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Genotoxicity Assessment of Five Tremorgenic Mycotoxins (Fumitremorgen B, Paxilline, Penitrem A, Verruculogen, and Verrucosidin) Produced by Molds Isolated from Fermented Meats

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ABSTRACT

A number of toxinogenic fungal species, particularly producers of tremorgenic mycotoxins, have been isolated from traditional fermented meats. Tremorgenic mycotoxins are a group of fungal metabolites known to act on the central nervous system, causing sustained tremors, convulsions, and death in animals. However, the mode of action of these mycotoxins has not been elucidated in detail, and their genotoxic capacity has hardly been investigated. Because genotoxicity is one of the most prominent toxicological end points in food safety testing, we assessed the genotoxicity of five tremorgenic mycotoxins (fumitremorgen B, paxilline, penitrem A, verrucosidin, and verruculogen) associated with molds found in fermented meats. The mycotoxins were tested in two short-term in vitro assays with the use of different genotoxic end points in different phylogenetic systems (the Ames *Salmonella*/mammalian-microsome assay and the single-cell gel electrophoresis assay of human lymphocytes). According to the results obtained in this study, all of the investigated mycotoxins except penitrem A exhibited a certain degree of genotoxicity. Verrucosidin appeared to have the highest toxic potential, testing positive in both assays. Verruculogen tested positive in the *Salmonella*/mammalian-microsome assay, and paxilline and fumitremorgen B caused DNA damage in human lymphocytes. The use of fungal starter cultures to avoid tremorgen contamination in fermented meats is recommended.

Tremorgenic mycotoxins are a group of fungal metabolites synthesized by common saprophytic molds of the genera *Penicillium* and *Aspergillus* and by some species of the genera *Claviceps* and *Neotyphodium* (*Acremonium*) *(4).* Although these mycotoxins are produced by unrelated fungal species, they all contain a modified indole moiety as a structural and biologically active feature. Depending on the number of nitrogen atoms per molecule, which might explain differences in the specific mode of action, tremorgenic mycotoxins can be divided in four subgroups: (i) those without N atoms (verrucosidin), (ii) those with a single N atom (paspalitrems, penitrems, janthitrems, and lolitrems), (iii) those with three N atoms (fumitremorgin-verruculogens), and (iv) those with four N atoms (tryptoquivalines) *(7, 43).* Tremorgenic mycotoxins have been shown to act on the central nervous system and to cause sustained tremors, convulsions, and death in animals *(27, 31, 38, 51, 52).* They have been associated with a number of grass-consumption-related neurotoxicoses in ruminants, commonly referred as staggers syndromes *(9).* The mode of action of these mycotoxins has not been elucidated in detail, but experimental and clinical findings have suggested interference with neurotransmitter release mechanisms *(23, 24, 43, 50).* Tremorgenic mycotoxins have been found in several important agricultural commodities, including silage, maize, and various forages *(10, 13),* as well as in foods spoiled by toxinogenic molds *(48).* Moreover, as shown in Table 1, a number of fungal strains capable of producing tremorgenic mycotoxins have been isolated from different traditional fermented meats.

Typical fermented meat products such as dry- and country-cured hams and sausages are traditionally produced in southern Europe. Fungal growth plays an important role in the appearance, flavor, and preservation of fermented foods, but it may also give rise to health hazards due to mycotoxin production. The risk posed by such hazards is generally minimized in industrial processes with the use of nontoxic fungal starters to control fermentation *(19, 22, 25).* Fungal starters, however, are not commonly used in traditional fermentation processes, which may lead to the development of a spontaneous mycoflora capable of producing undesirable metabolites. A number of studies have been undertaken to identify the mycoflora present on the surfaces of these products *(2, 14, 26, 33, 36, 37, 39, 46),* as well as to evaluate the risk of mycotoxin formation by the toxic fungal isolates (8, 18, 29, 35, 37, 39, 45, 47). Among the broad range of mycotoxins potentially produced in traditional fermented meats, there are some whose mutagenic and genotoxic effects are well known (aflatoxin, sterigmatocystin, and ochratoxin) *(11, 15).* In contrast, other my-

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TABLE 1. *Toxinogenic fungi isolated from traditional fermented meats and their associated tremorgenic mycotoxins*

