Acta Pharm. 55 (2005) 365–375 Original research paper

Mycotoxigenicity of clinical and environmental *Aspergillus fumigatus* and *A. flavus* isolates*

IVAN KOSALEC**
STJEPAN PEPELJNJAK

Department of Microbiology
Faculty of Pharmacy and Biochemistry
University of Zagreb, Zagreb, Croatia

Clinical isolates of fifty strains of *A. fumigatus* and 30 strains of *A. flavus* from immmunocompromised patients from the hematological unit were analyzed for mycotoxin production and compared with the same number of environmental isolates (from soil, compost, and air). Only 9 (18%) strains of *A. fumigatus* produced gliotoxin in a mean concentration 2.22 mg mL^{-1} (range 0.5–5 mg mL^{-1}). Aflatoxin B_1 was detected in 7 (23%) isolates (range from 0.02 to 1.2 mg L^{-1}) and aflatoxin G_1 in one (3%) of clinical *A. flavus* isolates (0.12 mg L^{-1}). In the group of environmental isolates, 11 (37%) were positive for aflatoxin B_1 production (range from 0.02 to 1.2 mg L^{-1}) and one for aflatoxin G_1 (0.02 mg L^{-1}). Bioautoantibiogram (»*bioassay in situ*«) on TLC plates against *Bacillus subtilis* NCTC 8236 showed that only gliotoxin-producing strains have bactericidal activity of R_f values corresponding to gliotoxin. The secondary-metabolite profiles of clinical and environmental *A. fumigatus* and *A. flavus* isolates were homogeneous, except for gliotoxin production, which was detected only in the group of clinical isolates of *A. fumigatus* (18%).

Keywords: Aspergillus fumigatus, A. flavus, gliotoxin, aflatoxin, mycotoxin, *in vitro* production, secondary metabolites, moulds

Received April 6, 2005
Accepted September 19, 2005

Saprophytic and thermophilic moulds of *Aspergillus* species are ubiquitary opportunistic fungi commonly isolated from immunocompromised individuals with acute leukemia, transplant recipients, patients with autoimmune diseases, and AIDS patients; these fungi are the major cause of high morbidity and mortality (1). In the immunocompetent host, *Aspergillus* species could colonize lung residual cavities or injured airways (*i.e.*, cystic fibrosis, tuberculosis, sarcoidosis). The most common clinical manifestation of infection by *Aspergillus* species is invasive aspergillosis with mortality higher than 90%. *A. fumigatus*, *A. flavus*, *A. niger* and *A. terreus* are frequently isolated from airways (nose, throat, bronchi) of such patients, and colonization could lead to invasive aspergillosis.

* A part of this work was presented at the 3rd Croatian Congress of Toxicology (with international participation, CROTOX), Plitvice (Croatia), May 26–29, 2004.

** Correspondence, e-mail: ikosalec@pharma.hr

Pathogenesis of aspergillosis is dependent on various factors of the host (immune status) and virulence factors of the pathogen. Except for the physical factors of fungi, such as their ability to grow at the temperature and pH of host tissues and small size of conidia (2), some putative virulence factors have been identified for different *Aspergillus* species. These include adhensins, pigment-secreted hydrolytic enzymes such as proteases, phospholipases, ribonucleases, restrictocin; production of catalases, superoxide-dismutases and production of the mycotoxins, low-molecular-weight, non-protein metabolites (3–5). One of the mycotoxins produced by *A. fumigatus* and *A. terreus* strains is gliotoxin, while aflatoxins are produced by *A. flavus*. These toxins could play a significant role in the pathogenesis of invasive aspergillosis. Gliotoxin is produced *in vivo* in tissues of animals infected with *A. fumigatus* (6–9), and it was recently detected in sera of patients with invasive aspergillosis (8).

Gliotoxin is a potent immunosuppressive mycotoxin and belongs to the epipolythiodioxopiperazine family with an active disulfide bridge in its structure. In the *in vitro* assays, gliotoxin inhibits phagocotosis by thymocytes, macrophages, induction of cytotoxic T cells, and stimulation of lymphocytes with mitogen (11). Gliotoxin can undergo redox cycling, generating oxygen radicals that cause oxidative damage to isolated DNA and induce apoptosis (12, 13).

Aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1) are mycotoxins produced by *A. flavus*, *A. parasiticus* and *A. nomius* strains. Aflatoxins also show a wide range of immunotoxic effects, they depress phagocitosis, intacellular killing and spontaneous superoxide production of macrophages (14–16). Aflatoxin B_1 also inhibits the production of the tumor-necrosis factor α (TNFα), interleukin-1 (IL-1) and IL-6 by lipopolysaccharide-stimulated macrophages (17).

