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Suppressive effects of *Aspergillus fumigatus* culture filtrates on human alveolar macrophages and polymorphonuclear leucocytes

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ABSTRACT: *Aspergillus* spp., especially *A. fumigatus* (*Af*) can colonize the airways and the lungs with localized underlying conditions and further invade the surrounding lung tissues, even in subjects without systemic predisposing factors, presumably by escaping the local host defences.

To clarify the mechanisms of colonization and invasion of *Af*, we investigated the *in vitro* effects of *Af* culture filtrates (ACFs) on the functions of human alveolar macrophages (AMs), and polymorphonuclear leucocytes (PMNs). ACFs were obtained by culturing clinically isolated *Af* in Medium-199 at 37°C for 5 days.

In the study of phagocytosis of *Af* conidia by human AMs, 52% of AMs ingested conidia during a 60 min incubation period in Medium-199. However, the percentage decreased to 24% when incubated with a final concentration of 30% ACF in Medium-199. With respect to the antichemotactic activity on human PMNs, 3% ACF was sufficient for significant suppression, and 30% ACF completely inhibited the migration of PMNs. In addition, phorbol myristate acetate (PMA)-induced O_2^- release from PMNs was significantly suppressed in Medium-199 which included 12.5% ACF or more. The antichemotactic activity of ACF was partially abolished by trypsin or chicken egg ovomacroglobulin. When ACF was separated into two fractions (molecular weight >10 and <10 kDa) by dialysis and centrifugation through CL-LGC filters, both fractions retained the antichemotactic activity.

We conclude that *Af* produce several antiphagocytic factors, which can be responsible for the colonization of *Af* in the bronchopulmonary tissues and allow this species to invade surrounding lung tissues in pulmonary aspergillosis by suppressing local host defences.

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Aspergillus fumigatus (*Af*), the commonest pathogenic Aspergillus species in humans, is known to cause a wide spectrum of illness ranging from saprophytic colonization of the bronchial trees to invasive and disseminated disease [1]. Although an acute progressive process occurs most commonly in systemic immunocompromised hosts, *Af* frequently colonizes lung residual cavities or injured airways, such as those in healed tuberculosis, bronchiectasis or cystic fibrosis, and occasionally chronic invasion of the surrounding lung tissues occurs even in patients without apparently compromised systemic host defences [1]. In contrast, pathogenic fungi other than Aspergillus species rarely colonize and invade the bronchopulmonary tissues in similar conditions. However, the mechanisms of colonization and local invasion by *Af* are still poorly understood.

SCHAFFNER and co-workers [2] demonstrated selective protection against conidia of Aspergillus by macrophages and against mycelium of Aspergillus by polymorphonuclear leucocytes (PMNs). They concluded that these lines of defence co-operate in the control and elimination of the fungi [2]. Since the respiratory tract is regarded as the main entrance to the human body for conidia, alveolar macrophages (AMs) seem to play a vital role in preventing germination of conidia in the airways and the lungs. AMs are considered to ingest and kill conidia by nonoxidative mechanisms [3]. PMNs exert the second line of host defence against Aspergillus by killing the mycelial form that has evaded attack by AMs and has germinated in the lung tissue [4, 5]. As Aspergillus hyphae have a long, dichotomously branched, filamentous shape and are nonphagocytosable by a single cell, adhesion to the hyphae and release of the reactive oxygen intermediates and cationic peptides are considered to play the key roles in damage to the hyphal form by PMNs. The strong clinical association of severe neutropenia or dysfunction of PMNs with the high prevalence of invasive aspergillosis also suggests the importance of

PMNs in host defences against the fungi [6]. Thus, it appears that AMs and PMNs form a dual defence system against Aspergillus.

Recently, there have been several reports that some secondary metabolites of *Af*, as well as culture filtrates or cell extracts of *Af*, suppress the host defence mechanisms *in vitro* [7–13]. It has been speculated that mycotoxins, such as gliotoxin [10, 11, 13] and fumagillin, and extracellular proteases [14] produced by *Af* suppress the local host defences, and these suppressive effects may contribute to making colonization easier and, furthermore, leading to a chronic infectious process by *Af*. However, most previous workers who have investigated interactions between secondary metabolites of *Af* and phagocytes *in vitro* have used cells obtained from experimental animals, and not human phagocytes. Therefore, little is known about the virulence determinant of *Af* in human pulmonary aspergillosis. Consequently, it is necessary to clarify the interaction between *Af* and human phagocytes, especially AMs and PMNs, before any extrapolation can be made to human diseases.