Toxinogenic fungus	Traditional fermented meat(s)	Associated tremorgen(s)
Aspergillus caespitosus	Italian Parma and San Danielle hams *(14)*	Fumitremorgin-verruculogens *(6, 42)*
A. flavus	Italian Parma and San Danielle hams *(14)*, Spanish dry-cured ham *(26, 39)*, American country-cured ham *(47)*	Paspalitrems *(6)*
A. fumigatus	Italian Parma and San Danielle hams *(14)*, Spanish dry-cured ham *(26, 39)*, American country-cured ham *(47)*	Fumitremorgin-verruculogens and tryptoquivalines *(6, 40)*
Penicillium aurantiogriseum	Iberian country-cured ham *(33, 36)*, Spanish dried-cured ham *(35)*	Verrucosidin *(16)*
P. commune	Iberian country-cured ham *(36)*, Spanish country-cured sausages *(29)*, Tyrolean smoked-cured ham *(37)*, Italian dry-cured sausages *(2)*	Penitrems *(6)*
P. puberulum	Italian Parma ham *(46)*	Penitrems *(6)*
P. simplicissimum	Iberian country-cured ham *(33)*	Fumitremorgin-verruculogens *(32)*
P. viridicatum	Iberian country-cured ham *(33, 36)*	Penitrems *(17)*

cotoxins, such as tremorgenic compounds, have been less investigated.

Because genotoxicity is one of the prominent toxicological end points in food safety testing, we assessed the genotoxic potentia289l of five tremorgenic mycotoxins (fumitremorgen B, paxilline, penitrem A, verrucosidin, and verruculogen; see Fig. 1) associated with fungi isolated from fermented meats (see Table 1) by two short-term in vitro assays (the *Salmonella*/mammalian-microsome assay [with and without metabolic activation] and the single-cell gel electrophoresis assay of human lymphocytes).

MATERIALS AND METHODS

Materials. The mycotoxins paxilline, verruculogen, and penitrem A were obtained from Sigma Chemical Co. (St. Louis, Mo.), and verrucosidin and fumitremorgen B were kindly provided by the Federal Institute for Meat Research (Kulmbach, Germany). For use as positive controls, aflatoxin B_1, sodium azid, benzo(*a*)pyren, and 9-aminoacridine were obtained from Sigma, 2-nitrofluorene was obtained from Aldrich (Zwijndrecht, The Netherlands), and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine was obtained from Schuchard (Munich, Germany). Ultrapure agarose was supplied by GIBCO BRL: Life Technologies (Paisley, Scotland). RPMI 1640 cell culture medium was purchased from Sigma, and Aroclor was purchased from Supelco (Bellfonte, Pa.). All other reagents were of analytical grade.

***Salmonella*/mammalian-microsome assay.** The Ames *Salmonella*/mammalian-microsome assay was carried out with the histidine auxotroph *Salmonella* Typhimurium tester strains TA1535 (base pair substitution), TA1537 (frameshift mutation), TA1538 (frameshift mutation), TA98 (frameshift mutation), and TA100 (base pair substitution), kindly provided by Dr. Bruce Ames (University of California, Berkeley). The mutability of the selected strains was tested with positive reference compounds with and without metabolic activation, as described by Ames et al. *(1)*. Overnight cultures containing 1.2×10^8 cells prepared from stock cultures were used for all of the experiments. The mycotoxins were dissolved in 0.1 ml of dimethyl sulfoxide to final concentrations of 0.5, 5.0, 50, and 1,000 nmol per plate, respectively. Each dose was tested in triplicate with and without metabolic activation. The method for the assay is described in detail by Maron and Ames *(30)*. Briefly, 0.1 ml of the dimethyl sulfoxide (DMSO)–soluble compound was added to culture tubes containing 2.0 ml of top agar and 0.1 ml of the tester suspension strain. The cultures were then plated. For the microsomal activation assay, 0.5 ml of S9 mix was added to the culture tubes after the bacteria were added. The S9 mix was prepared from Arochlor 1254 pretreated male Wistar rats and diluted with an NADPH-generating system as described previously *(30)*. All plates were examined after 48 h.

Single-cell gel electrophoresis assay of human lymphocytes: lymphocyte isolation. For each experiment, peripheral lymphocytes were freshly separated from heparinized whole blood samples obtained from healthy nonsmoking donors aged 25 to 40 years (blood was kindly provided by the Academic Hospital of Utrecht, The Netherlands). Lymphocytes were isolated from blood by centrifugation over a ficoll density gradient after the blood had been diluted (1:1) with phosphate-buffered saline (PBS, pH 7.4; GIBCO BRL: Life Technologies). After centrifugation, the interphase layer was collected and cells were washed twice with PBS. The viability of cells after the isolation was determined by automatic counting. The cells were resuspended in PBS to a concentration of 10^6 cells per ml.