Recent studies suggest that there are differences between clinical and environmental isolates of *A. fumigatus* strains in the activity of phospholipase (18). The ability of *in vitro* production of mycotoxins, aflatoxin B_1, G_1 and sterigmatocystin by the *A. fumigatus* and *A. versicolor* strains isolated from surgically removed tissues of patients with pulmonary asperegilloma (19) induced us to check out the possibility of mycotoxin production of *A. fumigatus* and *A. flavus* strains isolated from immunocompromised patients, and to compare its production with environmental isolates.

EXPERIMENTAL

Reagents and solvents

Gentamicin, streptomycine sulphate, penicillin G, polisorbate 80, Czapek-Dox salts ($MgSO_4$ x 7 H_2O, $NaNO_3$, KH_2PO_4, KCl and $FeSO_4$), $AgNO_3$, $AlCl_3$ x 6 H_2O and triphenyl-tetrazolium chloride were purchased from Fluka, Germany. Anhydrous Na_2SO_4, chloroform, ethyl acetate, formic acid and ethanol (96%, *V/V*) were purchased from Kemika (Croatia).

Microbiological media, Sabouraud 2% (*m/V*) glucose agar, Müller-Hinton agar, yeast extract and sucrose were purchased from Merck (Germany).

Gliotoxin standard was purchased from Calbiochem, Germany and aflatoxins from Sigma, USA.

All solvents used were of analytical grade.

Isolation and cultivation of mould isolates

Fifty strains of *Aspergillus fumigatus* Fresenius and thirty strains of *Aspergillus flavus* Link were recovered from immunocompromised patients with various diseases from the Hematological unit. Moulds were recovered from swabs at 25 ± 2 °C on Sabouraud 2%-glucose agar (SGA) with 50 mg L^{-1} gentamicin. Environmental isolates of fifty *A. fumigatus* strains and thirty *A. flavus* strains were isolated from the soil (compost) and air using SDA with 50 mg L^{-1} streptomycin and 20.000 I.U. of penicillin G, after incubation at 37 ± 1 °C for 72 h. *A. fumigatus* and *A. flavus* strains were identified according to their cultural and microscopic characteristics on Czapek agar and SGA at 37 ± 1 °C (20, 21). After primary isolation, strains were kept on SGA slants at –20 °C.

At the time of analysis, the conidia were harvested from a 5-day-old culture at 37 ± 1 °C by adding 5 mL of sterile physiological saline containing 0.05% polysorbate 80 (Tween® 80) to each SDA slant. The culture was scraped with a loop and 1 mL suspension, containing approximately 1–5 x 10^7 conidia/mL, was used for cultivation on the medium for biosynthesis.

Cultivation on yeast-extract liquid medium for biosynthesis

All isolates of *A. fumigatus* and *A. flavus* strains were grown on yeast-extract liquid medium (YES) containing yeast extract 20 g, sucrose 40 g and distilled water to 1000 mL. YES medium was sterilized by autoclaving for 15 minutes at 121 °C. Erlenmayer flasks (250 mL) with 50 mL of YES were inoculated with 1 mL of 1–5 x 10^7 conidia suspension. Flasks were than incubated six days at 25 ± 2 °C. Flasks were shaken periodically.

Extraction and mycotoxin analysis

The biomass was washed with 50 mL of chloroform and cut up into small pieces with a mixer at 3.500 rpm. The biomass was then filtrated through a Whatman No. 1 filter paper and extracted using 2 x 25 mL of chloroform, and filtrated through anhydrous Na_2SO_4. Chloroform fraction was pooled and evaporated to dryness on a rotary evaporator at reduced pressure and at 60 °C. For gliotoxin quantification, dried extracts were dissolved in 500 μL of chloroform and stored at 4 °C until analysis. Detection of gliotoxin was performed using a combination of methods by Bauer *et al.* (6) and Land *et al.* (22) using thin-layer chromatography (TLC). TLC plates were used (20 x 20 cm, 0.25 mm thickness, Silica Gel Merck 60 GF with fluorescence indicator 254 nm) for the detection of gliotoxin and aflatoxins from biosynthesis in developing solvents toluene/ethyl acetate/formic acid, 5:4:1 (*V/V/V*). For gliotoxin visualization at UV 366 nm, developed plates were sprayed with 10% (*w/V*) aluminium chloride in 50% (*V/V*) ethanol and heated 10 minutes at 110 °C. Gliotoxin appeared as a bluish-green spot under UV 366 nm at R_f value 0.59. Another method was used to prove gliotoxin when plates were sprayed with

freshly prepared 10% (*m/V*) silver nitrate in 80% (*V/V*) ethanol. Gliotoxin then appeared as a brown spot in visible light at R_f value 0.59. Aflatoxins appeared as blue (aflatoxin B_1), and green (aflatoxin G_1) spots under UV 366 nm with or without spraying with 10% (*w/V*) aluminium chloride in 50% (*V/V*) ethanol at R_f values at 0.59 (B_1) and 0.66 (G_1).

