Evaluation of Mutagenic Potential of Mold Extracts Isolated from Buildings Using the Mouse Lymphoma Thymidine Kinase Gene Mutation Assay (MLA)

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Abstract

In recent years interest in mycotoxin-producing fungi growing in indoor environments has increased. Evidence of mutagenic potential of some molds or their mycotoxins is still equivocal. Much more information is available for single mycotoxins or their metabolites than for complex mixtures composed of different fungi products.

The aim of this study was to identify the mutagenic potential of extracts isolated from mold-attacked buildings using the mouse lymphoma assay (MLA).

Although a thin-layer chromatography analysis of extracts isolated from test buildings showed the presence of well-known mycotoxins (ochratoxin A and sterigmatocystin), the test extracts as well as control extracts did not reveal mutagenic potential after 3-hr exposure without or with metabolic activation as tested with MLA. Negative results were also obtained after 24-hr treatment with every test sample in the absence of S9 fraction. The data indicates that extracts prepared from control and test buildings under experimental conditions did not induce mutations affecting the expression of the thymidine kinase gene in the cultured L5178Y TK⁻ cells.

Keywords: indoor molds, swab extracts, damp buildings, mutagenicity, Mouse Lymphoma Assay (MLA)

Introduction

Interest in molds in indoor environments has increased since people became aware that exposure to them can cause a variety of deleterious health effects and symptoms, including allergic reactions. Although molds are not dominant allergen and outdoor molds, rather than indoor ones, are the most important, the growth of any kind of mold in a building environment should not be tolerated. Molds growing indoors destroy building materials and degrade indoor air quality, creating a special microclimate (distinctive offensive odours, spores and metabolites, e.g. mycotoxins) that is dangerous for human health [1, 2]. Mold spores are present in all indoor environments and cannot be eliminated [3]. As oligotro-
Mycotoxins can affect many target organs and systems, notably liver, kidney and nervous, endocrine and immune systems. The mechanisms of their toxicity include interference with cellular respiration, interference with carbohydrate and lipid metabolism or direct binding to DNA and RNA. Studies in animals exposed to mycotoxins and mold-produced secondary metabolites [1, 6]. Toxic effects of molds (with *Stachybotrys sp.*, *Apergillus sp.* or *Penicillium sp.* listed among the most dangerous species) are mainly associated with exposure to mycotoxins and mold-produced secondary metabolites [1, 6]. However, there is also data indicating the presence of toxic effects after exposure to extremely high concentrations of indoor mold spores, even if they do not contain mycotoxins [7].

Mycotoxins can affect many target organs and systems, notably liver, kidney and nervous, endocrine and immune systems. The mechanisms of their toxicity include interference with cellular respiration, interference with carbohydrate and lipid metabolism or direct binding to DNA and RNA. Studies in animals exposed to purified mycotoxins demonstrate that these compounds and/or their metabolites can be teratogenic or carcinogenic after ingestion as well as after inhalation [8]. Several epidemiological studies also reported increased incidence of leukaemia and respiratory cancer in people occupationally exposed by skin or respiratory tract to high level of mold spores and their mycotoxins [9-11]. In spite of many studies conducted so far, the evidence of mutagenicity of mycotoxins [12, 13] is still equivocal. Moreover, still much more information is available for single mycotoxins or their metabolites than for complex mixtures composed of different fungi products. Some research has demonstrated that mycotoxins acted as direct mutagens (e.g. aflatoxin B1) [14] or they first required cytochrome P450-dependent metabolism (e.g. ochratoxin A; [15]) or other modifications (e.g. nitrosylation [13]) to reveal mutagenicity. In other studies no mutagenic effect could be detected, neither in the bacterial nor in the mammalian test assays [16]. There is also no convincing evidence demonstrating that indoor-growing molds, usually consisting of more than one species, can share the same potential as laboratory culture strains and if so, what level of exposure can be adverse for humans.