Single-cell gel electrophoresis assay of human lymphocytes: lymphocyte treatment. DMSO solutions of the mycotoxins with final concentrations of 1, 100, and 10,000 nM for paxillin, penitrem A, and verruculogen and with final concentrations of 1, 100, and 3,333 nM for fumitremorgen B and verrucosidin were added to the lymphocyte suspension. The final concentration of DMSO was 0.1% for all incubations, including that of the negative control. The cells were incubated in the presence of the mycotoxins for 1 h at 4°C. Each experiment included two positive controls prepared through the exposure of lymphocytes to UV light for 2 min and incubation with H_2O_2 (25 nM) for 15 min.

Single-cell gel electrophoresis assay of human lymphocytes: lymphocyte viability. The viability of the lymphocytes after incubation with the mycotoxins was assessed by the MTT (dimethylthiazol diphenyl tetrazolium bromide) test as described by Denizat and Lang *(12)*, with minor modifications. Briefly, after 1 h of incubation, the medium was discarded and fresh RPMI medium containing 0.6 mg of MTT per ml was added to the cells. After 4 h of incubation, the medium was removed and the reaction was stopped by adding acetic isopropanol (HCl/isopropanol, 2:98 [vol/vol]). Formazan, a product formed in viable cells by the mitochondrial enzyme succinate dehydrogenase, was measured at 560 nm. The absorbance of cells treated with mycotoxins was

