoculated with the nine strains of *A. fumigatus* described above. They were each suspended in a flask containing 500 ml of medium 199 tissue culture fluid (Gibco Laboratories, Uxbridge, Middlesex, United Kingdom [U.K.]) and cultured without shaking at 37°C for 7 days. Two milliliters of culture fluid was aspirated from each flask every day, filtered (Millex-GV 0.22-μm-pore-size filter; Millipore, Bedford, Mass.), and stored at −70°C. After 7 days of culture, the fungal mycelium was removed by passing the culture fluid through gauze; then the culture fluid was filtered (Sterivex-GS 0.22-μm-pore-size Millipore filter unit). A proportion was aliquoted for use in characterization experiments, and the rest was freeze-dried and stored at −70°C. Spores (5×10^7) were also taken from potato dextrose agar slants which had been inoculated with two strains of *C. albicans* (CA-1 and CA-2) 3 to 5 days previously. They were each suspended in

a flask containing 500 ml of medium 199 and cultured and prepared in the same way to obtain the culture filtrates. Prior to experiments, the pH of each culture filtrate was measured with an F-22 pH meter (HORIBA Ltd., Kyoto, Japan) and adjusted to 7.4 (original pH ranged from 8.1 to 8.3) by addition of 1 M hydrochloric acid to standardize the ciliary function assay. This adjustment required the addition of no more than 0.5% of the original volume of each culture filtrate.

Effect of *A. fumigatus* culture filtrate on CBF. Human respiratory ciliated epithelium was obtained from the nasal mucosa of normal volunteers by a brushing technique with a cytology brush used without local anesthesia (34). This procedure was approved by the Ethics Committee of the Royal Brompton Hospital. Strips of ciliated epithelium were dislodged by brisk agitation of the cytology brush in medium 199. The suspension of the epithelial strips was then divided into three or four samples which were centrifuged at 200 × *g* for 5 min, and the medium 199 supernatant was aspirated. One pellet of epithelium was resuspended in medium 199 as a control, and the other pellets were suspended in test solutions. Each sample was transferred to a sealed microscope coverslip-slide preparation (2, 42). The slides were randomized at the beginning of each experiment so that the observer was unaware of the nature of the samples that were being tested in each experiment. The ciliated epithelium was viewed directly through a Leitz Dialux 20 phase-contrast microscope at ×320 magnification. CBF was measured as previously described by a photometric technique at 37°C (2, 42). Ten readings of CBF were taken from 6 to 10 epithelial strips at 1-h intervals for 6 h. If more than one reading of CBF was taken from an epithelial strip, the points were well separated on the strip. The position of the strips in the coverslip-slide preparation was recorded so that readings of CBF could be taken from the same epithelial strips at each time point. During each experiment, any ciliary dyskinesia (loss of normal coordinated pattern of ciliary beating), ciliostasis (complete cessation of ciliary beating), or epithelial disruption was noted. Epithelial disruption was assessed subjectively by the same investigator and scored from − to +++: − was normal smooth contour of the epithelial strip; + was undulation of the epithelium; ++ was undulation and extrusion of individual cells from the epithelium; and +++ was complete disorganization of the epithelium.

Characterization of the *A. fumigatus* culture filtrate. Samples of AF-1 culture filtrate were treated as follows prior to assay of their effect on CBF: (i) maintained at +4°C or room temperature for 24 h; (ii) maintained at 56°C for 30 min; (iii) boiled for 5 min in a water bath; (iv) frozen at −70°C for 24 h and then thawed; (v) diluted (2×, 4×, 8×, and 16×) with medium 199; (vi) dialyzed against 10 volumes of medium 199 (molecular weight exclusion, 12,000) with hourly changes of medium 199 for 6 h. In order to obtain the dialysate (molecular weight exclusion, 12,000), another sample of AF-1 culture filtrate was dialyzed against 10 volumes of water with hourly changes of water for 6 h. All changes of water were collected, freeze-dried, and then resuspended in water to the original volume of culture filtrate. The pH and osmolarity were checked before testing on cilia. The last treatment (vii) was extraction twice with 2 volumes of chloroform. The aqueous and chloroform layers were separated. The chloroform was removed by rotary evaporation at 25°C, and the chloroform layer was then reconstituted with 1/100 volume of ethanol and 99/100 volume of medium 199, so that the final volume of the chloroform extract was the same as that of the original culture filtrate.

