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Ochratoxin A-Induced Mutagenesis in Mammalian Cells Is Consistent with the Production of Oxidative Stress

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Abstract

Ochratoxin A (OTA) is a widespread mycotoxin in food and a powerful nephrocarcinogen in rats. The mutagenicity of OTA has been extensively investigated but with conflicting results, thus leaving open the mechanistic question for OTA carcinogenicity. Here, we examined the mutagenicity of OTA by using well-standardized mutation assays such as the hypoxanthine-guanine phosphoribosyltransferase (HPRT) assay in Chinese hamster V79 cells and the thymidine kinase assay in mouse lymphoma LY5178 cells. OTA-induced HPRT mutations were characterized at the molecular level. In V79 cells, OTA produced a dose- and time-related decrease in cell number as a consequence of the transitory cytostatic effect mediated by G_2/M cell cycle arrest. In both mutation assays, OTA was weakly mutagenic and this effect was independent of biotransformation. OTA-induced mutations were characterized by point mutations (48%) and a lack of a detectable reverse-transcription polymerase chain reaction product (52%). The pattern of OTA-induced point mutations was similar to that of spontaneous mutants, suggesting that OTA induced an increase of the endogenous oxidative metabolism but not covalent DNA adducts. Our data support a model where OTA is mutagenic *via* oxidative DNA damage induction.

Introduction

Ochratoxin A (OTA)[1] is a mycotoxin produced by several *Aspergillus* and *Penicillium* mold species (1,2) and is a worldwide contaminant of food and feedstuffs. OTA is a potent renal toxin in various species (3-7) and one of the most potent carcinogens in rats (7). OTA is classified as a possible human carcinogen by the International Agency for Research on Cancer (8).

Although the carcinogenic potential of OTA has been known since the eighties, the mechanism of OTA carcinogenicity is largely undefined. At present, there is still insufficient understanding of whether OTA acts as a direct genotoxic carcinogen or whether its carcinogenicity is related to indirect mechanisms. Direct DNA-binding of OTA and DNA adducts formation has been reported by using ^{32}P-postlabeling (9-11), but DNA-binding studies using radiolabeled OTA were unable to detect DNA binding of OTA (12-15). The strongest evidence in favor of OTA-induced DNA damage is the induction of DNA single-strand breaks and formamidopyrimidine-DNA glycosylase (Fpg) sensitive sites (that are a marker of oxidative base damage) both in cells in culture (16-20) and in rats and mice (21-24). In agreement with OTA-induced DNA

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damage is the induction of unscheduled DNA synthesis in primary human urothelial cultures (25,26), primary hepatocytes (27), and rat and mouse cell lines (28).

Controversial results have been reported on OTA genotoxicity by using different test systems, but in general, the results indicated low if any genotoxic activity. In the *Salmonella typhimurium* assay, OTA was not mutagenic either in the absence or in the presence of liver or kidney S9 fractions from rats or mice (29-32). On the other hand, in the same system, by using conditioned culture medium from OTA-exposed cultured rat hepatocytes (33) and mouse kidney S9 fraction (34), mutagenic activity by OTA was reported. Similarly, in mammalian cells, a lack of mutagenic activity was reported in the hypoxanthine-guanine phosphoribosyltransferase (HPRT) assay in V79 hamster cells as well as in the thymidine kinase (TK) assay in L5178Y $TK^{\pm}$ mouse lymphoma cells (29,35), but OTA was mutagenic in murine NIH-3T3 cells stably expressing specific human cytochrome P450 enzymes by using a shuttle vector as a tool for mutation analysis (36). Controversial results have also been published on cytogenetic effects by OTA, both *in vivo* and *in vitro*, by using different end points such as sister chromatid exchanges (SCE) (37-39) and micronuclei (37,38). A recent report indicated a small increase in the frequency of chromosomal aberrations in splenocytes from rats treated with OTA *in vivo*, but no evidence of OTA binding in kidney DNA from treated animals was obtained (23).