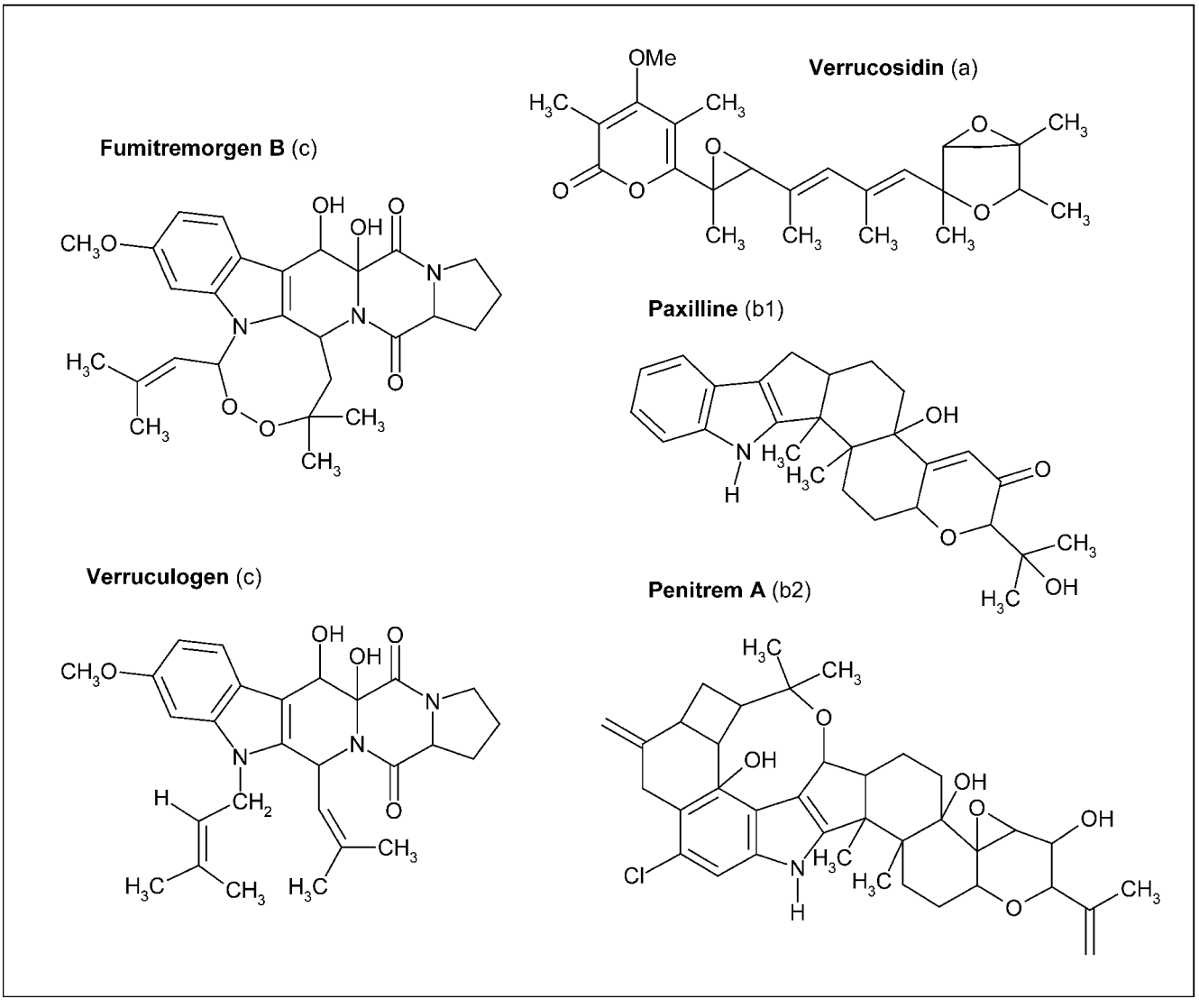

FIGURE 1. *Structures of verrucosidin, fumitremorgen B, verruculogen, paxilline, and penitrem A, which belong to the following subgroups of tremorgenic mycotoxins: (a) those having no N atoms, (b1) those having one N atom (paspalitrem), (b2) those having one N atom (penitrem), and (c) those having three N atoms.*

compared with the absorbance of those exposed to DMSO (negative control).

Single-cell gel electrophoresis assay of human lymphocytes: comet assay. Lymphocytes were resuspended in 200 μl of PBS and applied in the comet assay according to the alkaline protocol developed by Singh et al. *(44),* with minor modifications. Briefly, following incubation with mycotoxins, 100 μl of the cell suspension was mixed with 0.5% low-temperature-melting agarose and spread onto a frosted microscope slide placed on a cold glass plate. After solidification, the slides were immersed in a freshly prepared ice-cold lysing solution (2.5 M NaCl, 0.10 M Na_2EDTA, 10 mM Tris base, supplemented with Triton-X [pH 10]) and incubated at 4°C for 1 h in the dark. Following lysis, the slides were immersed in a cold alkaline buffer (0.3 M NaOH, 0.001 M EDTA [pH > 13]) for 20 min in the dark to unwind the DNA. Electrophoresis was conducted at room temperature in the same buffer for 20 min at 25 V and at 290 to 310 mA. Afterward, a neutralization buffer (0.4 M Tris-HCl, pH 7.5) was applied three times for 5 min on the resulting slides. Finally, the slides were dehydrated for 10 min in 96% ethanol and stored in a dark box at room temperature pending visualization.

Single-cell gel electrophoresis assay of human lymphocytes: comet analysis. The slides were stained with ethidium bromide (10 μg/ml) to visualize the comet images, which were examined with the use of computer-based image analysis (Colormorph). One hundred nonoverlapping comets per slide were randomly captured at a constant depth of the gel, avoiding edges and damaged regions. Another 100 cells were analyzed on a parallel slide prepared with an aliquot of the same cell sample. Therefore, 200 measurements were carried out for each mycotoxin concentration. Tail moment was the parameter used as an indicator of DNA damage. The tail moment is the tail length integrated over the intensity of the tail; thus, this parameter combines the amount of DNA in the tail with the distance of migration.

Single-cell gel electrophoresis assay of human lymphocytes: statistical analysis. Three tail moment values were obtained for each individual mycotoxin concentration, with each value representing the mean of the 200 cell measurements carried out in three independent experiments conducted for lymphocytes from three different donors. The influence of the two independent variables (concentration and donor) on natural logarithm–transformed DNA damage data (tail moment) was estimated by mul-

TABLE 2. *Mutagenicity of five tremorgenic mycotoxins as determined by the* Salmonella*/mammalian microsome assay with metabolic activation*