In the present study, the antiphagocytic effects of *Af* culture filtrates (ACF) on human AMs and PMNs were evaluated *in vitro*, and in this paper the *Af*-derived factors which might play important roles in suppressing human phagocytic functions are discussed.

Materials and methods

Preparation of fungal culture filtrates

A clinical isolate of *Af*, YN strain, was derived from the sputum of a patient with chronic necrotizing pulmonary aspergillosis. A strain of *Candida albicans* was also clinically isolated from a patient with oral candidiasis. After growth on slants of potato dextrose agar (PDA) (Difco Laboratories) for 5 days at 30°C, conidia (2×10^7) of YN strain of *Af* and spores (2×10^7) of *C. albicans* were cultured in flasks containing 300 mL of Medium-199 (Gibco RBL), without shaking, at 37°C. After 5 days of culture, the fungi were removed by passing the medium through gauze, and each culture filtrate was sterilized by passage through Millex-GV 0.22 µm Millipore-filters (Millipore Co.) and stored at -80°C. Before use in experiments, pH of the culture filtrates was adjusted to 7.4 with 1 M HCl. The sterility of the culture filtrates was tested by 3 days of culture on PDA plates at 37°C.

To assess the change in biological activities of the culture filtrates, a 5 mL aliquot of culture fluid was taken daily for 10 days from a flask containing 300 mL of Medium-199 and 2×10^7 conidia at the beginning of the culture, and stored at -80°C after sterilization through Millex-GV filters. It was found that antichemotactic activity became apparent at 3 days and reached a peak at 5 days after the beginning of the culture, just as the protein concentration reached a plateau (5 $\mu g\cdot mL^{-1}$). In this study, therefore, 5 day culture filtrates were used.

Cell preparations

Human AMs were isolated from the bronchoalveolar lavage (BAL) fluid of eight healthy adult volunteers who gave their informed consent. After centrifugation, the pellets were resuspended in Medium-199 containing 5% heat-inactivated AB serum. The cell suspensions were dispensed into Teflon tubes (Nalge Co.), which did not allow attachment to the tube wall [15]. More than 90% of the cell population was comprised of AMs. Fresh PMNs were prepared from heparinized venous blood obtained from an additional 10 healthy adult volunteers by sedimentation in 2% dextran, followed by centrifugation on a Ficoll gradient. Contaminating red blood corpuscles (RBCs) were lysed with hypotonic saline, and PMNs were washed and resuspended with Medium-199 in polypropylene tubes (Becton Dickinson Co.). Giemsa-stained cytospin preparations of the PMN suspensions typically showed more than 95% PMNs. These procedures were approved by the Ethics Committee of our department. In each experiment, the viability of the cells, tested by trypan blue exclusion, was found to be greater than 95% in the medium alone and greater than 90% in the medium with culture filtrates for 2 h.

Ingestion assay

Three hundred and fifty microlitres of Medium-199 containing floating AMs (1×10^6) and conidia of *Af*, YN strain (5×10^6) were mixed with 150 µL of ACF or Medium-199. Each mixture was incubated in a waterbath, with shaking, at 37°C for 15–60 min. Immediately after incubation, the mixtures were placed on ice to inhibit further phagocytosis and all tubes were washed twice with cold 0.1% ethylenediamine tetra-acetic acid (EDTA)-phosphate-buffered saline (PBS). Phagocytosis of conidia was assayed by the fluorescence quenching method [16]. Briefly, conidia prelabelled with fluorescein isothiocyanate (Sigma) were mixed with AMs. Immediately after incubation, each drop of suspension and trypan blue were placed together on a slide and examined under an ultra violet (UV) microscope (Nikon Fluophoto), and then the percentage of AMs ingesting conidia was calculated. Cells incubated with viable conidia were attached to the glass slides by the cytospin procedure and stained with May-Grünwald Giemsa. Five hundred AMs per slide were analysed for ingestion of conidia under light microscopy. The phagocytic index showed the average number of conidia ingested by 100 AMs.