Because of the inconsistency in the literature with regard to OTA genotoxicity and in particular to mutagenesis, the objective of this study was to re-evaluate the mutagenic potential of OTA in mammalian cells by taking into account the pleiotropic effects of OTA. Therefore, in the same cell system (i.e., V79 hamster cells) cytotoxicity, cell cycle effects, DNA synthesis, and HPRT mutation frequency and type were analyzed. Mutation frequency was also analyzed in LY5178 mouse cells using the TK locus as target. Here, we provide evidence that OTA has a strong effect on cell cycle progression and is weakly mutagenic in the absence of biotransformation and that the HPRT-induced mutation spectrum is similar to the spontaneous mutation spectrum.

Materials and Methods

Caution

OTA is hazardous and should be handled with care

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[1]Abbreviations:

OTA ochratoxin A

HPRT hypoxanthine-guanine phosphoribosyltransferase

TK thymidine kinase

PCR polymerase chain reaction

RT-PCR reverse transcription-PCR

RONS reactive oxygen and nitrogen species

FBS fetal bovine serum

Chemicals

OTA (O1877), ethylmethanesulfonate (EMS), methylmethanesulfonate (MMS), and dimethylbenzanthracene (DMBA) were purchased from Sigma-Aldrich (Taufkirchen, Germany). OTA dilutions were prepared in dimethyl sulfoxide (DMSO); the final solvent concentration at the time of the experiments was 1% (v/v). Vehicle control treatments were carried out with medium containing 1% DMSO.

Cell Lines and Culture Conditions

V79 Chinese hamster lung fibroblasts were grown in Eagle minimal essential medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 1 mM nonessential amino acids, 50 U/mL penicillin, 50 μg/mL streptomycin, and 10% fetal bovine serum (FBS). L5178Y $TK^{\pm}$ mouse lymphoma cells (ATCC code CRL-9518) were grown in RPMI 1640 minimal medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM nonessential amino acids, 50 U/mL penicillin, 50 μg/mL streptomycin, F68 pluronic, and 10% horse serum (heat-inactivated). Cells were grown at 37 °C in a 5% carbon dioxide atmosphere (100% nominal humidity) and routinely tested for mycoplasma contamination.

Determination of Cell Number

Cytotoxicity after OTA exposure was determined by a cell lysis procedure (40). Briefly, 8×10^4 V79 cells/well were seeded in six-well dishes. The following day, cells were exposed in triplicate to a range of OTA concentrations up to 188 μM. After 3, 6, 12, or 24 h treatment times, cells were counted using a Coulter counter. The counts were corrected for coincidence and dilution using the coincidence correction chart supplied with the instrument. The means ± standard deviation (SD) were used to construct cell growth curves representing the percentage of surviving cells relative to untreated cells. The IC_{50} values (defined as the concentration of agent required for 50% growth inhibition) were determined from the cell growth curves.

Flow Cytometric Cell Cycle and DNA Synthesis Analysis

Cell cycle and DNA synthesis, as assessed by staining with propidium iodide (PI) and incorporation of bromodeoxyuridine (BrdU) (both from Sigma-Aldrich), respectively, were analyzed simultaneously using a modification of the protocol described by Hoy et al. (41). Briefly, after treatment, cells were pulse labeled with 45 μM BrdU for 15 min. Cells were fixed with 70% cold ethanol overnight. To denature DNA, samples were incubated for 45 min in 3 N HCl, and 0.1 M sodium borate, pH 8.5, was added to neutralize the acid. Pellets were then incubated for 40 min with the primary antibody anti-BrdU (Dako Italia S.p.A.). After that, samples were incubated at room temperature for 30 min with the secondary antibody anti-BrdU-fluorescein (Technogenetics Gruppo Bouty, Italy). Pelleted cells were resuspended in PBS containing 5 μg/mL PI and 0.15 μg/mL RNase. A total of 10000 events were acquired and analyzed by flow cytometry (FACScan Cytometer) using the Cell Quest software (Becton Dickinson). The cell cycle distribution was determined from the resulting biparametric PI/BrdU-FITC dot plots. Cell cycle phases were designated as G_0/G_1 (2*n* DNA content with no BrdU incorporation), S (variable DNA content with BrdU incorporation), and G_2/M (4*n* DNA content without BrdU incorporation).