Mycotoxin concn (nmol/plate)	No. of His$^+$ revertants per plate for tester strain[a]				
	TA-1535	TA-1537	TA-1538	TA-98	TA-100
Negative control					
0	27	9	30	56	117
Fumitremorgen B					
0.5	28	9	28	53	108
5	27	7	30	52	113
50	30	10	35	50	112
1,000	23	11	38	97	111
Paxilline					
0.5	26	9	30	56	104
5	30	12	29	50	134
50	28	11	33	54	116
1,000	32	13	32	52	118
Penitrem A					
0.5	25	11	35	50	109
5	27.5	9	37	51	112
50	24.5	11	34	50	111
1,000	34.0	11	36	83	121
Verrucosidin					
0.5	31	11	95	50	109
5	35	11	146	54	122
50	28	14	163	52	132
1,000	31	11	**175**	62	105
Verruculogen					
0.5	33	11	39	46	245
5	39	11	36	50	230
50	33	10	33	49	242
1,000	**122**	**247**	**111**	**486**	249
Positive control					
A, B[b]	**109** (B)	**53** (B)	**130** (B)	**193** (A)	**380** (A)

[a] Mean value for two independent experiments conducted in triplicate. Values in boldface type indicate mutagenicity according to OECD guidelines *(3)*.

[b] A, aflatoxin B_1 (80 nmol per plate); B, benzo(*a*)pyren (1,000 nmol per plate).

TABLE 3. *Mutagenicity of five tremorgenic mycotoxins as determined by the* Salmonella*/mammalian microsome assay without metabolic activation*

Mycotoxin concn (nmol/plate)	No. of His$^+$ revertants per plate for tester strain[a]				
	TA-1535	TA-1537	TA-1538	TA-98	TA-100
Negative control					
0	26	11	31	42	119
Fumitremorgin B					
0.5	25	8	22	31	165
5	34	9	23	37	136
50	34	11	24	40	128
1,000	b	b	b	b	b
Paxilline					
0.5	25	14	22	41	86
5	27	10	23	42	204
50	30	10	27	43	216
1,000	43	9	33	43	b
Penitrem A					
0.5	41	7	43	27	195
5	41	8	41	37	227
50	39	11	40	38	105
1,000	30	13	55	49	110
Verrucosidin					
0.5	25	7	24	41	105
5	27	10	24	41	120
50	28	14	25	47	130
1,000	30	15	34	49	b
Verruculogen					
0.5	46	7	19	47	149
5	47	9	17	54	176
50	72	8	25	47	**303**
1,000	**95**	**152**	39	**338**	101
Positive control					
C, D, E, F[b]	**1,120** (C)	**700** (D)	b (E)	**550** (F)	**796** (C)

[a] Mean value for two independent experiments conducted in triplicate. Values in boldface type indicate mutagenicity according to OECD guidelines *(3)*. b, concentration was cytotoxic to bacteria.

[b] C, sodium azide (15.3 mmol per plate); D, 9-aminoacridine (26 nm per plate); E, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (50 nmol per plate); F, 2-nitrofluorene (9 nm per plate).

tiple linear regression analysis for each mycotoxin. The significant effects observed were evaluated by the Bonferroni posttest ($P < 0.05$ and $P < 0.01$) to compare values with the expected average difference.

RESULTS

***Salmonella*/mammalian-microsome assay.** Tables 2 and 3 present the results obtained by the *Salmonella*/mammalian-microsome assay with and without metabolic activation, respectively, for the five tested mycotoxins. The mycotoxin verruculogen induced an increase in the number of revertants in tester strains TA-1535, TA-1537, TA-1538, and TA-98 with metabolic activation by the S9 mix. The number of colonies obtained fitted the paradigm of a true mutagenic compound established in the OECD guidelines *(3)* only when the highest concentration of verruculogen (1,000 nmol per plate) was assessed. Without metabolic activation, 1,000 nmol of verruculogen per plate was mutagenic for strains TA-1535, TA-1537, and TA-98 but caused bacterial growth inhibition for strain TA-100. For strain TA-100, however, a mutagenic response was observed at a lower concentration (50 nmol per plate).

With verrucosidin, a significant dose-dependent increase in the number of revertants was observed for strain TA-1538 in the presence of S9 mix. The highest concentration of the toxin inhibited bacterial growth in TA-100 when tested without metabolic activation.