Taking into account the available data, the issue of real health hazard of indoor molds is still open for discussion. The growing controversy over mutagenic/carcinogenic potential of molds seems to be of special importance, not only from public health but also from an economic point of view. This study was undertaken to provide additional data on indoor mold mutagenicity with a major assumption to reflect the real exposure conditions as much as possible. To this end, extracts prepared from three separate mold-attacked flats as well as from a flat without such symptoms were assessed using microwell adaptation of the *in vitro* mouse lymphoma thymidine kinase gene mutation assay (MLA).

### Materials and Methods

Chemicals and Reagents

Trifluorothymidine (TFT), benzo[a]pyrene (B[a]P; ≥96% HPLC) and methyl methanesulfonate (MMS; ~99% GC), thymidine, hypoxanthine, methotrexate and glycine were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

TFT was prepared under subdued lighting at a concentration of 400 µg/ml in RPMI medium without serum (R0 medium) then filter sterilized, foil-wrapped and stored in aliquots at -20°C. Any residues of TFT were discarded and not frozen again. All platelets in selective medium containing TFT were carried out under subdued lighting. B[a]P (used as positive control in MLA with metabolic activation) was dissolved in DMSO at a concentration of 3 mg/ml and stored at -20°C, whereas MMS (positive control in MLA without metabolic activation) was reconstituted at a concentration of 1 mg/ml in R0 in the dark immediately before use. The 100xTHG (containing thymidine, hypoxanthine and glycine at the following concentrations: 0.3 mg/ml, 0.5 mg/ml and 0.75 mg/ml) and 100xTHMG (methotrexate at concentration of 0.1 mg/ml added to 100xTHG solution) stocks were prepared in R0 and stored at -20°C.

Cell Culture

L5178Y TK+/- mouse lymphoma cells (American Type Culture Collection; ATCC#CRL9518) were grown in suspension in RPMI 1640 medium (Gibco BRL) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich Chemical Company) and 200 µg/ml sodium pyruvate (Sigma-Aldrich Chemical Company) and 10% heat-inactivated horse serum (Gibco BRL) (R10 medium). The cells were assayed for survival and mutant frequency in the medium with 20% heat-inactivated horse serum (R20 medium). The population doubling time of un-
treated cells in suspension culture was 10 hours. Before each experiment, L5178Y TK+/- cells were cleansed of spontaneous TK-/- mutants by 24-hour incubation of 25 ml of cells at 107 cells/ml in R10 containing 3 µg/ml thymidine, 5 µg/ml hypoxanthine, 0.1 µg/ml methotrexate and 7.5 µg/ml glycine (THMG). Then, 5 ml of the culture was centrifuged to remove the methotrexate and cultured for 2-3 days in 150 ml of R10 supplemented with thymidine, hypoxanthine and glycine (THG) at the same concentrations.

Before experiment the cell line was screened for Mycoplasma sp. infection using indicator cell line 3T6 cells (ATCC#CCl-96) and MycoTech Kit (Gibco BRL) according to the manufacturer’s protocol.

Test Sample Preparation

Samples were collected from residential dwellings in Łódź (sample characteristics are shown in Table 1). Smears were taken from an area of 25 cm² (test samples) or 50 cm² (control) of building partitions. Three samples were taken from three separate mold-attacked flats and one (control) from the flat without fungi contamination symptoms. The flats, where walls were infected by molds and the growth was at least up to 0.5 m above the floor, were considered as mold-attacked ones. Moreover, dwellers living in such a flat complained of health discomfort.

The samples taken to 10 ml of phosphate-buffered saline (PBS; pH 7.4) were subjected to sonification (Ultrasonic Homogenizer 4710, Cole-Parmer Instrument Co, Chicago) and then filtration (pores 0.22 µm; Sartorius). Such prepared extracts were aliquoted and kept at -20°C until the day of assay. Freeze-dried (lyophilized) samples were used for the mouse lymphoma assay.