Extraction and purification of cilioinhibitory factors from culture filtrate. Lyophilized culture filtrate of AF-1 was reconstituted in distilled water (10- to 50-mg/ml final concentration on different occasions; pH 8.5) for gel filtration. Gel filtration of 2 ml of reconstituted AF-1 culture filtrate was undertaken on Sephadex G-50 (70 by 1.5 cm; Pharmacia, Piscataway, N.J.) eluting at 45 ml/h with distilled water. The eluate was monitored in a UV spectrometer at 280 nm. Each fraction (7.5 ml) was collected, and every four fractions were pooled, freeze-dried, and reconstituted with medium 199 for bioassay.

The reconstituted lyophilized culture filtrate of AF-1 was also extracted twice with 2 volumes of chloroform. Chloroform was removed by rotary evaporation at 25°C. High-performance liquid chromatography (HPLC) was carried out on a dual-pump system (Waters Associates, Chester, U.K.). Samples were initially chromatographed on a μBondapak C_{18} column (30 by 0.8 cm) eluting at 2 ml/min with a 30-min linear gradient of acetonitrile-water-heptafluorobutyric acid (30:70:0.1 to 70:30:0.1). A second HPLC step was then carried out on an Ultrasphere octyldecyl silane column (30 by 0.46 cm) eluting isocratically at 1 ml/min in acetonitrile-water-trifluoroacetic acid (36:64:0.1). The HPLC eluate was monitored in a UV spectrometer at 280 and 254 nm. In each case, solvents were removed under vacuum and the samples were resuspended in 50 μl of HPLC-grade ethanol–5 ml of medium 199 for bioassay.

Analysis of cilioinhibitory factors. UV spectroscopy was carried out in methanol on a Perkin-Elmer 551 spectrophotometer. Mass spectrometry was carried out on a Finnigan 4500 mass spectrometer in the fast atom bombardment mode or a VG-Quattro II mass spectrometer in the electrospray mode. The primary ionizing beam for fast atom bombardment-mass spectrometry was xenon, and either glycerol or glycerol-thioglycerol (1:1) was used as the matrix.

Proteolytic and elastolytic activities. General proteolytic activity was assessed by the method described by Burnett et al. (5), using purified human fibronectin (Sigma, Poole, Dorset, U.K.) labelled with sodium-^{125}I (ICN Flow, High Wycombe, Bucks, U.K.). The activity was estimated by comparing the amount of fibronectin degraded by test samples with that degraded by a standard solution of human neutrophil elastase (HNE) and was expressed as micrograms of HNE-equivalent per milliliter. The lower limit of detectable proteolytic activity by this method was 0.05 μg of HNE per ml.

The elastolytic activity of samples was determined in microtiter plates with *N*-succinyl-ala-ala-ala-*p*-nitroanilide (1 mg/ml; Sigma) as previously described (6). Activity was measured by determining the change in A_{410} and was quantified by extrapolation from a standard curve of increasing concentrations of HNE. The lower limit of detectable elastase activity by this method was 0.3 μg of HNE per ml.

***A. fumigatus* mycotoxins.** *A. fumigatus* mycotoxins [gliotoxin, bis-dethio-bis-(methylthio)-gliotoxin, fumagillin, and helvolic acid] were purchased from Sigma Chemical Co. These substances were dissolved in HPLC-grade methanol at 1 mg/ml, stored at −70°C, and appropriately diluted in medium 199.

Statistical analysis. The mean CBFs of control and test preparations were compared at 6 h by the unpaired Student *t* test. The statistics were two tailed.

RESULTS

The effect of nine culture filtrates obtained from clinical isolates of *A. fumigatus* on CBF were examined. The results are shown in Table 1. Eight of the nine culture filtrates of *A. fumigatus* caused a significant fall in CBF, with five of the nine culture filtrates causing a >50% fall in CBF. One culture filtrate (AF-2) did not slow CBF significantly. Eight of the nine culture filtrates (AF-1 and -2 and AF-4 through -9) produced moderate to marked disruption of epithelial integrity. Ciliary dyskinesia was observed in some epithelial strips after 1 to 6 h of incubation in all nine *A. fumigatus* culture filtrates, but this was not a uniform observation. In contrast, medium 199 alone and the culture filtrates of two strains of *C. albicans* had no effect on ciliated epithelium during a 6-h experiment. The cilioinhibitory activity began to appear within 5 days of culture (data not shown), and the activity was elevated further at 7 days. The culture filtrate of AF-1 caused a concentration-dependent slowing of CBF during the 6-h experiment (Fig. 1).