TABLE 4. *Comet assay of peripheral lymphocytes of three donors exposed to five different tremorgenic mycotoxins for 1 h*

Concn (nM) of compound	Tail moment for donor[a]			Statistical significance[b]
	1	2	3	
Negative control				
0	0.180	0.223	0.116	
Fumitremorgen B				
333	0.736	2.928	0.965	**
10	0.154	0.622	0.258	
0.1	0.230	0.633	0.175	
Paxullin				
1,000	0.552	0.362	0.571	**
10	0.599	0.462	0.502	**
0.1	0.157	0.155	0.240	
Penitrem A				
1,000	0.155	0.695	0.152	
10	0.131	0.622	0.276	
0.1	0.187	0.437	0.262	
Verrucosidin				
333	0.781	2.114	0.446	*
10	0.124	0.736	0.284	
0.1	0.131	0.665	0.414	
Verruculogen				
1,000	0.294	0.653	0.220	
10	0.242	0.605	0.392	
0.1	0.370	0.610	0.211	
Positive controls				
H_2O_2 (10 nM/15 min)	3.180	2.854	5.535	**
UV (2 min)	22.485	19.358	23.16	**

[a] Mean for 200 cells.
[b] ANOVA 2 factors were concentration and donor (Bonferroni comparison). *$P < 0.05$; **$P < 0.01$.

No mutagenicity was detected for fumitremorgen B, paxilline, or penitrem A for any of the tested strains at any of the concentrations assayed with or without metabolic activation. Evidence of bacterial growth inhibition was observed for all *Salmonella* strains when the highest concentration of fumitremorgen B was used without metabolic activation, and evidence of bacterial growth inhibition was observed for TA-100 when the highest concentration of paxilline was used.

Single-cell gel electrophoresis assay. Comet assay analyses of DNA damage were carried out for the five mycotoxins at three different concentrations (1, 100, and 10,000 nM for paxillin, penitrem A, and verruculogen and 1, 100, and 3,333 nM for fumitremorgen B and verrucosidin). The results of previous experiments had indicated that these concentrations did not induce any significant cytotoxicity according to the MTT test (data not shown).

Table 4 summarizes the DNA damage (expressed as average of tail moments) observed in human lymphocytes from three different donors exposed to several concentrations of the mycotoxins investigated. The results show significant increases in DNA damage to cells exposed to 3,333 nM of fumitremorgen B ($P < 0.01$), 10,000 and 100 nM of paxillin ($P < 0.01$), and 3,333 nM of verrucosidin ($P < 0.05$).

Statistical analyses revealed a significant difference ($P < 0.05$) between the DNA damage produced by fumitremorgen B and verrucosidin for donor 2 and the damage produced for the other donors. This effect can be also deduced from the data presented in Figure 2, which illustrates in a histogram format the tail moment distribution for the lymphocytes of the three donors exposed to fumitremorgen B.

DISCUSSION

Mycotoxin production by toxinogenic molds is mediated by several factors, such as substrate, temperature, light, water activity, pH, and oxygen *(28).* The low water activity (due to a high salt concentration) and high-protein content of fermented meats are conditions that are favorable for fungal growth but seem to be less favorable for mycotoxin production *(6).* However, at least during certain periods of the ripening process, mycotoxin production cannot be excluded. For example, the formation of the mycotoxin och-

