

Cytotoxicity of *Aspergillus* Fungi Isolated from Hospital Environment

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Abstract

The majority of mycotoxins produced by *Aspergillus* fungi are immunosuppressive agents, and their cytotoxicity may impair defense mechanisms in humans. The objective of the study was evaluation of the cytotoxicity of fungi isolated from an environment where inpatients with impaired immunity were present. The materials comprised 57 fungal strains: *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus flavus*, *Aspergillus versicolor* and *Aspergillus ustus* isolated from hospital rooms in Cracow. The cytotoxicity of all the strains was evaluated using the MTT test (3-(4,5-dimethylthiazol-2-yl) 2,5 diphenyltetrazolium bromide). To emphasize the differences in cytotoxicity among the particular strains, variance analysis (ANOVA) and Tukey's difference test were used. Out of 57 *Aspergillus* strains tested, 48 (84%) turned out to be cytotoxic. The cytotoxicity was high (+++) in 21 strains, mainly in *A. fumigatus*. The least cytotoxic were *A. niger* fungi, this being statistically significant ($p < 0,05$). To protect a patient from the adverse effects of mycotoxins, not only his or her immunity status should be evaluated but also the presence of fungi in hospital environment and their cytotoxicity should be monitored (possible exposure).

Key words: *Aspergillus* sp., cytotoxicity, environment

Introduction

There is evidence that a number of *Aspergillus* fungi present in hospital environment produce toxins (Burr, 2001; Ben-Ami *et al.*, 2009; Klich *et al.*, 2009). Their secondary metabolites such as ochratoxin A, aflatoxins, trichotecins or sterigmatocystine are toxic for various cellular structures and interfere with key processes like RNA and DNA synthesis (Ciegler and Bennet, 1980). Depending on fungal species and/or strain, mycotoxins differ in their specificity and influence on target cells, cellular structures and processes in them (Steyn, 1995; Pitt, 2000). It should be also kept in mind that not all moulds produce mycotoxins, and their production depends on culture medium, life cycle and environmental conditions (Pitt *et al.*, 2000; Kelman *et al.*, 2004). To assess the ability of a given strain to produce mycotoxins, the presence of other moulds in the environment should be taken into account as well as their influence on mycotoxin production intensity. It has been documented that the fungi incubated in monocultures in laboratory setting

lose their mycotoxin production potential (Fischer and Dott, 2003; Jarvis and Miller, 2005). The ability of fungi to produce mycotoxins is hard to evaluate because there is a no possibility to analyze the mycotoxins produced in the tissues of living organisms. The relationship between disease (or predisposition to it) and the level of exposure (detection of pathogenic spores) as well as symptoms and signs characteristic of experimental lesions produced by mycotoxins may contribute to establishing the noxiousness of fungi to human health. The objective of the study was evaluation of the cytotoxicity of *Aspergillus* fungi isolated from hospital environment.

Experimental

Materials and Methods

The materials comprised mould strains belonging to the *Aspergillus* genus, isolated from the environment, mainly from indoor air in hospital rooms at a number of hospitals in Kraków in the years 2007–2008.

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Primarily, the mycological flora was evaluated in hospital ward environment (neonatal intensive care unit, medical intensive therapy unit, the wards of chemotherapy and radiotherapy). The fungi were sampled using a MAS 100 device (Merck) from the indoor air in various hospital rooms. The species of particular strains were detected on the basis of thorough macroscopic and microscopic analysis. Out of all of the fungi isolated, 57 *Aspergillus* strains were randomly chosen for further examination. The chosen strains belonged to six species: *A. fumigatus* (21 strains), *A. niger* (14 strains), *A. ochraceus* (13 strains), *A. flavus* (5 strains), *A. versicolor* (3 strains) and *A. ustus* (a single strain). The fungi were divided into groups: each group comprised different species. The cytotoxicity test MTT (3-(4,5-dimethylthiazol-2-yl) 2,5 diphenyltetrazolium bromide) was performed separately for each strain. To evaluate the total cytotoxicity of the fungi, swine kidney cells (SK) were used because they are sensitive to most mycotoxins. The test is based on the reduction of yellow MTT tetrazolium salt to violet formazan, insoluble in water. The reduction occurs in the presence of intact SK, not damaged by mycotoxins. The intensity of reaction is proportional to the amount of metabolically active SK. When the SK are infected with moulds producing mycotoxins, their mitochondria fail to reduce tetrazolium salt into formazan. Therefore, when the SK are damaged by mycotoxins, the reaction is less intensive or does not occur, which can be measured photometrically as more or less intensive change of colour. Thus, the reduction or the absence of the reaction gives evidence of cytotoxicity of the fungal strain tested. (Hanelt et al., 1994).