The cilioinhibitory activity in the culture filtrate of AF-1 was characterized, and the results are shown in Table 2 ($n = 6$ for each series of experiments). This activity was stable at −70°C but was partially inactivated over 24 h in solution above 4°C. The cilioinhibitory activity was completely abolished by boiling for 5 min. Dialysis removed approximately 50% of the cilioinhibitory activity, and this could be recovered in the dialysate, which suggested the presence of both high- and low-molecular-weight cilioinhibitory factors. Approximately 50% of the cilioinhibitory activity of a culture filtrate was extractable into chloroform; between pH 3 and 8.5, the extraction was not pH dependent.

When the culture filtrate was fractionated by Sephadex G-50 gel filtration chromatography, cilioinhibitory activity was evenly split between the high- and low-molecular-weight fractions (Fig. 2). The high-molecular-weight fractions were tested for both proteolytic and elastolytic activities. At concentrations sufficient to cause ciliotoxicity, there was no evidence of enzymatic activity.

The chloroform extract of the strain AF-1 culture filtrate was purified further by reverse-phase HPLC on a semipreparative μBondapak C_{18} column. One major peak of cilioinhibitory activity was observed eluting at 14 min and was associated with a UV-absorbing species ($A_{280}/A_{254} = 0.82$; Fig. 3). This activity coeluted with the known *A. fumigatus* product, gliotoxin, but was well separated from both fumagillin and helvolic acid. The full UV spectrum in methanol of the ciliotoxin showed a broad absorbency centered around 266 to 268 nm which was similar, but not identical, to that of authentic gliotoxin (λ_{max} at 268 nm); the absorbency curve was somewhat flattened, implying the presence of other UV-absorbing material in the ciliotoxic fraction. The active fraction was separated further on an analytical Ultrasphere octyldecyl silane column into a number of UV-absorbing substances, one of which coeluted with gliotoxin (fraction II) and its metabolite, bis-dethio-bis(methylthio)-

TABLE 1. Effect of culture filtrates of *A. fumigatus* and *C. albicans* on human CBF in vitro

Strain	CBF (Hz)[a]		Epithelial disruption[b]
	1 h	6 h	
A. fumigatus			
AF-1			
Culture filtrate	13.9 (1.3)	2.6 (3.4)**	+++
Control	14.4 (1.2)	13.5 (1.7)	−∼+
AF-2			
Culture filtrate	13.6 (1.5)	11.7 (2.7)	++
Control	14.4 (1.2)	13.5 (1.7)	−∼+
AF-3			
Culture filtrate	13.3 (1.1)	10.7 (2.9)*	+
Control	14.4 (1.2)	13.5 (1.7)	−∼+
AF-4			
Culture filtrate	12.8 (1.4)	8.4 (2.5)*	++
Control	14.4 (1.2)	13.5 (1.7)	−∼+
AF-5			
Culture filtrate	13.5 (1.3)	11.4 (1.9)*	++
Control	14.4 (1.2)	13.5 (1.7)	−∼+
AF-6			
Culture filtrate	13.9 (1.4)	4.3 (3.1)**	+++
Control	14.6 (1.3)	14.4 (1.9)	−
AF-7			
Culture filtrate	12.6 (2.1)	2.9 (2.6)**	+++
Control	14.6 (1.3)	14.4 (1.9)	−
AF-8			
Culture filtrate	13.8 (1.4)	4.0 (2.8)**	+++
Control	14.6 (1.3)	14.4 (1.9)	−
AF-9			
Culture filtrate	12.9 (2.3)	3.0 (2.6)**	+++
Control	14.6 (1.3)	14.4 (1.9)	−
C. albicans			
CA-1			
Culture filtrate	13.2 (1.1)	13.2 (2.4)	−
Control	13.6 (1.4)	13.0 (1.9)	−∼+
CA-2			
Culture filtrate	12.9 (1.5)	13.2 (1.6)	−∼+
Control	13.6 (1.4)	13.0 (1.9)	−∼+

[a] All values represent the mean (n = 10) CBF ± standard deviation in culture filtrates compared with mean CBF standard deviation in medium 199 at 1 and 6 h. *, $P < 0.01$, and **, $P < 0.001$, compared with control.