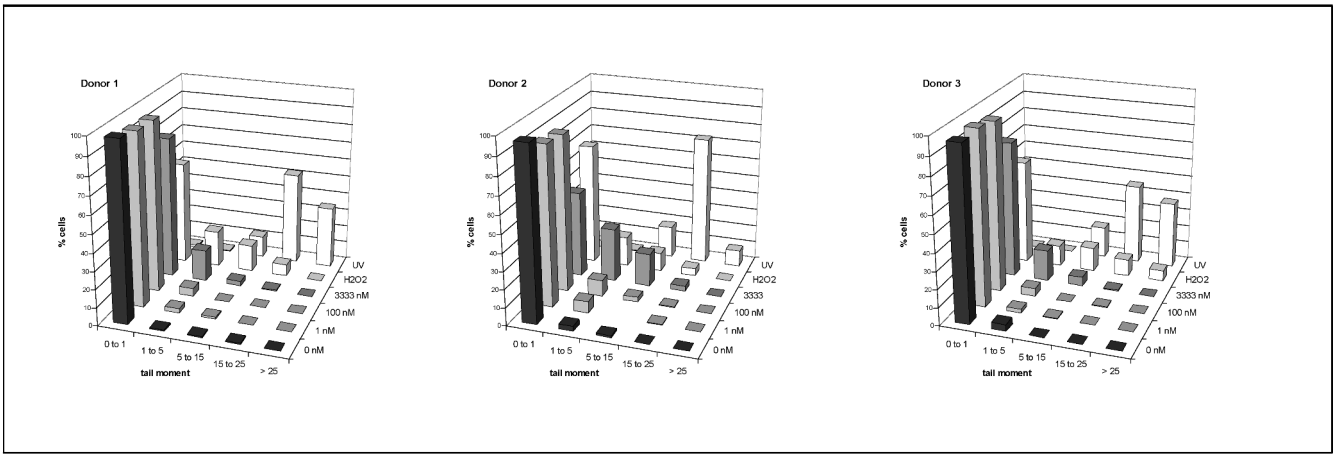

FIGURE 2. *Representative distribution histograms of tail moment measures for lymphocytes from three donors exposed to fumitremorgen B. As the distributions move to the right, the amount of DNA damage increases.*

ratoxin A in fermented meats has been reported *(45)*. Cantoni et al. *(8)* detected ochratoxin A in the casing and in the outer 1 cm of a fermented sausage, but not in the inner part. The presence of tremorgenic mycotoxins in commercial fermented meats has not yet been investigated, perhaps owing to both the lack of validated analytical methods for the detection of these toxins in meat products and the lack of evidence of toxic effects and potential public health risks arising from these toxins even at low concentrations. However, experimental data have demonstrated the formation of tremorgenic mycotoxins in raw sausages *(41)* and cheese *(34)* inoculated with toxinogenic molds. Moreover, for a *Penicillium polonicum* strain isolated from dried cured ham, Nuñez et al. *(35)* reported growth and verrucosidin production in a high-protein meat-based medium.

To assess the genotoxic capacity of these potential contaminants, we used two short-term in vitro assays directed at different genotoxic end points in different phylogenetic systems: the *Salmonella*/mammalian-microsome assay, a well-accepted tool for the assessment of the mutagenicity of chemicals, and the single-cell gel electrophoresis assay of human lymphocytes, a commonly used technique to evaluate DNA damage.

According to our findings, all investigated tremorgens except penitrem A exerted genotoxic effects to a certain degree. The mycotoxin verrucosidin appeared to have the highest genotoxic potential, testing positive in both assays. In the *Salmonella*/mammalian-microsome assay, verrucosidin induced frameshift mutations in TA-1538 after metabolic activation. This need for bioactivation is supported by previous experiments showing that after being metabolized by rat hepatocytes, verrucosidin almost completely inhibited phytohemagglutinin-induced mitosis in cultured lymphocytes in the sister chromatid exchange test *(20)*. On the other hand, the results of the comet assay demonstrated a genotoxic potential of the molecule, since one of the known limitations of blood lymphocytes is their inadequate metabolic capacity. In contrast, the mycotoxin verruculogen was clearly mutagenic in the *Salmonella*/mammalian-microsome assay, inducing base pair substitutions and frameshift mutations with and without metabolic activation in most of the tested strains, but did not induce DNA damage in the comet assay. Verruculogen also failed to induce mitosis after being metabolized by rat hepatocytes in the sister chromatid exchange test, as reported earlier *(21)*. Finally, paxilline and fumitremorgen B exerted significant genotoxicity in the comet assay but no mutagenicity in *Salmonella* Typhimurium. The present study's results on the effects of fumitremorgen B in the *Salmonella*/mammalian-microsome assay are in agreement with previous findings *(49)*. The observed differences between the degrees of DNA damage caused by fumitremorgen B and verrucosidin in lymphocytes of different donors cannot be explained but might be attributable to differences in the ages and health statuses of the donors *(5)*.

The in vitro mutagenic and genotoxic effects of tremorgenic mycotoxins described here should be confirmed by in vivo experiments. At present, only verruculogen has been tested in vivo, and results of that test indicated that this mycotoxin did not inhibit cell replication in bone marrow cells of NMRI mice *(21)*.

In conclusion, the data presented here provide evidence for the genotoxicity of four of the five tremorgenic mycotoxins tested. Considering that molds capable of producing these toxins have been isolated from different traditional fermented meats, their occurrence in these products should be more thoroughly investigated. However, since the variability encountered among traditional fermentation processes complicates such assessments, the most reliable option for the avoidance of any public health risk would be to replace the traditionally used spontaneous flora with tested starter cultures.

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