The analysis comprised the evaluation of a test sample (Petri dish with the mould on Czapek medium) and a control sample (Petri dish with Czapek medium). The SK were grown in medium containing antibiotics (penicillin and streptomycin, Sigma Aldrich) and calf fetal serum (Sigma Aldrich) in the Hera Cell incubator with carbon dioxide, manufactured by Heraeus (5% CO₂, 37°C, 98% humidity). The number of SK was 2.2×10^5 . The ranges of testing concentrations were prepared in ratio 1:2 and amounted from 31.251 to 0.061 cm²/ml. The value was expressed in terms of cm²/ml, where the area of the Petri dish from which the moulds were extracted together with the medium, was measured in square centimetres.

The quantitative evaluation of cytotoxicity was performed using a microslide spectrophotometer (Elisa Digiscan reader, Asys Hitech GmbH, Austria) and the programme MikroWin 2000 (Mikrotek Laborsysteme GmbH, Germany). The readings were made at the wave-length of 510 nm. All of the absorption values below 50% of the threshold activity were considered as toxic. So, the borderline toxic concentration was evaluated on the basis of the dilution i.e. the mean

inhibitory concentration IC₅₀ was equal to the smallest sample (in cm²/ml) which was toxic to the SK. The cytotoxicity was considered as low (+) when the values were within the range of 31.251 > 15.625 > 7.813 [cm²/ml], intermediate (++) for the values > 3.906 > 1.953 > 0.977 [cm²/ml], and high (+++) for > 0.488 > 0.244 > 0.122 > 0.061. The lack of cytotoxicity was reported when the absorption value exceeded 31.251 [cm²/ml] (Gareis, 1994).

The cytotoxicity was tested in the Department of Physiology and Toxicology, Institute of Experimental Biology at the Casimirus the Great University in Bydgoszcz, Poland.

To evaluate differences among the particular groups of fungi, the fungi were divided into four groups. The first group – I (21 strains) comprised fungi belonging to the *A. fumigatus* species, the second group – II (14 strains) *A. niger*, the third group – III (13 strains) *A. ochraceus*, and the fourth group – IV (9 strains) comprised the remaining strains (*A. flavus*, *A. versicolor* and *A. ustus*). Descriptive statistics were used, and the mean values and standard deviations were calculated. To show the differences of cytotoxicity of the species tested ANOVA test was used. As the mean toxicities were different in particular groups, the Tuckey's post hoc test was used to find out between which groups the differences were significant. The value of $p < 0,05$ was assumed as the borderline of significance (Armitage, 1971).

Results

The amounts of fungi and fungal species varied from one room to another. The mean numbers of fungi in the particular wards varied from 5 to 2370 c.f.u. × m⁻³. The fungi were most abundant in the neonatal intensive care unit: the mean number of colonies on a single Petri dish reflected 530 c.f.u. × m⁻³ in the indoor air. The lowest number of fungi was detected in the chemotherapy ward: 29 c.f.u.m⁻³ (Table I). The dominating genera were *Penicillium*, *Cladosporium* and *Aspergillus*. The highest percentage of *Aspergillus* was isolated in the neonatal intensive care unit: 38%. The moulds were the most abundant in this environment. The lowest percentage of *Aspergillus* was detected in air-conditioned intensive therapy ward: 22%. The total number of *Aspergillus* strains isolated was too high to evaluate cytotoxicity in all of them. The strains chosen to cytotoxicity test originated from three hospital wards because it was assumed that strains from the same ward might be similar genotypically.

Out of the 57 *Aspergillus* strains tested, 48 (84%) turned out to be cytotoxic. They belonged to the following species: *A. fumigatus* 19/21, *A. niger* 8/14, *A. ochraceus* 12/13, *A. flavus* 5/5, *A. versicolor* 3/3,

Table I
Results of cytotoxicity tests

Ward	Number of samples	Numbers of fungal colonies c.f.u.×m ⁻³				Percentage of fungi				Number of <i>Aspergillus</i> strains examined for cytotoxicity	Number of strains with documented cytotoxicity
		Minimum	Maximum	Mean	Median	A	C	P	I		
Radiotherapy	130	5	360	39,5	17,5	32%	9%	31%	28%	16	12
Chemotherapy	130	5	130	29	20	29%	8%	29%	34%	20	16
Neonatal ICU	30	50	2370	530	260	38%	18%	32%	12%	12	12
Intensive therapy	48	5	170	40	30	22%	17%	38%	23%	9	8

ICU – intensive care unit, A – *Aspergillus* sp., C – *Cladosporium* sp., P – *Penicillium* sp., I – other fungal