In certain cases, two-dimensional TLC (20 x 20 cm) was used for confirmation of the detected mycotoxins.

Gliotoxin and aflatoxins were semi-quantitatively analyzed on TLC plates comparing of the fluorescence under UV 366 nm of gliotoxin from extracts and the fluorescence of gliotoxin standard solution (or standard solution of aflatoxins) after developing and spraying the plates with 10% (*m/V*) aluminium chloride in 50% (*V/V*) ethanol and heating for 10 minutes at 110 °C.

Bioautoantibiogram on thin-layer chromatography plates against Bacillus subtilis

For localization of bactericidal compounds from extracts in the biosynthesis after separation by TLC, plates were left 3 days at room temperature (mobile phase evaporated completely). TLC surface was sprayed with sterile physiological saline and 15 mL of Müller-Hinton agar melted at approximately 50 °C and inoculated with 1 mL of the suspension of *Bacillus subtilis* NCTC 8236 cells (10^7 CFU mL^{-1}) was poured on the surface. The inoculated plates were stored in a humid atmosphere, and incubated overnight at 37 ± 1 °C. The zone of inhibition around compounds separated from extracts was visualized by spraying the bioautography-plates with 1% (*m/V*) triphenyl-tetrazolium chloride (TTC) solution in sterile distilled water. Only living bacterial cells hydrolyzed TTC to red colored formazan. The zone of growth inhibition was clearly visible around the bactericidal compound on the TLC plate.

Statistical analysis

Concentrations of gliotoxin and aflatoxins were calculated as mean ± SE. Numbers of metabolites from environmental and clinical isolates were compared by the *t*-test following the Mann Whitney *U* post-test using the GraphPad Prism Software, GraphPad Software, San Diego, CA.

RESULTS AND DISCUSSION

Cultivation in YES liquid medium and mycotoxins production analysis

Most of the clinical strains of *Aspergillus fumigatus* and *A. flavus* were isolated from immunosuppressed patients with acute leukemia: twenty-seven (54%) of 50 clinical *A. fumigatus* strains and seventeen (56%) of 30 *A. flavus* strains. The other *A. fumigatus* and *A. flavus* strains were isolated from patients with Hodkgin's and non-Hodkgin's diseases, liver and heart operations, and other diseases listed in Tables I and II.

All of 100 *A. fumigatus* isolates were analyzed by thin-layer chromatography (TLC) for gliotoxin production.

Table I. Source of clinical isolates of A. fumigatus *strains*

Isolate code[a]	Diagnosis	Number of isolates
6407, 6409, 6410, 6412, 6449, 6387, 6388, 6391, 6394, 6397, 6403, 6404, 6459, 6460, 6462, 6464	Myeloid leukemia	16
6392, 6396, 6398, 6398B, 6408, 6413, 6414, 6434, 6434B, 6448, 6458	Lymphatic leukemia	11
6400, 6402, 6406, 6411, 6460B, 6467	Hodgkin's disease	6
6395, 6405, 6415, 6386, 6389	Heart surgery	5
6393, 6463, 6465	Non-Hodgkin's disease	3
6390, 6457, 6466	Bronchopneumonia	3
6399, 6401	Liver transplantation	2
6470, 6470B	Plasmacytome, lung embolia	2
6445	Aplastic anemia	1
6468	Chronic sinusitis	1
		Total 50

[a] The isolation code is the number of fungal strains from the Collection of Microorganisms of the Depertment of Microbiology, Faculty of Pharmacy and Biochemistry, University of Zagreb.

Table II. Source of clinical isolates of A. flavus *strains*

Isolate code[a]	Diagnosis	Number of isolates
6417, 6419, 6420, 6421, 6435, 6435B, 6438, 6449, 6449B, 6453, 6454, 6613, 6613B	Myeloid leukemia	13
6416, 6418, 6436, 6437, 6455, 6612	Hodgkin's disease	6
6434, 6439, 6441, 6609	Lymphatic leukemia	4
6390, 6607, 6607B	Heart surgery	3
6451, 6453, 6611	Non-Hodgkin's disease	3
6440	»Immunodeficiency«	1
		Total 30

[a] The isolation code is the number of fungal strains from the Collection of Microorganisms of the Department of Microbiology, Faculty of Pharmacy and Biochemistry, University of Zagreb.