Identification of Mold Species in Swab Samples Isolated from Buildings

Molds were isolated from surfaces of building partitions with visible fungal growths. Smears were taken from an area of 25 cm² (test samples) or 50 cm² (control) of building partitions. The samples were taken to 100 ml of 0.85% NaCl and then seeded on 2% MEA medium and DG-18 agar medium. After incubation at 28°C for 7 days, the mold species were identified and number of colony-forming units (CFU) per 100 cm² of surface was estimated [17, 18].

Thin-Layer Chromatography (TLC)

Samples were collected from surfaces of building partitions with visible fungal growths. Smears were taken from an area of 25 cm² to 10 ml of methanol: chloroform (2:1).

Thin-layer chromatography was performed according to Frisvad et al. [19]. Briefly, 5 µl of each extract collected from wall surface as well as standard mycotoxins (ochratoxin A and sterigmatocystin) were spread on TLC plates (Silica Gel 60, Merck). After developing in TEF (toluene: ethyl acetate: formic acid, 5:4:1), the chromatograms were observed under UV light (360 nm) before and after development with AlCl₃ solution and compared with the standards. Standard mycotoxin solutions used for TLC were the following: aflatoxin B1 – 100 µg/ml (500 ng/5µl sample), ochratoxin A – 200 µg/ml (1000 ng/5µl sample), sterigmatocystin – 20 µg/ml (100 ng/5 µl sample).

S9 and S9-Mix Preparation

The mouse lymphoma mutagenicity assay was performed in two variants: with and without metabolic ac-

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Table 1. Mold species and their metabolites detected in swab samples isolated from buildings.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amount of molds in test sample [CFU/100 cm²]</th>
<th>Mold species detected in test sample</th>
<th>Metabolites detected in test sample (TLC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract from control building</td>
<td>2 x 10³</td>
<td>Penicillium chrysogenum (100%)</td>
<td>-</td>
</tr>
<tr>
<td>Extract from test building (1)</td>
<td>7.46 x 10⁵</td>
<td>Aspergillus niger (81%)</td>
<td>Ochratoxin A and other metabolites*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Penicillium chrysogenum (8%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cladosporium cladosporioides (10%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Penicillium sp. (probably P. expansum) (1%)</td>
<td></td>
</tr>
<tr>
<td>Extract from test building (2)</td>
<td>1.46 x 10⁶</td>
<td>Alternaria alternata (46%)</td>
<td>Other metabolites*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cladosporium cladosporioides (44%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Penicillium chrysogenum (9%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Penicillium granulatum (1%)</td>
<td></td>
</tr>
<tr>
<td>Extract from test building (3)</td>
<td>1.5 x 10⁶</td>
<td>Aspergillus versicolor (83%)</td>
<td>Sterigmatocystin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aspergillus niger (6%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Penicillium sp. (probably P. expansum) (11%)</td>
<td></td>
</tr>
</tbody>
</table>

* – other metabolites, different from ochratoxin A and sterigmatocystin
tivation. Post-mitochondrial fraction (S9) from male rat liver (outbred I mp: WIST) after induction of microsomal enzymes by Aroclor 1254 was prepared as described by Ames et al. [20]. The protein concentration of the S9 fraction (determined by Lowry’s method) was 5.3 mg/ml [21]. The S9-mix was prepared just prior to use by mixing S9 with 180 mg/ml glucose 6-phosphate disodium salt (G-6-P), 25 mg/ml β-Nicotinamide adenine dinucleotide phosphate disodium salt (NADP) and 150 mM KCl in the ratio 2:1:1:1. The concentration of S9-mix was 5% during treatment and the final concentration of S9 was 2%.