Table II
Distribution of fungal species in relation to their cytotoxicity

No	Species	N (%)	Cytotoxicity			
			None	+	++	+++
1	<i>Aspergillus fumigatus</i>	21 (37%)	2	1	7	11
2	<i>Aspergillus niger</i>	14 (25%)	6	3	3	2
3	<i>Aspergillus ochraceus</i>	13 (23%)	1	3	2	7
4	<i>Aspergillus flavus</i>	5 (8%)	0	2	2	1
5	<i>Aspergillus versicolor</i>	3 (5%)	0	0	3	0
6	<i>Aspergillus ustus</i>	1 (2%)	0	1	0	0
	Total	57 (100%)	9	10	17	21

N – Total number of fungi; (+) – low cytotoxicity; (++) – intermediate cytotoxicity; (+++) – high cytotoxicity

and a single strain *A. ustus*. Only nine strains were not cytotoxic (16%). The absorption value >31.251 [cm²/ml] (lack of cytotoxicity) was most frequently found in *A. niger* (six strains). Twenty one strains tested revealed high cytotoxicity (+++), most often *A. fumigatus* (11 strains), and *A. ochraceus* (7 strains). Intermediate cytotoxicity was found in seventeen strains, mainly *A. fumigatus*. Ten strains revealed low cytotoxicity, mainly *A. niger* and *A. ochraceus* (Table II). The ANOVA test revealed differences of the patho-

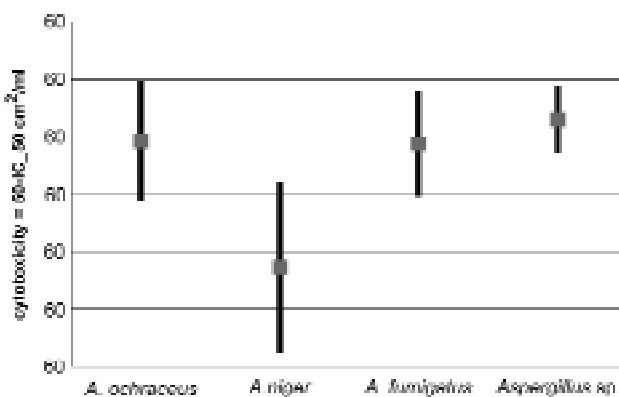


Fig. 1. Intervals of confidence 95% CI and mean value of *Aspergillus* cytotoxicity estimated as difference between reference level equal to 50 and measured value of IC 50 cm²/ml.

Legend: the squares at the middle represent the mean value of the estimated cytotoxicity, and the vertical lines represent the size of the confidence interval of the estimated cytotoxicity.

genicity within the particular fungal species ($p = 0,03$; $p < 0,05$). The least cytotoxic was the *Aspergillus niger* species: its mean value 32.71 and SD 27.97 were the highest among the fungi tested. The Tuckey's post hoc test, performed following ANOVA, revealed that the cytotoxicity of *A. niger* is significantly lower than those of *A. fumigatus* and *A. ochraceus* ($p < 0,05$). The cytotoxicity of the other *Aspergillus* fungi (group IV) did not differ significantly from the three species mentioned above, which may be a result of too small number of fungi in the samples. Moreover, the confidence interval was calculated for each of the species tested; these are presented in Fig. 1.

Discussion

Moulds can grow mainly in the environment in which humidity exceeds 45%, the temperature is within the range of 5°C–35°C (optimum 18°–27°C). and water activity above 0.8. Such environment is conducive to the production of secondary metabolites – mycotoxins. *Penicillium* and *Aspergillus* are dominating mould species in the rooms where water activity is around 0.85 (Jarvis, 2003). The *Aspergillus* genus is the most pathogenic to living organisms, however, it globally produces less mycotoxins than *Stachybotrys* even though the former dominates in the environment. This may not be true in all the *Aspergillus* species, because sterigmatocystin produced by *A. versicolor* may contribute to 1% of its biomass when water activity is equal to one (Fog Nielsen, 2003). We managed to isolate only three *A. versicolor* strains and to evaluate their cytotoxicity, which was intermediate in all cases. Such a small number of isolates resulted from the fact that this fungal species is rarely isolated indoor and is present mainly in colder regions such as mountains or polar areas. The species most frequently isolated from indoor air are *A. fumigatus* and *A. niger*. Just these two species were most often isolated in our study and examined for cytotoxicity.