Only 9 strains (18%) of *A. fumigatus* from the group of clinical isolates produced gliotoxin after 6 days of incubation in YES liquid medium at 25 ± 2 °C. Gliotoxin producing strains were isolated from patients with leukemia (5 isolates), and other strains from patients undergoing heart surgery (2 isolates), and one isolate each from patients with Hodgkin's disease and non-Hodgkin's disease (Table I). Semi-quantitative analysis of gliotoxin concentration in YES liquid medium showed that the concentration of gliotoxin depended on the *A. fumigatus* strain and ranged from 0.5 to 5 mg mL^{-1}, with the mean concentration of 2.22 mg mL^{-1}.

Apart from gliotoxin production in 9 strains, fifteen different metabolites were isolated and visualized on developed TLC plates from the chloroformic extract of *A. fumigatus* culture in YES liquid medium. R_f values of these metabolites in clinical and environmental isolates are presented in Fig. 1.

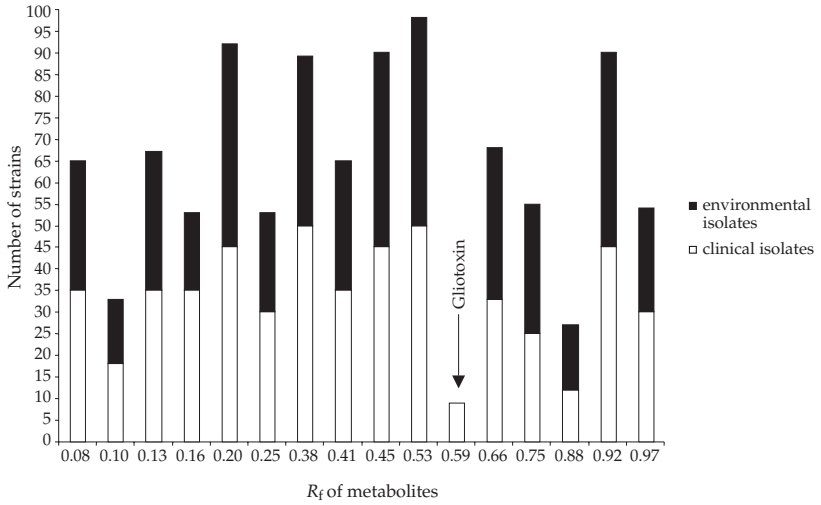

Fig. 1. Frequency of 16 separated secondary metabolites (with R_f values from 0.08 to 0.97) on TLC plates between clinical and environmental isolates of *A. fumigatus* strains (gliotoxin is marked, all other metabolites are unknown).

None of 50 environmental *A. fumigatus* isolates produced gliotoxin, nor did any of the examined 60 *A. flavus* strains. No production of aflatoxins in *A. fumigatus* strains was detected either.

Incidence of these metabolites in groups of clinical and environmental isolates was compared, and no statistic difference was recorded. Analysis of secondary metabolites indicates homogenic distribution in both groups of *A. fumigatus* isolates.

Sixty strains of *A. flavus* were examined for aflatoxins production. After 6 days at 25 ± 2 °C eleven of the 30 environmental strains (37%) produced aflatoxin B_1 in a concen-

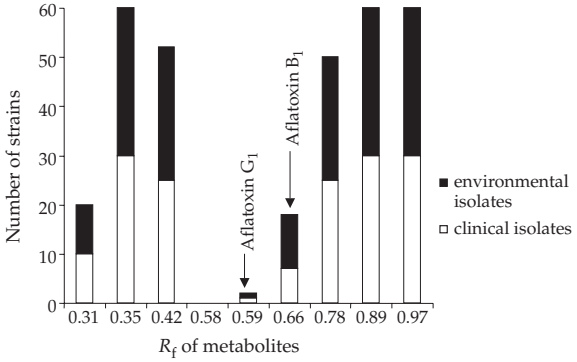

Fig. 2. Frequency of 9 separated secondary metabolites (with R_f values from 0.31 to 0.97) on TLC plates between clinical and environmental isolates of *A. flavus* strains (aflatoxins are marked, all other metabolites are unknown).