Metabolic Activation System

S9 tissue homogenate fractions were prepared from kidneys and livers of young male Sprague-Dawley rats that had received prior treatment with phenobarbital and β-naphthoflavone to induce high levels of xenobiotic metabolizing enzymes. S9 tissue fractions were controlled for protein content (Lowry method) and aminopyrine demethylase activity and checked in an Ames test with the indirect mutagens 2-aminoanthracene and benzo(a)pyrene, using *S. typhimurium* tester strain TA100.

HPRT Mutation Assay

On the day before the experiment, 75 cm^2 flasks were inoculated with 2×10^6 V79 cells for each treatment group. The cells were allowed to attach overnight prior to treatment. On day 0, cells were treated with different concentrations of OTA for 3 h (in the presence and in the absence of kidney/liver S9 fraction) in serum-free medium or 24 h in complete medium (containing 10% FBS). In the case of 3 h of treatment, the treatment medium was removed and replaced by fresh complete medium for an additional 21 h. At the end of the incubation period, 200 cells were plated in each of three 60 mm tissue culture Petri dishes to evaluate cytotoxicity. Cells were incubated for 6-9 days to allow the phenotypic expression. On days 6 and 9, 1×10^5 cells were plated in each of five 100 mm Petri dishes per experimental group in selective medium containing 6-thioguanine (6-TG) (at 7.5 μg/mL). Cells from each culture were also plated at 200 cells per 60 mm Petri dish in triplicate in the absence of selective drug to determine plating efficiency (PE). After 2 weeks of incubation, colonies were counted and the mutation frequency was calculated [mutation frequency = number of colonies/(number of seeded cells × PE) × 10^6 cells]. The PE of cells treated with solvent was set 100%. A 10 mM concentration of EMS and 10 μg/mL DMBA (experiments in the presence of S9 metabolism) were used as positive controls. Historical control data from HPRT mutation assays (n = 62) carried out in the same laboratory were used for comparison.

TK Mutation Assay

The TK assay in L5178Y $TK^{\pm}$ mouse lymphoma cells was performed by using the microtitration technique developed by Cole et al. (42). Duplicate cultures were prepared for each experimental group, with the exception of the positive controls. An amount of 10 μg/mL MMS or 30 μg/mL 2-AA (experiments in the presence of S9 metabolism) was used as a positive control. Briefly, 10×10^6 cells (0.5×10^6 cells/mL) were treated (day 0) for 3 h with various concentrations of OTA or with solvent (1% DMSO) in the absence or in the presence of a kidney S9 fraction. Cells were allowed to grow for 48 h and then seeded in selective medium containing 3 μg/mL trifluorothymidine at a density of 2×10^3 cells per well in four 96 well plates for each experimental group. To determine the cloning efficiency, cells were plated at a density of 1 cell per 200 μL per well of two 96 well plates for each experimental group at days 0 and 2. After 1-2 weeks of incubation, colonies were counted. Colonies were classified as large when they extended to more than one-fourth of the diameter of the well (43).

The following formulas were used:

Cloning efficiency, CE = -ln (ys/ns)/Number of cells per well (ys = Number of wells without clones; ns = Total number of wells) *Relative survival*, %RS = CE × Cell count factor (Cell count factor = treated post-treatment cell count/control post-treatment cell count). Cell survival was expressed as percentage of control cells not exposed to OTA

Total Growth, TG = (number of cells at day 1/number of cells seeded at day 0) × (number of cells at day 2/number of cells seeded at day 1)

Relative Total Growth, RTG = TG compound/TG solvent × 100

% *Relative Total Growth*, %RTG = RSF × (relative CE compound day 2/CE solvent day 2)

Mutation frequency, MF = [CE (mutant)/CE (viable)] × 10^6

Statistical analysis was performed according to UKEMS guidelines (44). Historical control data from TK mutation assays (n = 76) carried out in the same laboratory were used for comparison.