The Mouse Lymphoma Assay (MLA)

Mutagenicity assessment was carried out in 96-well microtiter plates following OECD TG 476 [22]. The cells were exposed to test or control samples for 3 hours (without or with metabolic activation) and 24 hours (without metabolic activation). Calculations of the parameters of plating for survival and then, after expression time, plating for viability and TFT resistance were performed as described earlier [23]. Briefly, for the 3-hr exposure, 1x10^5 exponentially growing cells (2x10^5 cells/ml; placed in 50 ml centrifuge tubes) were centrifuged and resuspended in 10 ml of R10 medium. Then, 9 ml of R0 medium containing a suitable amount of test extract (a solid residue remaining after freeze drying (lyophilizing) of 0.6 ml of each extract was reconstituted in medium R0), negative control (solvents: a. medium R0, b. 0.001% DMSO, c. freeze dried PBS extract treated in the same way as the test samples), or positive control (a. 3 µg/ml B[a]P in presence of S9-mix or b. 10 µg/ml MMS in absence of S9-mix) was added. Additionally, 1.0 ml of S-9 mix or 30 mM KCl was added to metabolic activated and non-activated cultures, respectively. A final volume of treatment medium was 20 ml and it contained a decreased amount of horse serum (5%) to protect the activity of mutagens.

For the 24-hr exposure (culture flasks without shaking), 6x10^5 cells in 40 ml of total volume of treatment medium containing 10% of horse serum were used. Cells were exposed for 24 hrs to the test samples that showed negative results after 3-hr treatment.

After 3 or 24-hr incubation, cultures were centrifuged at 200 x g for 5 min, the cells washed twice with R10 medium and resuspended in 20 ml of R10 medium. Cell densities were determined using haemocytometer and adjusted to 2x10^5/ml. Cells were transferred to flasks for growth through the expression period or diluted to be plated for survival.

For the plating for survival the samples were diluted to density of 8 cells/ml and 0.2 ml of each culture was placed into two 96-well plates (192 wells; on average 1.6 cell/well). The plates were incubated (37°C, 5% CO_2) for 11-13 days. Wells that did not contain viable clones were identified using microscope and counted.

For the plating for 5-trifluoro-thymidine (TFT) resistance, to the cell cultures (adjusted to 1x10^5/ml at the end of the expression period) TFT (400 µg/ml) was added to give the final concentration of 4 µg/ml. Cells (0.2 ml of each suspension) were placed into four 96-well plates (384 wells; 2x10^5 cells/well). The plates were incubated (37°C, 5% CO_2) for 11-13 days and wells containing small (less than a quarter of the diameter of the well) and large colonies were identified using a microscope and counted.

Calculations of MLA Results and Statistical Analysis

Plating efficiency (PE), percentage relative survival (%RS), suspension growth (SG), relative total growth (%RTG) and mutant frequency (MF) were calculated as previously described in details by Clements [23]. In brief:

Plating efficiency (PE)

\[ PE = \frac{P}{n} \]

where: \( P = \text{number of colonies} \)

Relative PE2 = PE_{test} at day 4 / PE_{solvent} at day 4

Percentage relative survival (%RS)

\[ \%RS = \left( \frac{PE_{test}}{PE_{solvent}} \right) \times 100 \]

Adjusted%RS = %RS × (Number of test sample-treated cells at day 1 / Number of solvent-treated cells at day 1)

Suspension growth (SG)

\[ SG = \frac{N_{test}}{N_{solvent}} \]

Relative suspension growth (RSG) = SG_{test} / SG_{solvent}

Relative total growth (%RTG)

\[ \%RTG = RSG \times \text{relative PE2} \]

Mutant frequency (MF)

\[ MF = \frac{PE_{mutant}}{PE_{2}} \times 10^6 \]

Number of TFT-resistant cells per 1 x 10^6 cells
Mutation index (MI)

\[ MI = \frac{MF_{\text{test}}}{MF_{\text{control}}} \]

Bartlett’s test of homogeneity of variance was used to determine if the results had equivalent variances at the \( p<0.05 \) level. If the variances were not significantly different, the results were compared using a standard one-way analysis of variance (ANOVA). If the results did not have equivalent variances at the \( p<0.05 \) level, then the Kruskal-Wallis test was used to assay the differences in result means.