tration from 0.024 to 1.2 mg L^{-1}. In one of the 30 strains aflatoxin G_1 production was also detected in a concentration of 0.02 mg L^{-1}. Similar concentration was detected in the group of clinical isolates of *A. flavus* strains. Seven of the 30 strains (23%) produced aflatoxin B_1 in concentrations from 0.02 to 1.2 mg L^{-1}. One isolate produced also aflatoxin G_1 in a concentration of 0.12 mg L^{-1}. Concentrations of aflatoxin B_1 in clinical and environmental isolates were compared, but no differences were found. The number of metabolites from chloroformic extracts of *A. flavus* strains in YES liquid medium at 25 ± 2 °C was compared. Six different metabolites, besides aflatoxins, were found in extracts. Analysis of the frequency of metabolites showed no differences between clinical and environmental isolates. They had a homogenic distribution of metabolites as did the extracts from *A. fumigatus* strains (Fig. 2).

Antibacterial activity of metabolites using the bioautoantibiogram assay

After the TLC analysis of gliotoxin and aflatoxins, other TLC plates were used for the bioautography assay against *Bacillus subtilis* NTCT 8236. In the group of clinical *A. fumigatus* isolates, only gliotoxin producing strains had a clear zone of inhibition around the R_f value of gliotoxin (Fig. 3). No zones were noticed on TLC plates with separated metabolites in chloroformic extracts from environmental isolates of *A. fumigatus* strains or in any *A. flavus* strains tested. For confirmation of inhibition zones corresponding to gliotoxin, the chloroformic solution of gliotoxin (50 μg mL^{-1}) was used.

Aspergillosis is a serious opportunistic infection that can occur in immunosuppressed patients. Despite the development of new antifungal drugs which may lower the incidence of *Aspergillus* infections in transplant patients, mortality of patients with leukemia and AIDS due to such infections is still very high (> 90%) (23). Aspergillosis is most commonly caused by the fungi *A. fumigatus, A. flavus, A. niger* and *A. terreus.* These fungi can produce low-molecular-weight secondary metabolites such as gliotoxin (*A. fumigatus* and *A. terreus*) and aflatoxins (*A. flavus*), and the connection between mycotoxin production *in situ* and the pathogenicity of aspergillosis is still unknown. It could be hypothesized that the production of potent toxic metabolites of asperille such as gliotoxin and aflatoxins at the site of mycosis could potentially play a significant role in the pathogenesis of aspergillosis and other fungal diseases. Although gliotoxin is secreted in infected tissues of man and animals, by *A. fumigatus* and the yeast *Candida albicans*, no research has been done to find the differences between mycotoxin production by isolates of strains colonized in airways of immunocompromised patients and environmental isolates from soil and air.

In a study of Aufauvre-Brown *et al.* (24), a difference in virulence was found between clinical and environmental isolates in an immunosupresed mouse model and clinical isolates were more virulent. Authors also suggest that these differences could be due to the inter-mouse variation and immunossuppresion rather than to differences in virulence of *A. fumigatus* strains. In another study, the in *vitro* comparison of extracellular phospholipases (high-molecular-weight secreted metabolites) secretion of clinical and environmental isolates showed stronger activity of phospholipases in clinical isolates of *A. fumigatus* strains (18). Hobson (25) discussed the inhibition of human neutrophil phagocytosis by the spore diffusates from *A. fumigatus* and *A. terreus*. In this study, spore diffusates, which do not contain mycotoxins such as gliotoxin or other secondary metab-

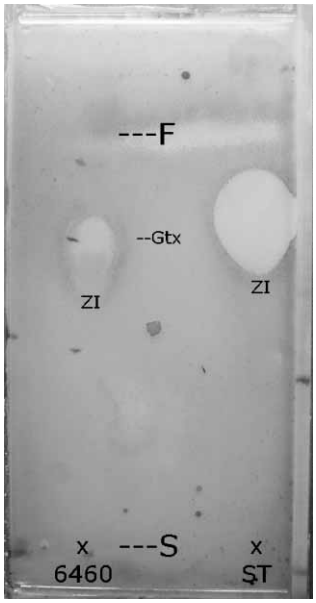

Fig. 3. Bioautoantibiogram (»bioassay *in situ*«) *on TLC plates against Bacillus subtilis* after spraying with TTC (Legend: S – start, F – front, 6460 – extract of *A. fumigatus* strain 6460, ST – standard solution of gliotoxin 50 ng mL^{-1}, Gtx – R_f values of developed gliotoxin, ZI – growth inhibition zones).

olites, inhibited phagocitosis from 89.7 to 94.6%, and equivalent inhibitory effects were found for both clinical and environmental isolates of *A. fumigatus.*

Our study compares the production of secondary metabolites *in vitro* of clinical and environmental *A. fumigatus* isolates and we found no differences in the number of metabolites. The secondary-metabolites profiles were homogenous, without a significant difference in production. A small, but significant number (9 out of 50.18%) of clinical *A. fumigatus* isolates, mainly from patients with leukemia, produced gliotoxin in an average concentration of 2.22 mg mL^{-1} in YES liquid medium after 6 days of incubation. The same results were found for the mycotoxin production ability of clinical and environmental isolates of *A. flavus* strains. Similar concentrations of aflatoxins B_1 and G_1 were found in both groups.