[b] −, normal smooth contour of epithelium; +, undulation of epithelial surface; ++, undulation and extrusion of individual cells; +++, complete disorganization of the epithelium.

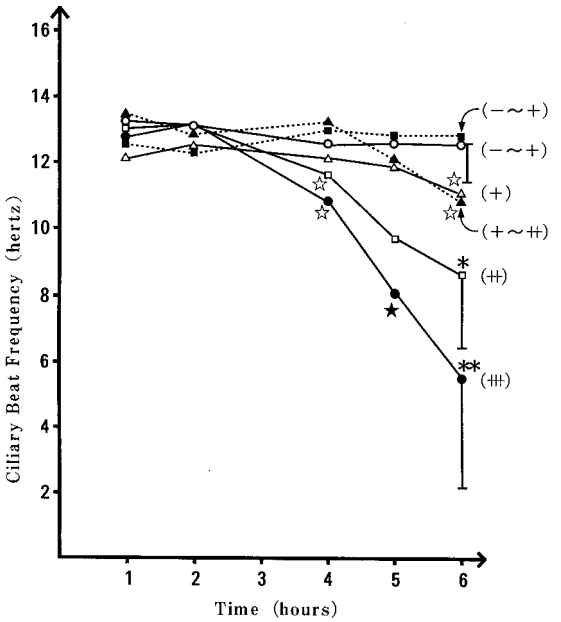

FIG. 1. Effect of *A. fumigatus* culture filtrates on CBF. Mean (n = 10) CBF was measured for 6 h with different dilutions of culture filtrate from *A. fumigatus* AF-1: neat culture filtrate (●), diluted 2× (□), diluted 4× (▲), diluted 8× (△), diluted 16× (■), and control medium 199 alone (○). The degree of epithelial disruption is indicated in parentheses (see Table 1). ☆, ciliary dyskinesia first observed; ★, ciliostasis first observed. Bars represent 1 standard deviation. $P < 0.01$ (*) and $P < 0.001$ (**), compared with control (medium 199).

gliotoxin (fraction I) (Fig. 3, inset). When fractions I and II were mixed separately with authentic gliotoxin and its metabolite and rechromatographed on HPLC, each mixture generated a single symmetrical UV-absorbing peak, confirming coelution. Fraction I was indistinguishable from bis-dethio-bis (methylthio)-gliotoxin in terms of both its fast atom bombardment-mass spectrum {ions at m/z 357 [$M + H^+$], 309 [$-CH_3SH$], 263 [$-(SCH_3)_2$], 261 [$-(CH_3SH)_2$], 245, 243, 233, and 215} and its desorption electron impact mass spectrum (m/z 309 [$M^+ - CH_3S$], 261 [$-CH_3S-CH_3SH$], 251, 243, 233, and 215). With the amount of material available, it was not possible to confirm the presence of gliotoxin in fraction II by fast atom bombardment-mass spectrometry. Purified samples were also analyzed by positive ion electrospray mass spectrometry. Fraction I generated a spectrum which was indistinguishable from that of bis-dethio-bis(methylthio)-gliotoxin with ions at m/z 379 ($M + Na^+$), 357 ($M + H^+$), 309 ($-CH_3SH$), 243, and 214. Within the electrospray mass spectrum of fraction II, ions could be distinguished at m/z 349 ($M + NA^+$), 327 ($M + H^+$), 301, and 245, indicating the presence of gliotoxin. Based on UV peak heights post-HPLC, and assuming that extraction yields are 20 to 30%, we estimate that the concentration of gliotoxin in culture filtrates is between 0.5 and 1 μg/ml. A more accurate estimate of gliotoxin concentration will require the development and validation of a suitable HPLC- or gas chromatography-mass spectrometry-based assay.

The ratio of gliotoxin to bis-dethio-bis(methylthio)-gliotoxin (fraction II/fraction I) varied between culture filtrates; in some cases, there were equimolar amounts of the two materials, while in other samples, the metabolite was in 10- to 100-fold excess. The ratio was not affected by shaking the fungal culture, although this did increase the total amount of gliotoxins produced considerably. In one preparation, there was also evidence of a more polar ciliostatic factor eluting before gliotoxin, with similar spectral properties, suggesting that it was another metabolite.