Test Acceptance Criteria [23]

The assay is considered as valid if the following criteria are fulfilled:

- The mutant frequency for solvent control (MF) is above 60 mutants per \( 10^6 \) viable cells but not more than three times the historical mean value.
- The plating efficiency (PE) and relative plating efficiency (PE2) for solvent controls is between 60-140% and 70-130%, respectively.

The test sample is considered to be mutagenic in the assay if all the following criteria are fulfilled:

- the assay is valid
- the mutant frequency at one or more doses is significantly greater than that of the negative control \( (p<0.05) \)
- there is a significant dose-relationship as indicated by the linear trend analysis \( (p<0.05) \).

Results

Characteristics of Test Samples

Four samples were isolated from four different buildings (one from control and three from mold-attacked buildings) and the extracts were prepared as described in Materials and Methods. The amount of molds in each sample was expressed in colony-forming units (CFU/100 cm²). The most common genera of fungi identified (\textit{Alternaria}, \textit{Penicillium}, \textit{Cladosporium} and \textit{Aspergillus}) and their amounts in the individual samples isolated by swab sampling are illustrated in Table 1. The mold metabolites in each sample were separated using thin layer chromatography (TCL) and the results were compared with distribution patterns of ochratoxin A and sterigmatocystin that were used as reference mycotoxins (Table 1).

The Mutagenicity of Extracts Isolated from Buildings in the Mouse Lymphoma Assay

In the first set of experiments, L5178Y TK+/- cells were exposed to test extracts for 3 hours without or with metabolic activation. As can be seen in Table 2 the values of critical parameters for the evaluation of the mutagenicity with MLA to be valid were all in the correct range for each control sample. All collected data including the number of empty wells onto survival and viability plates as well as the number of small and large colonies counted during TFT resistance measurements was used to calculate the percentage relative survival (\( \%RS \)), relative total growth (RTG), mutant frequency (MF) and mutation index (MI). As a result of conducted experiments, neither negative control nor test extracts revealed mutagenic potential after 3-hr exposure without or with metabolic activation (MI below 2). In contrast, 3-hr exposure to methyl methanesulfonate (10 µg/ml) or benzo[a]pyrene (3 µg/ml) used as positive controls showed considerable mutagenic effect (MI 8.8 and 7.3, respectively). Since the 3-hr exposure gave negative results, the cells were subsequently treated with the extract samples for 24 hours as recommended by OECD TG 476 [22]. This prolonged exposure in the absence of S9-mix also gave negative results for each sample. Methyl methanesulfonate showed again strong mutagenic activity (MI 5.0). Results calculated after 3- and 24-hr treatments are shown in Table 3 and 4, respectively.

Discussion

Indoor growth of mycotoxin-producing molds is associated with adverse health effects in humans [24, 25].

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Results after 3-hour exposure</th>
<th>Results after 24-hour exposure</th>
<th>Recommendation [50]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated cells</td>
<td>Negative control (PBS)</td>
<td>Negative control (DMSO)</td>
</tr>
<tr>
<td>PE [%] (plating efficiency)</td>
<td>73 (-S9)</td>
<td>67 (-S9)</td>
<td>67 (+S9)</td>
</tr>
<tr>
<td></td>
<td>72 (+S9)</td>
<td>85 (+S9)</td>
<td>67 (+S9)</td>
</tr>
<tr>
<td>PE2 [%] (relative plating efficiency)</td>
<td>91 (-S9)</td>
<td>108 (-S9)</td>
<td>- (- S9)</td>
</tr>
<tr>
<td></td>
<td>106 (+S9)</td>
<td>97 (+S9)</td>
<td>84 (+S9)</td>
</tr>
<tr>
<td>MF (x 10^6) (mutant frequency)</td>
<td>69 (-S9)</td>
<td>63 (-S9)</td>
<td>- (- S9)</td>
</tr>
<tr>
<td></td>
<td>73 (+S9)</td>
<td>66 (+S9)</td>
<td>62 (+S9)</td>
</tr>
</tbody>
</table>
Due to their toxicity, inhalation of extremely high concentrations of indoor mold spores is considered a health hazard even if the spores do not contain mycotoxins [7]. However, data demonstrating that exposure at levels commonly encountered in most mold-attacked buildings can be harmful for occupants or workers are still scarce. Building occupants can be exposed through inhalation to complex mixtures of fungi and their mycotoxins (but also to bacteria, endotoxins and other antigens). A single mold species may produce several different mycotoxins and, conversely, different mold genera may produce the same mycotoxin [6].