This is in agreement with earlier observations that environmental isolates do not produce gliotoxin or produce it less frequently than other metabolites. For example, Land *et al.* (22) found no gliotoxin producing strains in 100 isolates of *A. fumigatus* from sawmills, but other mycotoxins such as fumitremorgen B and verruculogen were detected in 32% of strains using biosynthesis in YES medium. Tepšič *et al.* (26) detected verruculogen in 40% of *A. fumigatus* isolates from a saltern, and less frequently other metabolites. Gliotoxin was not detected in 50 strains of *A. fumigatus*. The same results were obtained by Fischer *et al.* (26), who did not detect gliotoxin in *A. fumigatus* strains isolated from air around a compost facility. It is interesting that gliotoxin can be identi-

fied in building materials (spruce wood, gypsum boards, chipboards) when these materials are contaminated with *A. fumigatus* (27).

In the tissues infected with *A. fumigatus* or the yeast *Candida albicans*, most of the isolated strains produce gliotoxin. For example, in women with vaginitis caused by *C. albicans*, 32 of 50 isolates produce gliotoxin (28), and Bauer *et al.* (6) detected gliotoxin in a bovine udder infected with *A. fumigatus*. In recent studies of Lewis *et al.* (10) gliotoxin was detected in the sera of patients with invasive aspergillosis, which indicates that gliotoxin, and probably other mycotoxins, are produced *in vivo*.

Our results showed that a small number (18%) of *A. fumigatus* strains isolated only from immunocompromised patients produce gliotoxin *in vitro*. Higher percent of aflatoxigenic *A. flavus* strains was recorded in groups of environmental and clinical isolates, 37 and 23%, respectively.

After developing TLC plates, isolated metabolites from chloroformic extracts were analyzed for antibacterial activity against *Bacillus subtilis* NCTC 8236. Only extracts with gliotoxin showed inhibition zones around the R_f value of gliotoxin (Fig. 3). Other extracts of *A. fumigatus* and *A. flavus* strains had no active antibacterial metabolites, which indicates that the bioautoantibiogram (»bioassay *in situ*«) on developed TLC plates against *B. subtilis* is suitable for detection of gliotoxin in extracts, or that it could be used as the confirmation assay for the presence of gliotoxin in combination with TLC.

It is known that the genetic factor of fungi and environmental conditions (substrate composition with precursors of mycotoxins, pH of the substrate, O_2/CO_2 ratio) could play an important role in mycotoxin production. The production and possible toxic effects of mycotoxins in infected tissues should be investigated, as well as the secreted concentration and biotransformation of these metabolites. Besides gliotoxin and aflatoxins, the *in situ* production of other low-molecular-weight metabolites of *Aspergillus* spp. isolated from immunosuppressed patients is in progress in our laboratory.

CONCLUSIONS

In this study we presented the *in vitro* production of toxic secondary metabolites – mycotoxins from the culture of *Aspergillus fumigatus* and *A. flavus* strains isolated from immunocompromized patients without invasive aspergillosis. Results of mycotoxin production, including gliotoxin and aflatoxin B_1 and G_1, were compared with the environmental isolates (from soil). The secondary-metabolite profiles of clinical and environmental *A. fumigatus* and *A. flavus* isolates were homogeneous, except for the gliotoxin production, which was detected only in the group of clinical isolates of *A. fumigatus* (18%). The few, but significant gliotoxinogenic *A. fumigatus* strains could contribute to the invasiveness of gliotoxin-producing *A. fumigatus* of *A. terreus* strains. From the epidemiological point of view, even our study was limited to the *in vitro* production of mycotoxins; further studies have to compare the *Apergillus* strains that cause invasive aspergillosis in immunocompromised patients, because the source of spores is the environment. The answer to the virulence of clinically important *Aspergillus* strains must include secondary metabolites with toxic activity against the host's cells and immunity and other factors of fungi independent of the factors of compromised hosts.