The effects on human nasal ciliary beating of three commercially available *A. fumigatus* mycotoxins, fumagillin, gliotoxin, and helvolic acid, were examined (n = 6 for each series of experiments) (Table 3). At high concentrations (10 μg/ml), each toxin caused complete ciliostasis and marked epithelial disruption. The effects on CBF were dose dependent, with relative potencies of gliotoxin>fumagillin>helvolic acid; gliotoxin was active at 0.2 μg/ml (0.6 nM). The metabolite of gliotoxin, bis-dethio-bis(methylthio)-gliotoxin, which was present in all culture filtrates, showed no cilioinhibitory activity at 10 μg/ml.

TABLE 2. Characterization of cilioinhibitory factor activity in culture filtrate of *A. fumigatus* AF-1[a]

Experimental conditions	CBF ± SD (Hz) after 6 h		% Ciliary slowing	Epithelial disruption[b]	
	Control	Test		Control	Test
Untreated	13.6 ± 1.5	3.1 ± 3.2**	77.2	−	+++
24 h at −70°C	12.9 ± 2.1	3.6 ± 3.2**	72.1	−	+++
24 h at 4°C	12.9 ± 2.1	7.4 ± 3.1*	42.6	−	+++
24 h at room temp	12.9 ± 2.1	9.0 ± 3.1*	30.2	−	+++
56°C for 30 min	13.6 ± 1.5	10.1 ± 3.7*	25.7	−	+~++
Boiled for 5 min	13.6 ± 1.5	13.5 ± 1.7	0.7	−	−
After dialysis	13.1 ± 1.8	9.0 ± 4.8*	31.3	−	+~++
In dialysate	13.1 ± 1.8	8.7 ± 3.1*	33.6	−	++
After chloroform extraction	12.0 ± 1.4	8.5 ± 2.2*	29.2	−~+	++
In chloroform extract	12.0 ± 1.4	8.0 ± 3.3*	33.3	−~+	++~+++

[a] The CBF ± standard deviation of human nasal epithelium in medium 199 (control) was compared with the culture filtrate of AF-1 (test) before and after treatment in a number of experimental conditions. *, $P < 0.01$, and **, $P < 0.001$, compared with control.

[b] −, normal smooth contour of epithelium; +, undulation of epithelial surface; ++, undulation and extrusion of individual cells; +++, complete disorganization of the epithelium.

DISCUSSION

The conidia of *A. fumigatus* occur commonly in the air, especially during autumn and winter. They may cause sensitization in asthmatic subjects, colonize lung cavities or diseased airways, or occasionally invade the lung tissue, most commonly, after lung infarction or in the immunosuppressed (4, 26). A much more indolent form of this disease, in which pulmonary invasion occurs slowly over weeks, months, or even years, is now recognized (3, 13). The mechanisms by which *A. fumigatus* colonizes the respiratory mucosa are not known. In some conditions, such as bronchiectasis and cystic fibrosis (8, 43), mucociliary clearance is already impaired prior to *A. fumigatus* colonization, and the fungus most commonly colonizes airways in which the defenses are already reduced. However, the production of cilioinhibitory substances which reduce the efficacy of the mucociliary system will delay clearance of inhaled conidia from the airway and remove the mechanical barrier of ciliary beating which prevents inhaled particles reaching the epithelial surface. Once *A. fumigatus* has become established in the airway, fungus-induced epithelial damage will further impair the mucociliary system and may release nutrients for the fungus. Epithelial damage would also provide a route of invasion.

Studies of postmortem lung specimens show that *A. fumigatus* is present more commonly than one would expect from the prevalence of its conidia in the air (23). This supports the idea that *A. fumigatus* may have special properties among the aerospora which allow it to colonize the lung of appropriate hosts long enough to initiate allergic and infective conditions. Macrophages and neutrophils are well recognized as important host defenses against *A. fumigatus* (9, 19, 35), and the fungus is known to produce a number of factors with antiphagocytic activity (22, 31).