Latex beads (Fluoresbrite carboxylate microspheres, 2.2 µm in diameter; Polysciences Inc.) were added to the AMs ($2\times10^6\cdot mL^{-1}$) suspensions pretreated with culture filtrates (20 and 50% of ACF, and 50% of *C. albicans* culture filtrates) at a ratio of 10:1 for beads:AMs, and then incubated for 30 min at 37°C. The controls were placed on ice throughout the incubation period. Phagocytosis of beads was examined by flow cytometric analysis performed with a fluorescence-activated cell sorter

(FACScan) flow cytometer (Becton Dickinson Co.). The data were analysed on monoparametric histograms [17], and expressed as percentage of positive fluorescent cells. Percentages of phagocytosing cells were calculated by subtracting percentage fluorescence-positive cells in controls (<5%) from that of incubated cells.

Chemotaxis assay

Chemotaxis of PMNs under agarose was determined according to the method of Nelson *et al*. [18]. Briefly, six series of three wells, 3 mm in diameter and spaced 3 mm apart, were cut in agarose plates. Each of 10 µL of formyl-methionyl-leucyl-phenylalanine (FMLP, 10^{-7} M) (Sigma) and Hank's balanced salt solution (HBSS) was placed in the outer and inner wells, respectively. The centre wells contained 1×10^6 PMNs suspended in Medium-199 with culture filtrates (0, 3, 5, 10, 20 and 30%). The plates were incubated in a 5% CO_2 incubator for 2 h at 37°C, and then fixed with ethanol and stained with Giemsa. Quantification of migration was assessed by the linear distance which the cells had moved from the margin of the centre well towards FMLP.

ACF was centrifuged at 5,000×g at 4°C for 40 min through membrane filters (CL-LGC) (Millipore Co.) to obtain a fraction containing low molecular weight (MW) substances (<10 kDa) (fraction I). ACF was also dialysed in a dialysis tube (MW exclusion of 10 kDa) against 10 volumes of Medium-199 changed hourly for 6 h to obtain another fraction containing high MW substances (>10 kDa) (fraction II). Both fractions were reconstituted to the original volume with Medium-199. The antichemotactic activity of both fractions was assessed. Aliquots of ACF were treated with either trypsin (0.01 and 0.1 mg·mL^{-1}) or chicken egg white ovomacroglobulin (OMG) (0.3 and 3.0 mg·mL^{-1}) (a generous gift from Otuka Pharmaceutical Co. Ltd) for 30 min at 37°C, and their effects on the antichemotactic activity of ACF were examined.

Superoxide anion assay

Production of superoxide anion was assayed by determining superoxide dismutase (SOD)-inhibitable reduction of cytochrome C [19]. Briefly, 2.5×10^5 PMNs were preincubated in Medium-199 containing various concentrations of culture filtrates (0, 3, 6, 12.5, 25 and 50%) for 60 min at 37°C and then washed with PBS. In a final volume of 500 µL of HBSS containing cytochrome C (120 µmol) (Sigma) with or without 50 µg of SOD, phorbol myristate acetate (PMA, 100 ng·mL^{-1}) (Sigma) and PMNs were incubated in a shaking water bath at 37°C for 30 min. Finally, the cells were removed by centrifugation. The superoxide production was assessed in the supernatants as the difference in absorption from the control at 550 nm measured on a spectrophotometer (Hitachi U-3210), and amount of superoxide anion in nanomoles released per 10^6 PMNs was calculated. All experiments were performed in triplicate.

Assay of general proteolytic activity

Azocoll hydrolysis was analysed by the method of Chavir *et al*. [20]. Each assay tube contained 5 mg of azocoll in 50 mM Tris-HCl, pH 8.0, and the reaction was started by addition of ACF in a shaking water bath at 37°C for 30 min. The reaction of the solution was stopped by placing tubes on ice followed by centrifugation, and absorbance of the supernatant at 520 nm was recorded. One unit was defined as a change of 0.001 optical density (OD) at 520 nm per minute at 37°C, and 1 U activity corresponded to 13 µg protein. The protein concentration was determined by the Lowry method.

Statistical analysis

Data are presented as mean±sem. Statistical comparisons between the test solutions and control were performed using Student's t-test for paired data. A p-value of less than 0.01 was considered to be significant.