Mutant Colony Isolation and Molecular Analysis of HPRT Mutations

A total of 18 spontaneous and 21 OTA-induced HPRT mutant colonies were isolated from three independent experiments and grown to mass culture in medium supplemented with 7.5 μg/mL 6-TG. RNA isolation was performed from 5×10^6 cells by using Trizol reagent (Gibco, Invitrogen Corp., Carlsbad, CA) as described by the manufacturer. RNA reverse transcription and cDNA synthesis were obtained by using the kit SuperScript one-step reverse-transcription polymerase chain reaction (RT-PCR) with platinum Taq (Gibco, Invitrogen Corp.). About 3 μg of RNA was reverse-transcribed in 50 μL volume reactions. Thermal cycling for each RT-PCR amplification was conducted as follows: 30 min at 55°C for cDNA synthesis and 2 min of pre-DNA denaturation at 94°C, followed by 30 cycles of PCR amplification (15 s at 94°C, 30 s at 62 or 55 or 50°C, and 2 min at 72°C), and a final extension step for 8 min at 72°C. The quality of the RNA samples was checked by RT-PCR of β-actin (45). A negative control for detection of RNA contamination was run in each PCR reaction set. RT-PCR products were purified by using QIAquick PCR purification Kit (Qiagen, Valencia, CA) and subjected to DNA sequencing by using the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, United States). The reaction mixture (20 μL final volume) included 500 ng of the purified PCR product, 20 μM either primer, and 8 μL of BigDye Terminator. The sequencing program consisted of 5 min at 96°C for pre-DNA denaturation, followed by 30 cycles (96°C for 10 s, 50°C for 5 s, and 60°C for 4 min). Product purification was carried out by using the DyeExTM 2.0 Spin Kit (Qiagen). The samples were vacuum-dried, resuspended in 25 μL of template suppression reagent, and then heated for 3 min at 95°C. Sequencing samples were analyzed by using a Perkin-Elmer ABI Sequencer. Mutant HPRT sequences were compared to the wild-type HPRT sequence by using the “DNases” sequence analysis software.

Analysis of Genomic HPRT DNA

The isolation of genomic DNA was performed by using the Qiagen Blood & Cell Cultured DNA Mini Kit, as described by the supplier. DNA was air-dried and dissolved in TE, pH 8.0. The multiplex PCR for genomic HPRT analysis was performed as described by Xu et al. (46). All HPRT exons were simultaneously amplified, except exon 1, which was amplified separately, using 200 ng of genomic DNA in a 50 μL volume. A negative control without genomic DNA was performed in each multiplex PCR reaction set to test for the presence of contaminating DNA.

All PCR reactions were conducted by using the DNA Peltier Thermal Cycler, PTC-200 (MJ Research, MN). PCR products were analyzed by 1% agarose gel (BMA, BioWhittaker Molecular Applications, United States) electrophoresis.

Oligonucleotides Primers

The primers used for HPRT cDNA synthesis, amplification, and sequencing were vrl-16 (47), zee-1 (48), HP10, and EX92 (46). The primers used for β-actin cDNA synthesis and amplification were as described in ref 45. The primers used for multiplex PCR amplification were as described in ref 46.

Statistics

Statistical significance was evaluated using Student’s *t* test. $p < 0.01$ was considered as significant.

Results

OTA Exerts Its Cytotoxic Effect by Inducing G_2/M Cell Cycle Arrest in V79 Cells

V79 cells were exposed to a wide range of OTA doses (from 1 to 188 μM) for increasing periods of time (from 3 to 24 h), and the number of cells was determined. No cytotoxicity was detected after 3 and 6 h treatment times, whereas a clear dose- and time-dependent cytotoxic effect was observed at longer exposure times (Figure 1). The IC_{50} (dose causing 50% growth inhibition) values of 133 and 35 μM OTA were determined after 12 and 24 h of incubation time, respectively.