Our data suggest that the cilioinhibitory activity of *A. fumigatus* culture filtrates is associated with as yet unidentified high-molecular-weight species together with a known secondary metabolite of the organism, gliotoxin. The bis-dethio-bis-

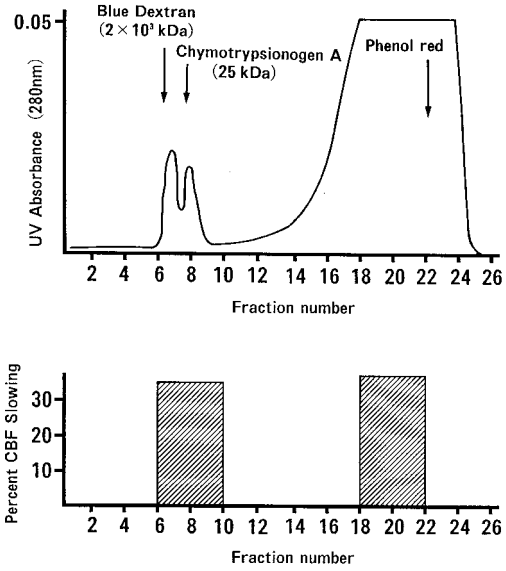

FIG. 2. Sephadex G-50 gel filtration of *A. fumigatus* AF-1 culture filtrate. Cilioinhibitory activity was evenly split between high- and low-molecular-weight fractions. The high-molecular-weight region contained two UV peaks: the first associated with the void volume (>35 kDa), and the second coeluted with chymotrypsinogen A (25 kDa). The low-molecular-weight region corresponded with a third UV peak which eluted immediately before phenol red (<1,000 Da).

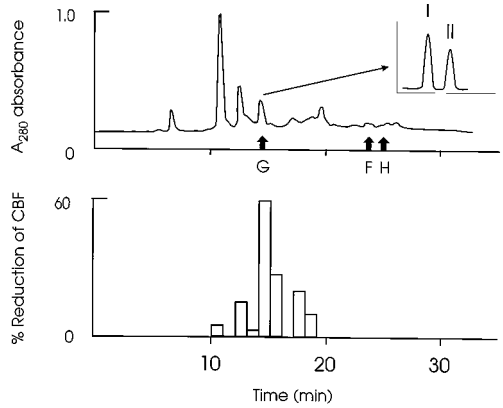

FIG. 3. Reverse-phase HPLC on a μBondapak C_{18} column of the chloroform extract of *A. fumigatus* AF-1 culture filtrate. The major peak of cilioinhibitory activity (fraction 14) coeluted with authentic gliotoxin (arrow G). Gliotoxin was well separated from fumagillin (arrow F) and helvolic acid (arrow H), which eluted at 23.6 and 24.8 min, respectively, but coeluted with its metabolite, bis-dethio-bis(methylthio)-gliotoxin. Isocratic reverse-phase HPLC on an Ultrasphere octyldecyl silane column (inset) separated the active fraction into fraction II, which coeluted with authentic gliotoxin, and fraction I, which coeluted with bis-dethio-bis(methylthio)-gliotoxin. Cilioinhibitory activity was associated with gliotoxin.

TABLE 3. Cilioinhibitory effects of *A. fumigatus* mycotoxins on human respiratory epithelium in vitro

Mycotoxin concn (μg/ml)	CBF ± SD (Hz) after 6 h		% Ciliary slowing	Epithelial disruption[b]	
	Control	Test[a]		Control	Test
Gliotoxin					
0.2	13.2 ± 1.8	10.8 ± 2.1*	18.2	−∼+	++
0.5	13.2 ± 1.8	9.9 ± 1.8*	25.0	−∼+	++
1	13.2 ± 1.8	6.2 ± 4.0**	53.0	−∼+	+++
2	11.8 ± 2.4	4.7 ± 2.7**	60.2	−	+++
5	13.1 ± 2.0	1.4 ± 1.8**	89.3	−∼+	+++
10	11.8 ± 2.4	Ciliostasis**	100	−	+++
Bis-dethio-bis(methylthio)-gliotoxin					
1	13.9 ± 0.9	14.3 ± 1.0	0	−	−
5	13.9 ± 0.9	14.2 ± 1.7	0	−	−
10	13.9 ± 0.9	13.5 ± 1.8	2.9	−	−∼+
Fumagillin					
1	13.1 ± 2.0	10.3 ± 2.6*	21.4	−∼+	+∼++
2	12.8 ± 2.4	8.7 ± 4.0*	32.0	+	++
5	13.1 ± 2.0	7.1 ± 2.1**	45.8	−∼+	++
10	12.8 ± 2.4	Ciliostasis**	100	+	+++
Helvolic acid					
1	13.1 ± 2.0	11.5 ± 2.7	12.2	−∼+	+
2	12.8 ± 2.4	10.4 ± 3.4*	18.8	+	++
5	13.1 ± 2.0	8.9 ± 2.0*	32.1	−∼+	++
10	12.8 ± 2.4	Ciliostasis**	100	+	+++

[a] *, $P < 0.01$, and **, $P < 0.001$, compared with control.
[b] See footnote *b*, Table 2, for explanation of symbols.