Considering general scarcity of data and often contradictory results on genotoxicity/mutagenicity of molds growing in buildings, this study was undertaken to provide more details on cytotoxic and mutagenic potential of indoor molds. Concentrations of mold spores detected in the extracts prepared from 4 selected buildings ranged from relatively low (2x10^3 CFU/100 cm^2 – control building) to quite high (1.5x10^8 CFU/100 cm^2). Genera of the molds detected in the extracts were diverse, including Aspergillus, Alternaria, Penicillium, and Cladosporium. Although well-known mycotoxins (ochratoxin A and sterigmatocystin) were identified, other unidentified metabolites were also detected in 2 out of 3 samples. Most fungi species detected in the swab samples are common contaminants (e.g. of food) proven to produce potentially toxic mycotoxins.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of colonies [small+large]</th>
<th>%RS (-S9)</th>
<th>RTG (-S9)</th>
<th>MF (x10^-6)</th>
<th>MI (-S9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>20+24 (-S9)</td>
<td>- (-S9)</td>
<td>- (-S9)</td>
<td>69 (-S9)</td>
<td>- (-S9)</td>
</tr>
<tr>
<td>PBS</td>
<td>32+18 (-S9)</td>
<td>- (-S9)</td>
<td>- (-S9)</td>
<td>63 (-S9)</td>
<td>0.9 (-S9)</td>
</tr>
<tr>
<td>MMS (10 μg/ml)</td>
<td>25+20 (-S9)</td>
<td>124 (-S9)</td>
<td>171 (-S9)</td>
<td>66 (-S9)</td>
<td>0.9 (+S9)</td>
</tr>
<tr>
<td>MMS (10 μg/ml)</td>
<td>- (-S9)</td>
<td>- (-S9)</td>
<td>- (-S9)</td>
<td>73 (+S9)</td>
<td>- (+S9)</td>
</tr>
<tr>
<td>Extract from control building</td>
<td>16+20 (+S9)</td>
<td>88 (-S9)</td>
<td>64 (-S9)</td>
<td>604 (-S9)</td>
<td>8.8 (-S9)</td>
</tr>
<tr>
<td>Extract from control building</td>
<td>100 (+S9)</td>
<td>- (-S9)</td>
<td>- (-S9)</td>
<td>- (+S9)</td>
<td>- (+S9)</td>
</tr>
<tr>
<td>Extract from test building (1)</td>
<td>21+23 (-S9)</td>
<td>109 (-S9)</td>
<td>81 (-S9)</td>
<td>65 (-S9)</td>
<td>0.9 (-S9)</td>
</tr>
<tr>
<td>Extract from test building (1)</td>
<td>119 (+S9)</td>
<td>- (-S9)</td>
<td>- (-S9)</td>
<td>47 (+S9)</td>
<td>0.6 (+S9)</td>
</tr>
<tr>
<td>Extract from test building (2)</td>
<td>31+16 (-S9)</td>
<td>119 (-S9)</td>
<td>96 (-S9)</td>
<td>78 (-S9)</td>
<td>1.1 (-S9)</td>
</tr>
<tr>
<td>Extract from test building (2)</td>
<td>106 (+S9)</td>
<td>- (-S9)</td>
<td>- (-S9)</td>
<td>115 (-S9)</td>
<td>1.6 (+S9)</td>
</tr>
<tr>
<td>Extract from test building (3)</td>
<td>25+27 (-S9)</td>
<td>82 (-S9)</td>
<td>94 (-S9)</td>
<td>70 (-S9)</td>
<td>1.0 (-S9)</td>
</tr>
<tr>
<td>Extract from test building (3)</td>
<td>17+16 (+S9)</td>
<td>91 (+S9)</td>
<td>72 (+S9)</td>
<td>47 (+S9)</td>
<td>0.6 (+S9)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of colonies [small+large]</th>
<th>%RS (-S9)</th>
<th>RTG (-S9)</th>
<th>MF (x10^-6)</th>
<th>MI (-S9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>30+19</td>
<td>- (-S9)</td>
<td>- (-S9)</td>
<td>69 (-S9)</td>
<td>- (-S9)</td>
</tr>
<tr>
<td>PBS</td>
<td>36+18</td>
<td>77</td>
<td>95</td>
<td>75</td>
<td>1.1</td>
</tr>
<tr>
<td>MMS (10 μg/ml)</td>
<td>125+82</td>
<td>31</td>
<td>103</td>
<td>346</td>
<td>5.0</td>
</tr>
<tr>
<td>Extract from control building</td>
<td>19+11</td>
<td>135</td>
<td>181</td>
<td>35</td>
<td>0.5</td>
</tr>
<tr>
<td>Extract from control building</td>
<td>18+14</td>
<td>93</td>
<td>121</td>
<td>45</td>
<td>0.7</td>
</tr>
<tr>
<td>Extract from test building (2)</td>
<td>37+42</td>
<td>103</td>
<td>90</td>
<td>90</td>
<td>1.3</td>
</tr>
<tr>
<td>Extract from test building (3)</td>
<td>23+26</td>
<td>104</td>
<td>100</td>
<td>70</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Table 3. The percentage relative survival (%RS), relative total growth (RTG), mutant frequency (MF) and mutation index (MI) after treatment of L5178Y Tk^-/+ cells with test extracts isolated from buildings or positive control substances (MMS and B[a]P) in the absence or presence of S9-mix for 3 hours.