Results

Effect of ACF on phagocytosis by human AMs

As shown in figure 1a and b, ingestion by AMs of conidia obtained from YN strain of *Af* was assessed in the presence or absence of ACF. In Medium-199 alone, 52% of AMs ingested conidia during the 60 min incubation period and the phagocytic index increased from 30±9.4 (15 min) to 126±27.0 (60 min) in a time-dependent manner. However, when co-cultured with 30% ACF, the percentage of AMs ingesting conidia was 24% and the phagocytic index was 42±11.9 at 60 min. The ingestion of conidia by AMs treated with 30% ACF was significantly suppressed compared with those treated with Medium-199 alone ($p<0.01$).

The effect of ACF on nonspecific phagocytosis by AMs using latex beads was also examined as shown in table 1. The percentage of AMs associated with latex beads and the mean fluorescence intensity were both decreased significantly ($p<0.01$) with 50% ACF treatment for 60 min. Treatment with the culture filtrates of *C. albicans* had no antiphagocytotic effect.

Effect of ACF on chemotaxis of human PMNs

As shown in figure 2, the migration distance of PMNs towards FMLP was reduced by ACF in a concentration-dependent manner, with 3% ACF being sufficient for the inhibitory effect ($p<0.01$). With 20% ACF, chemotaxis was within the range of random migration which was also further decreased, and PMNs treated with 30% ACF were almost immotile. The antichemotactic activity of ACF was completely abolished by autoclaving at 121°C for 10 min. The migration of PMNs towards ACF was

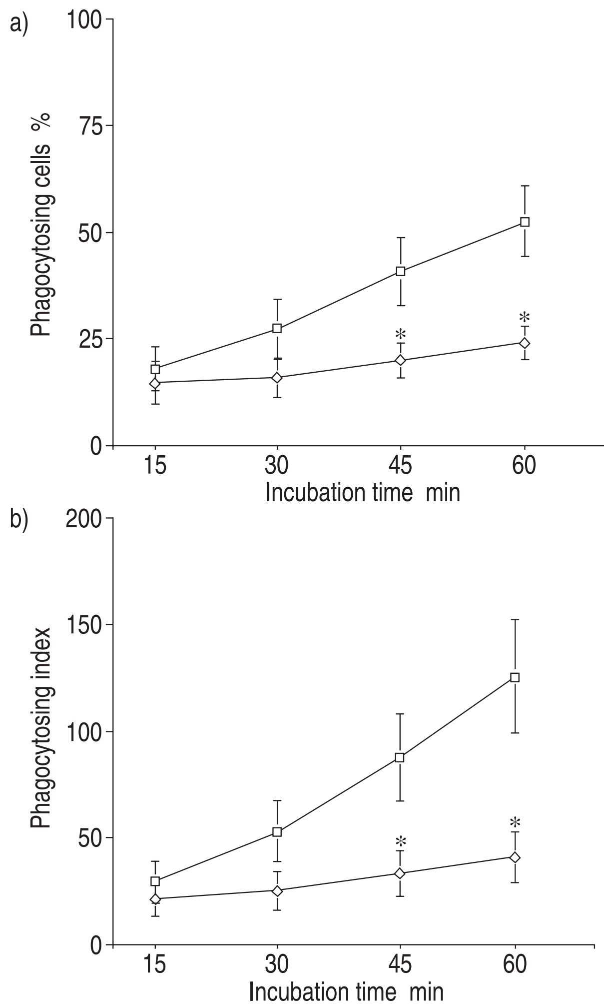

Fig. 1. – Phagocytosis of conidia by alveolar macrophages (AMs) in Medium-199 containing 30% ACF (—◊—) or Medium-199 alone (control) (—□—). a) Phagocytosis expressed as percentage phagocytosing cells (percentage of cells containing conidia); and b) phagocytosis expressed as phagocytic index (numbers of ingested conidia per 100 AMs) at each culture time-point (15, 30, 45 and 60 min). The results are presented as mean±SEM. Each experiment was repeated four times by using AMs from different volunteers. *: p<0.01 compared to control (Medium-199). ACF: *Aspergillus fumigatus* culture filtrates.

further examined and it was confirmed that ACF was not acting as a chemoattractant (data not shown).

On the other hand, culture filtrates of *C. albicans* at concentrations of 10–30% showed no significant inhibitory effect on the migration of PMNs. Moreover, as shown in figure 3, both low MW (fraction I) and high MW (fraction II) fractions retained antichemotactic activity (p<0.01), although the suppressive effect of fraction I was stronger than that of fraction II throughout the range of dilution of both fractions between 10–30% in Medium-199 (p<0.01). These results suggest that several factors in ACF contribute to its antichemotactic activity, and that low MW substances may be more potent than those with high MW as antichemotactic factors.