The effects on cell cycle progression and DNA synthesis after 24 h of exposure to OTA doses approaching the IC_{50} were then evaluated by flow cytometry (Figure 2). A simple visual inspection of PI fluorescence (A), which indicates the cell cycle distribution on the basis of DNA content, shows a dose-dependent effect of OTA on cell cycle. Cells treated at doses ≥35 μM accumulated at G_2/M phases with a reduction of cells in G_0/G_1 phases. The biparametric DNA-BrdU plots (B) produced a more detailed cell phase distribution analysis since the S-phase cells can be identified. Exposure to 20 μM OTA for 24 h did not drastically affect the cell cycle distribution of V79 cells, whereas a significant increase of the proportion of cells in G_2/M phase (approximately six-fold increase) was observed after treatment with 35 μM OTA for 24 h (C). No inhibition of DNA synthesis was observed suggesting that cells that were in G_0/G_1 or S phase at the time of OTA treatment continued to progress into cell cycle and arrested in G_2/M. These findings indicate that OTA specifically affects the cell cycle in V79 cells by inducing a G_2/M arrest without inhibition of DNA synthesis.

OTA Is Mutagenic in the Absence of Biotransformation in Mammalian Cells at Two Different Gene Loci

The mutagenic effects of OTA in the absence of biotransformation were analyzed by incubating cells with OTA for 24 h in growth medium containing 10% FBS. The results of three independent experiments are displayed in Table 1. An increase of mutation frequency over background was observed in all three experiments but in a narrow range of concentrations (from 20 to 35 μM) and with a decline in mutation frequency at higher doses (see experiment 3). The analysis of OTA-induced mutation frequency as a function of survival indicates the presence of a cytotoxicity threshold for mutagenicity and a decrease of mutation frequency at doses inducing more than 50% cell killing. The significance of the mutagenic effect of OTA was evaluated by comparing the induced mutation frequency within each experiment with that not only of the internal control (untreated cells) but also with a laboratory historical control (mean 10.5×10^6, 1% upper confidence limit 31.6, $n = 62$). A conservative estimation of approximately four-fold increase of mutation frequency over mean historical control by OTA was estimated.

To evaluate the role of metabolism in the mutagenic activity of OTA, V79 cells were treated with OTA doses ranging from 35 to 438 μM, for 3 h, both in the absence and in the presence of metabolic activation. S9 fractions were prepared from kidney and liver of rats induced with phenobarbital and β-naftoflavone. The treatment time was set to 3 h because incubation with S9 fraction for longer times was cytotoxic to the cells and OTA was added to free-serum medium. A representative HPRT mutation assay in the presence of kidney S9 fraction is shown in Table 2. OTA induced a detectable increase over background in mutation frequency (8.5-fold) only in the absence of the S9 fraction and in a narrow range of concentrations (35-80 μM). Similarly to what is shown in Table 1, at higher OTA doses, the mutation frequency decreased. When cells were incubated with the kidney S9 fraction, an increase in the spontaneous mutation frequency was observed, thus decreasing the sensitivity of the assay. However, no increase over spontaneous mutation frequency was observed after OTA treatment.

Similar results were obtained by incubating OTA in the presence of liver S9 fraction (data not shown). These results indicate that under these experimental conditions, there is no production of mutagenic OTA-derived metabolites.

To strengthen the observation of OTA-induced mutagenesis, the TK mutation assay in L5178Y $TK^{\pm}$ mouse lymphoma cells was performed. Cells were exposed to OTA for 3 h in the absence and in the presence of the S9 fraction from rat kidney (Table 3). A significant increase in mutation frequency over background was observed in the absence of the S9 microsomal fraction. The increase in mutation frequency was also confirmed when comparing data with the historical control (mean 90.2×10^6, 1% upper confidence limit 167.8, $n = 76$). In the presence of metabolic activation, the increase in mutation frequency was of the same order of magnitude as that observed in the absence of S9. Similarly to what was reported in hamster cells, OTA-induced mutagenic effects were detected in a narrow range of doses with a decrease in mutation frequency at higher doses (in the presence of S9). The TK mutant mouse lymphoma clones can be differentiated on the basis of their size in small and large colonies that identify different molecular events (49). An even distribution between large and small size colonies was observed among OTA-induced mutants as well as for spontaneous mutants, suggesting that both point mutations and large chromosomal rearrangements are induced in either case.