Table 4. The percentage relative survival (%RS), relative total growth (RTG), mutant frequency (MF) and mutation index (MI) after treatment of L5178Y Tk^-/+ cells with test extracts isolated from buildings or positive control substance (MMS) in the absence of S9-mix for 24 hours.
Molds belonging to *Aspergillus* sp., prevailing in test samples, were previously shown to produce different mycotoxins, including aflatoxins, sterigmatocystin and ochratoxin A [26, 27]. Sterigmatocystin, a metabolic precursor of aflatoxins (produced mostly by *A. versicolor* and *A. nidulans*), as well as ochratoxin A, were classified as potential human carcinogens (Group 2B IARC; [8, 28]) based on carcinogenicity studies in animals. However, the mutagenic potential of these mycotoxins is still contradictory. Some studies have revealed that ochratoxin A (OTA) had no mutagenic effect in the *Salmonella typhimurium* test systems used in conjunction with S9 fractions prepared either from liver or kidney of rats or mice [29]. Similarly, OTA was not mutagenic in the Ames assay or in the HPRT (hypoxanthine-guanine-phosphoribosyl-transferase) assay using mammalian V79 cells and metabolic activation with S9 fraction from rat liver [16]. In contrast, Obrecht-Pflumio et al. [30] found positive results in the Ames assay after using mouse kidney S9 fraction. Also, Henning et al. [31] showed the mutagenic effect of OTA using a conditioned culture medium from OTA-exposed cultured rat hepatocytes. Altogether, in spite of these rather contradictory results, it seems that biotransformation and, ultimately, mutagenicity of ochratoxin A depend on the type of metabolic activation (i.e. source of microsomes prepared from different organs and animal species) as well as on OTA concentration used for *in vitro* study.