Table 1. – Effect of culture filtrates on the association between alveolar macrophages (AMs) and latex beads

	AMs associated with latex beads %
Medium-199	57±11
20% of ACF	47±11
50% of ACF	37±11*
50% of Candida culture filtrates	54±4

The data are presented as mean±SEM of four experiments. *: p<0.01 compared with Medium-199. ACF: *Aspergillus fumigatus* culture filtrates.

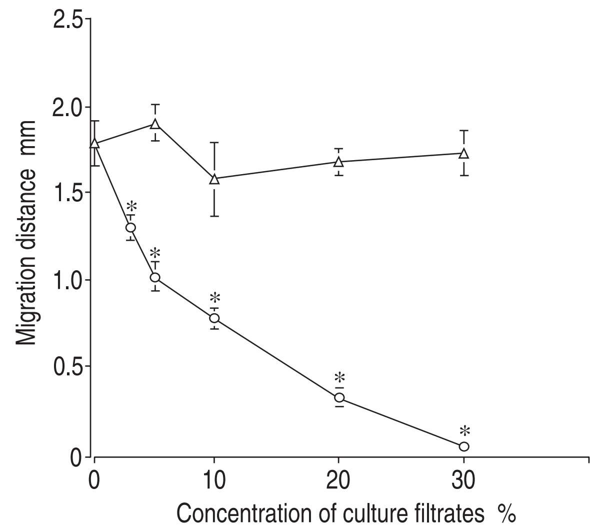

Fig. 2. – Effect of *Aspergillus fumigatus* culture filtrates on chemotaxis of human polymorphonuclear leucocytes (PMNs). Chemotactic activity was expressed as the linear distance (mm) the cells had migrated towards FMLP (10^{-7} M). The migration distance of PMNs treated with various concentrations of culture filtrates prepared from *A. fumigatus* (—○—) and *Candida albicans* (—Δ—) were plotted. The experiments were repeated four times, and data are presented as mean±SEM. *: p<0.01, compared to control (Medium-199). FMLP: formyl-methionyl-leucyl-phenylalanine.

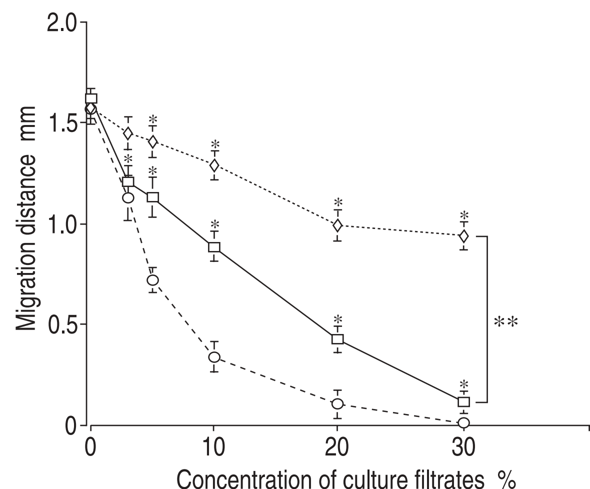

Fig. 3. – Effect of fraction I (MW <10 kDa) (—□—) and fraction II (MW >10 kDa) (····· ◊ ·····) on the migration of human polymorphonuclear leucocytes (PMNs). Although antichemotactic activity of fraction I and fraction II was reduced compared with that of original ACF (- - - ○ - - -), both fractions retained antichemotactic activity. The experiments were repeated three times and data are presented as mean±SEM. *: p<0.01, compared to control (Medium-199); **: p<0.01, comparison between fraction I and II. MW: molecular weight; ACF: *Aspergillus fumigatus* culture filtrates.