All together, these results indicate that OTA is a relatively weak mutagen in mammalian cells in two different gene loci *via* a mechanism that is independent of exogenous metabolic activation. Moreover, OTA is mutagenic at threshold cytotoxic doses, whereas at higher doses pathways other than mutagenesis are likely to be triggered.

OTA-Induced HPRT Mutational Spectrum Is Similar to the Spontaneous Mutational Spectrum

A total of 21 OTA induced HPRT mutants from two independent experiments and 18 spontaneous mutants were analyzed at molecular level by direct sequencing of HPRT cDNA (657 bp). Among OTA-induced mutant clones (Table 4), 10 (48%) gave rise to full-length cDNA products, whereas the remaining (52%) did not produce any RT-PCR product. Multiplex PCR analysis of genomic DNA of this class of mutants confirmed the presence of all nine exons. Among spontaneous mutants (Table 5), 12 (67%) presented full-length cDNA products and the remaining (33%) showed the occurrence of deletions/insertions. The types of molecular changes detected are shown in Tables 4 and 5. The OTA mutation spectrum (Table 4) is characterized by point mutations (10/21; 48%) equally distributed between transitions and transversions. These base substitutions involved different exons although mutations at the same position were detected. This was not unexpected when the increase of mutation frequency over background is relatively low. The spectrum of the spontaneous mutants (Table 5) showed the predominance of point mutations (7/18, 39%) as well as the occurrence of frameshifts, large insertions, and deletions (10/18; 56%). One mutant showed the wild-type sequence. Although the spontaneous mutation spectrum presented a large number of siblings, it well-represents the type of mutations detected in larger set of spontaneous mutants previously described (50) (Table 5).

Overall, OTA-induced base substitutions did not show the induction of specific types of mutations but were similar to those arising spontaneously, suggesting that this mycotoxin increases the process involved in spontaneous mutagenesis.

Discussion

Here, we show that OTA induces a significant increase of mutation frequency at two gene loci, HPRT and TK, *via* a mechanism that is independent of biotransformation. Both *in vitro* and *in vivo* studies in mammalian systems indicate that OTA is poorly metabolized and that the formation of electrophilic intermediates is unlikely (12,14). This is in line with the absence of

an effect of metabolic activation systems on OTA mutagenicity. The OTA-induced increase in mutation frequency (approximately four-fold over historical spontaneous mutation frequency) is modest and is characterized by a bell-shaped dose-response relationship with a decrease of mutation frequency at high OTA doses (>50% lethality). The reason for the decrease of mutation frequency at higher doses is not clear. However, a similar dose-response pattern has been described for the induction of fpg-sensitive sites by comet assay in kidneys of OTA-treated rats (17,23), as well as in different cell lines (51), suggesting a possible link between oxidative DNA damage and OTA-induced mutations. OTA has been shown to induce apoptosis both *in vivo* (52) and *in vitro* (51,53,54), including rat kidney and kidney-derived cells that are the target for OTA carcinogenicity. It has been hypothesized that the selective loss of heavily damaged cells by apoptosis might be responsible for OTA-induced nonlinear dose responses. Alternatively, it is well-known that the effects of reactive oxygen and nitrogen species (RONS) are characterized by different thresholds for various cellular markers (55). If OTA genotoxicity is mediated by RONS production, at sublethal doses, mutagenesis might prevail whereas at higher doses the cell antioxidant response might decrease the likelihood of DNA damage and targets other than DNA might become more relevant.