Acknowledgement. – We thank Dr. Marija Jandrlić from the Zagreb University Hospital (Department of Microbiology) for providing clinical isolates of *Aspergillus* strains.

REFERENCES

1. B. Dupont, M. Richardson, P. E. Verweij and F. G. M. Meis, Invasive aspergillosis, *Med. Mycol.* **38** (Suppl. 1) (2000) 215–224.
2. J. F. C. Tomee and H. F. Kauffman, Putative virulence factors of *Aspergillus fumigatus*, *Clin. Exp. Allergy* **30** (2000) 476–484.
3. L. H. Hogan, B. S. Klein and S. M. Levitz, Virulence factors of medically important fungi, *Clin. Microbiol. Rev.* **9** (1996) 469–488.
4. J. P. Latgé, *Aspergillus fumigatus* and aspergillosis, *Clin. Microbiol. Rev.* **12** (1999) 310–350.
5. S. Paris, D. Wysong, J. –P. Debeaupuis, K. Shibuya, B. Philippe, R. D. Diamond and J. –P. Latgé, Catalases of *Aspergillus fumigatus, Infect. Immunol.* **71** (2003) 3551–3562.
6. J. Bauer, M. Gareis, A. Bott and B. Gedek, Isolation of a mycotoxin (gliotoxin) from a bovine udder infected with *Aspergillus fumigatus*, *J. Med. Vet. Myocol.* **27** (1989) 45–50.
7. J. L. Richard and M. C. DeBey, Production of gliotoxin during the pathogenic state in turkey poults by *Aspergillus fumigatus* Fresenius, *Mycopathologia* **129** (1995) 111–115.
8. J. L. Richard, T. J. Dvorak and P. F. Ross, Natural occurrence of gliotoxin in turkeys infected with *Aspergillus fumigatus*, Fresenius, *Mycopathologia* **134** (1996) 167–170.
9. G. S. Bondy and J. J. Pestka, Immunomodulation by fungal toxins, *J. Toxicol. Environ. Health* **3** (2000) 109–143.
10. R. E. Lewis, J. Chi, N. P. Wiederhold, D. P. Kontoyiannis, X. Han and R. A. Prince, Detection of gliotoxin, an immunologically active metabolite secreted by *Aspergillus fumigatus*, in animals and humans with invasive aspergillosis (IA), Book of Abstracts of 43rd ICAAC (Interscience Conference on Antimicrobial Agents and Chemotherapy), Chicago, 14–17 September, 2003.
11. I. Kosalec and S. Pepeljnjak, The chemistry and biological effects of gliotoxin, *Arh. Hig. Rada Toksikol.* **55** (2004) 313–320.
12. P. Waring and J. Beaver, Gliotoxin and related epipolythiodioxopiperazines, *Gen. Pharm.* **27** (1996) 1311–1316.
13. P. Sutton, J. Beaver and P. Waring, Evidence that gliotoxin enhances lymphocyte activation and induces apoptosis by effects on cyclic AMP levels, *Biochem. Pharmacol.* **50** (1995) 2009–2014.
14. V. Cusumano, G. B. Costa and S. Seminara, Effects of aflatoxins on rat peritoneal macrophages, *Appl. Environ. Microbiol.* **56** (1990) 3482–3484.
15. G. J. Jakab, R. R. Hmieleski, A. Zarba, D. R. Hemenway and J. D. Groopman, Respiratory aflatoxicosis: suppression of pulmonary and systemic host defenses in rats and mice, *Toxicol. Appl. Pharmacol.* **125** (1994) 198–205.
16. D. M. Hinton, M. J. Myers, R. A. Raybourne, S. Francke-Varroll, R. E. Sotomayor, J. Shaddock, A. Warbritton and M. W. Chous, Immunotoxicity of aflatoxin B1 in inflammatory response in a chronic intermittent dosing study, *Toxicol. Sci.* **73** (2003) 362–377.
17. E. Y. Moon, D. K. Rhee and S. Pyo, *In vitro* suppressive effect of aflatoxin B_1 on murine peritoneal macrophage functions, *Toxicology* **133** (1999) 171–179.
18. M. Birch, D. W. Denning and F. D. Robson, Comparison of extracellular phospholipase activities in clinical and environmental *Aspergillus fumigatus* isolates, *Med. Mycol.* **42** (2004) 81–86.
19. S. Pepeljnjak, Z. Slobodnjak, M. Šegvić, M. Peraica and M. Pavlović, The ability of fungal isolates from human lung aspegilloma to produce mycotoxins, *Human Exper. Toxicol.* **23** (2004) 15–19.