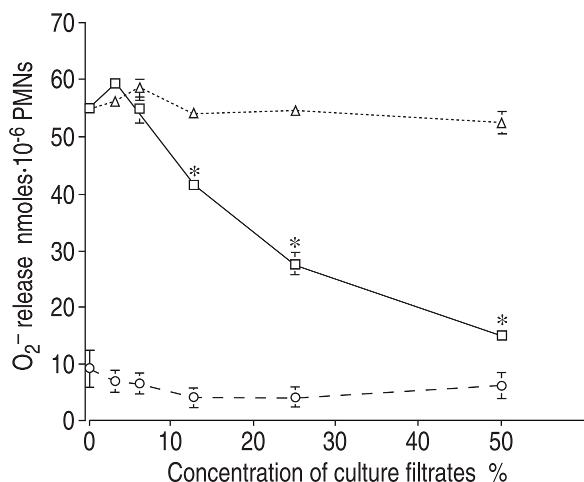

Fig. 4. – Effect of *Aspergillus fumigatus* culture filtrates (ACF) on superoxide anion release from human polymorphonuclear leucocytes (PMNs). Superoxide anion release (expressed as nmoles·10^{-6} PMNs·30 min) from human PMNs treated with various concentrations of culture filtrate prepared from *A. fumigatus* (□, ❍) and *Candida albicans* (Δ) were plotted. Spontaneous release of O_2^-, shown by dashed line (- - - ❍ - - -), was not affected by ACF treatment. PMA (100 ng·mL^{-1}) stimulated O_2^- release was suppressed by ACF treatment, as shown by a continuous line (—□—), without loss of cellular viability. PMA-stimulated O_2^- release in the culture filtrates of *C. albicans* (·····Δ·····) remained unchanged. The experiments were repeated 10 times, the data are presented as mean±SEM. *: p<0.01, compared to control (Medium-199). PMA: phorbol myristate acetate.

Effect of ACF on superoxide anion release from PMNs

Without stimulation, spontaneous O_2^- release was affected neither by *A. fumigatus* nor by *C. albicans* culture filtrates throughout the 60 min incubation period. When PMA (100 ng·mL^{-1}) was used as the stimulant, a short-term exposure within 45 min had no significant effect on superoxide generation by PMNs (data not shown). However, as shown in figure 4, after treatment for 60 min in 12.5–50% ACF, the superoxide anion release stimulated with PMA was significantly reduced in a concentration-dependent manner compared to that in Medium-199 (53.8±1.40 nmoles·10^6 AM·30 min in medium alone, 41.6±1.40 in 12.5% ACF and 14.5±1.24 in 50% ACF). ACF at concentrations between 3–6% appeared to augment O_2^- production slightly, but not significantly. ACF had the same effect on O_2^- release when PMA was replaced by opsonized zymosan as the stimulant (data not shown). Since ACF itself did not influence the O_2^- release of PMNs (data not shown), these results were not due to exhaustion of PMNs by ACF. In contrast, PMNs treated with 3–50% culture filtrates of *C. albicans* for 60 min showed no significant changes in O_2^- release in response to PMA or opsonized zymosan.

Effects of trypsin and OMG on biological activity of ACF

As shown in figure 5, the antichemotactic effect of ACF was reduced by 56% in the presence of 0.1 mg·mL^{-1} trypsin (p<0.01), or by 29.5% in the presence of 3 mg·mL^{-1} OMG (p<0.05). At these concentrations,

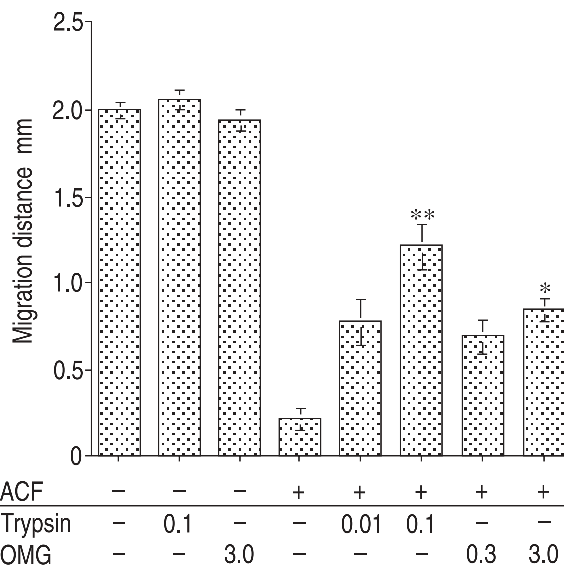

Fig. 5. – Partial inactivation of antichemotactic activity of *Aspergillus fumigatus* culture filtrates (ACF) by trypsin or ovomacroglobulin (OMG). ACF were pretreated with trypsin (0.01 and 0.1 mg·mL^{-1}) or OMG (0.3 and 3.0 mg·mL^{-1}) for 30 min at 37°C. Chemotactic activity of polymorphonuclear leucocytes (PMNs) was not affected either by trypsin or OMG, compared with control (Medium-199). Data are presented as mean±SEM of three separate experiments. *: p<0.05, compared with ACF; **: p<0.01, compared with ACF.