(methylthio) metabolite of gliotoxin was also present in *A. fumigatus* culture filtrates but possessed no cilioinhibitory activity. In other assay systems based on viral replication, modification of the S-S bridge in gliotoxin by either reduction or reduction and *S*-methylation also caused a reduction in bioactivity (40, 41) (Fig. 4). Whereas the dithiol can readily be reoxidized to the active S-S bridged form, *S*-methylation will irreversibly inactivate gliotoxin.

Two other *A. fumigatus* toxins, helvolic acid and fumagillin, also slowed CBF, although at much higher concentrations. There was no evidence for ciliostatic activity eluting on HPLC at the position of these two substances, implying that the low-molecular-weight ciliotoxic activity in culture filtrates is associated with gliotoxin. It is possible, however, that altered growth conditions of *A. fumigatus* in the airways in vivo may lead to the production of other ciliotoxins. Indeed, in one culture filtrate there was evidence of another more polar gliotoxin-like substance.

The mechanism by which gliotoxin alters CBF is not known.

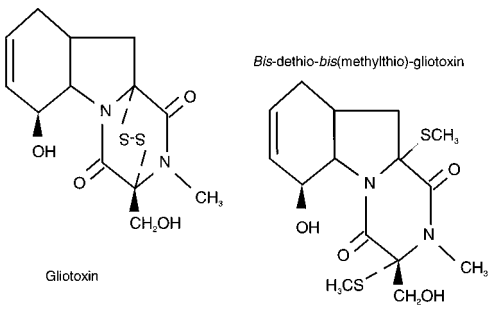

FIG. 4. Chemical structures of gliotoxin and its metabolite, bis-dethio-bis-(methylthio)-gliotoxin.

There is evidence showing that another chemically distinct low-molecular-weight ciliotoxin called pyocyanin, which is produced by *P. aeruginosa*, affects CBF via a reduction in intracellular cyclic AMP and ATP levels (17). Treatment with either the *A. fumigatus* culture filtrate or authentic gliotoxin also caused epithelial damage after 6 h. Although the disruption of the epithelium was similar to that described with pyocyanin (15, 42), the epithelial disruption caused by gliotoxin occurred concurrently with the slowing of CBF, suggesting that the two effects were closely related. It is known that gliotoxin can induce DNA damage through substrate cycling and the production of active oxygen species (11). Similarly, it has been suggested that the cellular damage caused by pyocyanin, which is distinct from the slowing effect on cilia, also occurs through this mechanism (17). DNA damage has also been proposed as the mechanism by which hydrogen peroxide slows ciliary beating (12). The consequence of oxidative damage to DNA is activation of the DNA repair enzyme, which utilizes NAD as a cofactor. This could lead to interference with energy metabolism in the cell because of depletion of intracellular NAD, which is an essential cofactor in the Krebs cycle. Normal mitochondrial generation of ATP is necessary for ciliary beating (18, 36).

Apart from its action on cilia, gliotoxin has a number of other biological activities. For example, it inhibits phagocytosis by rodent macrophages, adherence to plastic by human peripheral blood monolayers, the bactericidal activity of peritoneal macrophages, and the basal rate of hydrogen peroxide production by human neutrophils (10). These effects occur at concentrations of gliotoxin similar to those which cause slowing of CBF (10, 22). The effect of gliotoxin on phagocytes is not associated with cell death, and in the case of macrophages it is associated with absence of cellular projections, suggesting an effect on the cell membrane or cytoskeleton (10), both of which, like ciliary beat, may be energy-dependent events. Glio-

toxin also inhibits RNA synthesis (33) and possesses immunosuppressive activity in vivo (38).

We conclude that substances which slow ciliary beat and cause disruption of epithelial integrity are released by *A. fumigatus* in culture. These include gliotoxin and other uncharacterized high-molecular-weight factors. If these factors are produced in sufficient quantity in vivo, they may play important roles in the pathogenesis of infection of the airways by *A. fumigatus.*

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