It is reported in many papers that *A. fumigatus* is the most pathogenic *Aspergillus* species (Bennett,

2009; Krishnan *et al.*, 2009; Albrecht *et al.*, 2010). This finding was confirmed by Kamei *et al.* (2002) who analyzed the virulence of *A. fumigatus*, *A. flavus*, *A. niger* and *A. terreus* and their influence on murine macrophages. The macrophages treated with 1% *A. fumigatus* filtrate were seriously damaged, up to necrobiosis while the damage caused by similar filtrates of *A. niger* was lighter and that caused by *A. flavus* and *A. terreus* was almost undetectable. Those data are consistent with our findings because *A. fumigatus* was the most frequently highly cytotoxic (+++) as compared with other species, and only two strains out of 21 were not toxic to SK. On the other hand, only eight out of fourteen *A. niger* strains were cytotoxic, but their toxicity was the lowest as compared with other species. All of the *A. flavus* strains were cytotoxic in our study. Only five strains were tested, perhaps too few to come to a conclusion.

The MTT test focused on *in vitro* influence of fungi on living cells is performed with swine, sheep or lamb cell cultures. Stec *et al.* (2007) investigated the influence of mycotoxins such as aflatoxin B, ochratoxin, patuline, citrinine and zelarenon on various cell cultures. They found out that SK fibroblasts were most sensitive to mycotoxins, however, the intensity of the yellow tetrazolium salt reduction to formazan depended on the kind of toxin. It appears that the choice of SK in our study was appropriate, and it could be expected that the majority of strains isolated in our study might be pathogenic to living organisms. In further trials carried out by Stec *et al.* (2007) cell cultures responded in different ways to treatment with various mycotoxins. Therefore, it appears essential to determine the kind of mycotoxins produced by highly mycotoxic fungi. It is not always possible. In our previous study (Gniadek and Macura, 2003) performed in 2001 with 21 *A. flavus* strains isolated from the environment at social welfare homes were examined for aflatoxins B₁, B₂, G₁ and G₂ using high performance liquid chromatography – HPLC. Aflatoxins were not detected in any of the strains tested. The strains were not tested using MTT, however we were not sure that they were not pathogenic. The isolated *A. flavus* strains could produce the so called “masked mycotoxins”, *e.g.* aflatrem, aspergilliac acid, cyclopiacetic acid, kojic acid, maltoryzin, paspalicin or highly toxic sterigmatocystine. It should be kept in mind that fungi are able to produce mycotoxins in presence of other fungi (toxic metabolites are released only in a cross-reaction with toxins secreted by other fungi). (Fischer *et al.*, 2000). Such a conclusion may be confirmed by the findings of Murtoniemi *et al.* (2005), who examined the influence of cytotoxicity of various fungi (*Stachybotrys chartarum*, *Streptomyces californicus*, *A. versicolor* and *Penicillium spinulosum*) isolated from wet plaster bars on murine macrophages. They have shown that

some microcultures, after prolonged growth on wet cardboard sheets, were mutually stimulated or caused a synergic growth of cytotoxic fungi (especially *S. chartarum* and *A. versicolor*), but they did not cause inflammatory reaction in the cells tested.

Variable environmental conditions may stimulate fungi to produce mycotoxins, *e.g.* gliotoxin produced by *A. fumigatus* (Ben-Ami *et al.*, 2009; Kwon-Chung and Sugui, 2009). This substance inhibits T-cell activity, stimulates macrophage apoptosis and is included into pathogenesis of severe allergic rhinitis, bronchial asthma and allergic alveolitis in form of farmer’s lung, baker’s lung as well as allergic broncho-pulmonary alveolitis (ABPA). The study carried out by Watanabe *et al.* (2004) gives evidence that a good access to oxygen stimulated *A. fumigatus* to produce toxic gliotoxins and increased the general cytotoxicity of the fungi. The conditions in the rooms in our study were conducive to fungal growth: the mean temperature was 24°C, and humidity around 40%.

The indoor environment is an active ecosystem that changes in the course of time and as a result of changing temperature and humidity, and in the presence of other microorganisms. The toxin production by moulds is influenced by those factors and depends also on the fungal culture age, the stage of sporulation and the access to nutrients (Kelman *et al.*, 2004). The examination of the environment of a patient diagnosed with a mycotoxin infection is most often performed after the initial phase of the disease and may not reflect the exposure to harmful substances present in the course of the disease (Gniadek *et al.*, 2005; Hardin *et al.*, 2003). On the basis of the measurements of environmental factors which appeared after the infection, the real exposure at the onset of the disease may be only estimated. Therefore, it is a reasonable prophylactic measure to monitor mycological cleanliness of the environment where immunocompromised patients are present and to evaluate the cytotoxicity of the fungi known as pathogenic.

Conclusions. 1. The majority of the fungi *Aspergillus* in the environment of the rooms tested were cytotoxic and most often ($p < 0,05$) were non-*A. niger*. 2. To protect patients from harmful influence of mycotoxins, the immunity status of the patients should be evaluated and the presence of fungi in the environment should be monitored, including their cytotoxicity testing (possible exposure).

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