neither trypsin nor OMG *per se* had any effect on PMN chemotaxis. OMG was reported to be a broad-spectrum protease inhibitor and to suppress the activities of the extracellular proteases produced by *Serratia marcescens* and *Pseudomonas aeruginosa* [21]. Proteinase activity with azocoll as the substrates of the untreated ACF was 1 U·mL^{-1}. These results suggest that protein and proteinase account, at least partially, for the antichemotactic activity of ACF.

Comparison of the biological activities of ACF and gliotoxin

The biological activity of ACF was compared with that of gliotoxin, a secondary metabolite of *Af*, which was reported by EICHNER and co-workers [10, 11] to inhibit rodent macrophage functions. It was also confirmed that gliotoxin (Sigma) inhibited chemotaxis of human PMNs at 0.1 and 0.3 µg·mL^{-1}, and that the former concentration of gliotoxin had antichemotactic activity equivalent

Table 2. – Effects of gliotoxin on chemotaxis and superoxide anion release of human polymorphonuclear leucocytes (PMNs)

Gliotoxin µg·mL^{-1}	Migration distance mm	O_2^- release nmoles·10^{-6} PMNs
0	2.05±0.05	58.1±0.69
0.1	0.73±0.12*	64.2±0.98
0.3	0.27±0.03*	52.5±1.49*
1.0	ND	33.3±1.55*

Values are presented as mean±SEM. ND: not determined. *: p<0.01.

to that of 5–10% ACF. However, a higher concentration of gliotoxin (0.3 μg·mL^{-1}) was required to suppress O_2^- release from human PMNs stimulated with PMA (table 2).

Discussion

We have demonstrated that ACF interfered with ingestion both of Aspergillus conidia and latex beads by human AMs. In addition, ACF reduced migration of human PMNs towards FMLP and suppressed superoxide anion release from PMNs stimulated with PMA, whilst spontaneous O_2^- release was not affected. We have also confirmed that ACF obtained from four other clinical isolates of *Af*, in addition to the YN strain, had the same sort of effects on phagocytic functions (data not shown), whereas, no such inhibitory activities were observed with *C. albicans* culture filtrates.

As pulmonary aspergillosis results from inhalation and deposition of conidia, AMs are considered to form the first line of defence against the fungal spores [22]. Both in humans and in experimental animals, neutropenia and dysfunction of PMNs are the greatest risk factors for invasive aspergillosis [6, 23]. Cellular and humoral immunity are not considered to contribute to the host defence in aspergillosis [3, 4, 17, 22]. Alveolar macrophages and neutrophils appear to be the primary components of the host defence network, and play essential roles in eradication of the fungus [3, 4, 6, 24]. Phagocytic functions, such as ingestion, migration and release of reactive oxygen intermediates, which appear to be responsible for the killing activity, are important host defence mechanisms against *Af*. Evasion of these functions may be the principal tools by which *Af* persist in the sites of colonization and proliferate further.

AMs killed conidia more efficiently than peritoneal macrophages of rabbits, which suggests that the anatomical site of macrophages influences their activities against *Af* [3]. Some groups have analysed the interactions between Aspergillus and phagocytes, and have shown that several fungal metabolites interfere with phagocytic function. However, these previous studies mainly used phagocytes obtained from experimental animals [7–11, 25]. Therefore, corresponding experiments on human AMs and PMNs are necessary before any extrapolation can be made to human pulmonary aspergillosis. In the present study, we examined the interactions between human phagocytes and ACF *in vitro*. Our results suggest that *A. fumigatus* releases factors which impair the functions of human AMs and PMNs, and consequently escapes the local host defences. It is meaningful that ACF of the YN strain itself suppressed the ingestion of conidia derived from this strain by AMs.