One of the most abundant mold strains detected in the test extracts was *Alternaria* sp. Some studies demonstrated that the major metabolite produced by the mold, alternariol (AOL), was able to induce DNA single-strand and double-strand breaks as well as to induce DNA repair synthesis and inhibition of DNA replication in *Escherichia coli* [32]. Furthermore, AOL induced DNA strand breaks in cell-free systems [33] and in mammalian hepatocytes [34]. A long-term exposure to low levels of AOL was linked with oesophageal cancer [34]. Although the mutagenic potential of AOL in bacterial test systems is still contradictory [13, 35], a concentration-dependent increase of mutations at the hypoxanthine-guanine phosphoribosyltransferase (HPRT) locus of Chinese hamster V79 cells as well as at the thymidine kinase (TK) locus of mouse lymphoma LY5178Ytk+/- cells was observed upon treatment of the cells with non-toxic and moderately cytotoxic concentrations of AOH [36].

Fungi strains found in the test samples in minority, namely *Penicillium* sp. (*P. granulatum* and *P. expansum*) were also previously shown to produce genotoxic compounds, e.g. patulin (PAT) [37, 38, 39]. The evidence for carcinogenicity of PAT in experimental animals was considered inadequate by the IARC [40]. Assessment of genotoxic activity of PTA in short-term assays showed differences in susceptibility of different cell systems. For example, PTA did not increase revertant frequency in the Ames test using several strains of *S. typhimurium* [40, 41], but it showed a mutagenic effect in *Saccharomyces cerevisiae* and in *Bacillus subtilis* [40]. Chromosome aberrations and gene mutations were induced by PAT in FM3A cells, C3H mouse mammary carcinoma cell line [42] but not in human peripheral blood lymphocytes [43]. A concentration-dependent increase in HPRT mutations was observed upon treatment of the cells with non-cytotoxic concentrations of PAT [37].

During analysis of health effects of indoor molds potential protective properties of some fungi products also need to be taken into consideration. For example, *Penicillium chrysogenum* detected in the sample from control building is considered non-toxic and some strains of *P. chrysogenum* are even used in food processing, e.g. as starters in dry-cured hams [44]. Furthermore, there are data indicating that some components of *Aspergillus niger* cell wall, e.g. carboxymethyl-chitin-glucan possess antimutagenic properties against cyclophosphamid-induced mutagenicity in mice [45]. It is postulated that the effects probably result from antioxidative capability of the compounds to scavenge reactive oxygen species [46]. Other studies also demonstrated that derivatives of beta-D-glucans isolated from fungi could adsorb some mycotoxins hence reducing their bioavailability and protecting animals against adverse effects [47].

Taking the above data together, it is obvious that mechanisms underlying the genotoxic effects of mycotoxins are very complex and the nature of factors responsible for inconsistencies observed in mutagenicity tests are largely unknown. Even less clear are the mechanisms of interactions between mammalian cells and different mycotoxins produced by a mixture of molds.

In the present study, no increase in the mutagenic effect of the test extracts in comparison to appropriate controls was observed. Among possible reasons for lack of such effect, too low final concentration of mycotoxins could be listed. This in turn, could result from the method of extraction used. Although, there are different methods available for isolation of mold extracts, the method based on swab extraction in PBS is, in the opinion of the authors, the most relevant, considering conditions of in vivo inhalation exposure. Considering data on potential protective properties of some mold products it cannot be excluded that such products (identification of which in isolated extracts was not performed in details) counteracted promutagenic effects of mycotoxins leading to abolition of mutagenicity.

In conclusion, our study demonstrated that extracts isolated from mold-attacked flats as well as from the flat without such symptoms did not induce mutations affecting the expression of the thymidine kinase gene in the L5178Y TK-/- cells used when tested under the study conditions. In further investigations using other cell types, metabolic activation systems such as mouse kidney S9 fraction [30] or prostaglandin synthase [48] are needed to clarify potential toxicity of indoor molds. It seems that the mutagenic effect of mold extracts may largely depend on organ-specific metabolism of mycotoxins [16]. On the other hand, interactions between pre- and antimutagenic metabolites and/or components of fungi should not be ruled out [46, 47, 49].
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