20. *Atlas of Clinical Fungi*, 2nd ed. (Eds. G. S. deHoog and J. Guarro), Centraalbureau voor Schimmelcultures, Universitat Rovira i Virgili, Baard-Delft 2000.
21. M. A. Klich, Identification of common *Aspergillus* species, Centralbureau voor Schimmelcultures, Utrecht, 2002.
22. C. J. Land, H. Lundström and S. Werner, Production of tremorgenic mycotoxins by isolates of *Aspergillus fumigatus* from sawmills in Sweden, *Mycopathologia* **124** (1993) 87–93.
23. P. Sutton, N. R. Newcombe, P. Waring and A. Müllbacher, *In vivo* immunosuppressive activity of gliotoxin, a metabolite produced by human pathogenic fungi, *Infect. Immunol.* **62** (1994) 1192– 1198.
24. A. Aufauvre-Brown, J. S. Brown and D. W. Holden, Comparison of virulence between clinical and environmental isolates of *Aspergillus fumigatus*, *Eur. J. Clin. Microbiol. Infect. Dis.* **17** (1998) 778–780.
25. R. P. Hobson, The effects of diffusates from the spores of *Aspergillus fumigatus* and *A. terreus* on human neutrophils, *Naegleria gruberi* and *Acanthomoeba castellanii*, *Med. Mycol.* **38** (2000) 133–141.
26. K. Tepšič, N. Gunde-Cimerman and J. C. Frisvad, Growth and mycotoxin production by *Aspergillus fumigatus* strains isolated from a saltern, *FEMS Microbiol. Lett.* **157** (1997) 9–12.
27. G. Fischer, T. Muller, R. Schwalbe, R. Ostrowski and W. Dott. Exposure to airborne fungi, MVOC and mycotoxins in biowaste-handling facilities, *Int. J. Hyg. Environ. Health* **203** (2000) 97–104.
28. S. M. Nieminen, R. Kärki, S. Auriola, M. Toivola, H. Laatsch, R. Laatikainen, A. Hyvärinen and A. von Wright, Isolation and identification of *Aspergillus fumigatus* mycotoxins on growth medium and some building materials, *Appl. Environ. Microbiol.* **68** (2002) 4871–4875.
29. D. T. Shah and B. Larsen, Clinical isolates of yeast produce a gliotoxin-like substance, *Mycopathologia* **116** (1991) 203–208.

S A Ž E T A K

Mikotoksinogenost kliničkih i okolišnih *Aspergillus fumigatus* i *A. flavus* sojeva

IVAN KOSALEC i STJEPAN PEPELJNJAK

Analizirana je mikotoksinogenost pedeset kliničkih sojeva vrste *A. fumigatus* i trideset sojeva vrste *A. flavus* izoliranih iz imunokompromitiranih ispitanika u hematološkom odjelu. Kao usporedba, izoliran je isti broj okolišnih sojeva iz zemlje, komposta i zraka. Utvrđeno je da samo 9 sojeva (18%) vrste *A. fumigatus* tvori gliotoksin sa srednjom vrijednošću 2,22 mg mL^{-1} (u rasponu od 0,5 do 5 mg mL^{-1}). Tvorba aflatoksina B_1 utvrđena je u 7 sojeva (23%) (raspon od 0,024 do 1,2 mg L^{-1}) i u jednog kliničkog izolata (3%) *A. flavus* vrste (0,12 mg L^{-1}). U skupini okolišnih sojeva utvrđena je tvorba aflatoksina B_1 u 11 sojeva (37%) (u rasponu od 0,024 do 1,2 mg L^{-1}) i u jednog soja tvorba aflatoksina G_1 (0,024 mg L^{-1}). Bioautoantibiogramom na tankoslojnoj kromatografskoj ploči s bakterijskom vrstom *Bacillus subtilis* NCTC 8236 utvrđeno je da samo gliotoksinogeni sojevi pokazuju baktericidnu aktivnost oko R_f vrijednosti koja odgovara gliotoksinu. Profili sekundarnih metabolita kliničkih i okološnih sojeva *A. funmigatus* i *A. flavus* vrsta su bili homogeni, osim za tvorbu gliotoksina koji je utvrđen samo u skupini kliničkih izolata *A. fumigatus* sojeva (18%).

Ključne riječi: Aspergillus, *A. fumigatus*, *A. flavus*, gliotoksin, aflatoksin, mikotoksin, tvorba *in vitro*, sekundarni metaboliti, plijesni

Farmaceutsko-biokemijski fakultet, Sveučilišta u Zagrebu, Zagreb