Washburn *et al.* [25] reported that *Af* produced a soluble extracellular inhibitor of the alternative complement pathway, and showed that this material interfered with C3b-dependent phagocytosis and killing. Schaffner [22] reported that resident AMs ingested conidia in the absence of antibodies, thus permitting phagocytosis of conidia by AMs in an environment where serum components were not readily available. Since the conidia used in our study were not opsonized, it seems that the inhibitory effect of ACF on phagocytosis was not due to the mechanism reported by Washburn *et al.* [25].

Eichner and co-workers [10, 11] reported that a 3 day culture of *Af* generated metabolites with antiphagocytic activity, namely inhibition of phagocytosis of carbon particles by mouse peritoneal exudate cells, and adherence to plastic surfaces by rodent peritoneal and alveolar macrophages, and also inhibited the basal rate of H_2O_2 production by human PMNs. These activities were stable against digestion by trypsin and protease, and the factors responsible had a MW less than 0.5 kDa. They identified one of the biologically active components as gliotoxin [10, 11]. Gliotoxin has been isolated from cultures of *Af* as well as other fungi, including *Gliocladium fimbriatum*, *Penicillium obscurum*, and *Trichoderma lignorum*, and belongs to the epipolythiodioxopiperazine group. Most members of this group are known to possess some biological activities [26].

We also confirmed that gliotoxin interfered with migration of PMNs and PMA-induced O_2^- release from PMNs. However, at least some of the antichemotactic components of ACF consisted of protein and were associated with the high MW fraction (>10 kDa) as well as the low MW fraction.

Robertson and co-workers [7–9] reported that *Af* spore diffusates, obtained from 3 h culture supernatants, reduced the migration of human PMNs, the spreading of mouse peritoneal exudate cells, and the spontaneous O_2^- release from mouse peritoneal exudate cells or BAL cells of *Corynebacterium parvarum*-treated rats. The spore diffusates were obtained after only 3 h of culture, whilst gliotoxin and ACF used in the present study were obtained after 3–5 days of culture, when mycelial growth would be abundant. In human tissues, *Af* grows almost exclusively in the mycelial form. Consequently, to progress from simple colonization of *Af* to semi-invasive or invasive pulmonary aspergillosis, the latter factors are considered to be more important than the spore diffusates obtained after short-term culture.

Several researchers have reported the isolation and characterization of extracellular elastinolytic proteases from *Af*. These enzymes appeared to be serine proteases [27] or metalloproteases [28]. Kothary *et al.* [29] reported that elastase activity was correlated with virulence for mice, and Rhodes *et al.* [30] reported that all isolates involved in human invasive aspergillosis displayed elastinolytic activity *in vitro*. Although they suggested that digestive exoenzymes degrade the structural barriers in the host, they did not comment on the relationships between elastase and phagocytic functions.

The results of the present study suggest that part of the antichemotactic activity is associated with protease. Recently, Holden and co-workers [31] established a protease gene-disrupted mutant of *Af*, and reported no differences between mutant and wild-type strain in terms of mortality or histological features in murine models for aspergillosis. We have tested culture filtrates of the mutant strain (a kind gift from D.W. Holden), which

lacked extracellular proteolytic activity. We confirmed that culture filtrates of the mutant strain exhibited anti-chemotactic activity and suppressed PMA-induced O_2^- release, although the activities were lower than that of the culture filtrates of the wild-type strain (data not shown).

Thus, we conclude that several factors participate in the antiphagocytic activity of ACF. Such candidates may be low MW substances, probably including gliotoxin, and high MW substances, such as proteases and other unidentified substances. It will be an important future work to identify the substances in ACF and to clarify the mechanisms of antiphagocytic effects.

We have previously shown that most ACFs of clinically isolated *Af*, including the YN strain, slow ciliary beat frequency associated with marked disruption of human respiratory ciliated epithelium, probably leading to impairment of the mucociliary clearance, which is the most important first line of defence in the airways [12]. AMITANI *et al*. [13] isolated the cilioinhibitory substances from ACF, and identified one of the potent substances as gliotoxin. Consequently, *Af* must be capable of circumventing the elaborate defence network both in the airways and the lungs in order to persist and proliferate there.

The final role of PMNs in host defence against Aspergillus is in hyphal damage. Additional studies to determine whether ACF suppresses the hyphal damage by PMNs are currently in progress. The demonstration of the antiphagocytic substances in human tissues infected by *Af* will also be required to clarify the pathogenetic mechanisms of colonization and invasion of *Af* in the lungs.

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