We show that OTA severely affects cell cycle by arresting cells at G_2/M phases. Similar effects were described in several kidney cell lines (51,56,57), as well as in rats (52,58) and pigs treated with OTA (59) where the G_2/M cell cycle arrest is also suggested by the marked appearance of giant nuclei in P3 epithelial cells. Altogether, these findings suggest that OTA interferes with molecules involved in cell division as suggested by inhibition of microtubule assembly in human kidney cells (57).

In contrast with our findings, previous studies conducted in V79 (35) and L5178Y $TK^{\pm}$ (29) cells reported that OTA was not mutagenic. However, the comparison with our study is difficult because of different treatment conditions that might impede the detection of the effect if the critical threshold for OTA-induced mutagenesis is not achieved. There is one report of mutagenic activity of OTA in mammalian cells by using murine cells stably expressing human cytochromes and a shuttle vector as a tool for mutation analysis (36). The mutagenic activity that we detect is independent of metabolic activation; however, it should be taken into account that the mechanism of mutagenesis of a gene carried out by a shuttle vector might be different from those of intrachromosomal genes.

Mutational spectra have been instrumental for the comprehension of the mechanism of action of mutagenic compounds. The molecular characterization of OTA-induced mutations, although limited by the small number of mutants analyzed, indicates that this mycotoxin induces two types of mutational events: point mutations and lack of cDNA products. The analysis of genomic DNA confirmed the presence of all nine exons of the HPRT gene in this second class of mutants. This phenomenon has been previously described among both spontaneous and induced HPRT mutants (60,61), but the mechanisms involved remain to be elucidated. Point mutations were targeted at both GC and AT base pairs and were substantially similar to those arising as spontaneous mutants. G > A mutations are compatible with deamination events whereas G > C and A > C transversions might be explained by oxidative DNA damage. However, to draw more definitive conclusions, a larger number of mutants should be analyzed. It is interesting to mention that, similarly to OTA, reactive species derived from NO have been shown to induce an increase of spontaneous mutagenesis (62). OTA indeed induces oxidative and nitrosative stress (17,51,63-69), and as expected, antioxidants are able to prevent its toxic effects (70-74). Different mechanisms for oxygen and nitrogen radical production by OTA have been proposed as follows: (i) oxido-reduction mechanisms directly involving OTA and Fe^{3+} (68,75), (ii) perturbation of Ca^{2+} homeostasis (65,66), (iii) generation of hydroquinone/quinone species from OTA oxidation (64,76-78), (iv) OTA-mediated reduction of antioxidant cellular defenses (73), and (v) induction of the expression of inducible

nitric oxide synthase (iNOS) (79) that is dominantly expressed during inflammatory reactions. The generation of the hydroquinone metabolite of OTA (OTHQ) deserves further comment. It has been proposed that this electrophile participates in OTA-mediated DNA adduction and genotoxicity (78). Its production requires pig kidney microsomes or cell types with the capacity for oxidative biotransformation. In our cell system, OTA-induced mutation frequency was not affected in the presence of S9 fraction, indicating that OTA does not undergo conversion by cytochrome P450 to mutagenic metabolites. Moreover, the lack of mutation specificity as inferred from OTA-induced mutational spectrum strongly suggests that no specific DNA adducts are induced. Therefore, our data do not support the involvement of OTHQ in OTA-mediated mutagenicity.

In agreement with a potential role for oxidative stress in OTA toxicity and genotoxicity, recent toxicogenomic data indicate that OTA affects the expression of genes pertaining to the inflammatory and oxidative stress response systems both *in vitro* and *in vivo* (79-81).

In conclusion, in this study, we provide the first evidence that OTA is mutagenic at cytotoxic doses in mammalian cells independently on biotransformation. Molecular analysis of OTA-induced mutants indicates that this mycotoxin increases spontaneous mutagenesis in agreement with the induction of endogenous RONS over the normal oxidative metabolism. Our results support a model where OTA leads to mutation by production of free radicals. This mechanism together with the pleiotropic effects of OTA on protein synthesis, cell cycle dynamics, and signal transduction pathways might contribute to cancer initiation.

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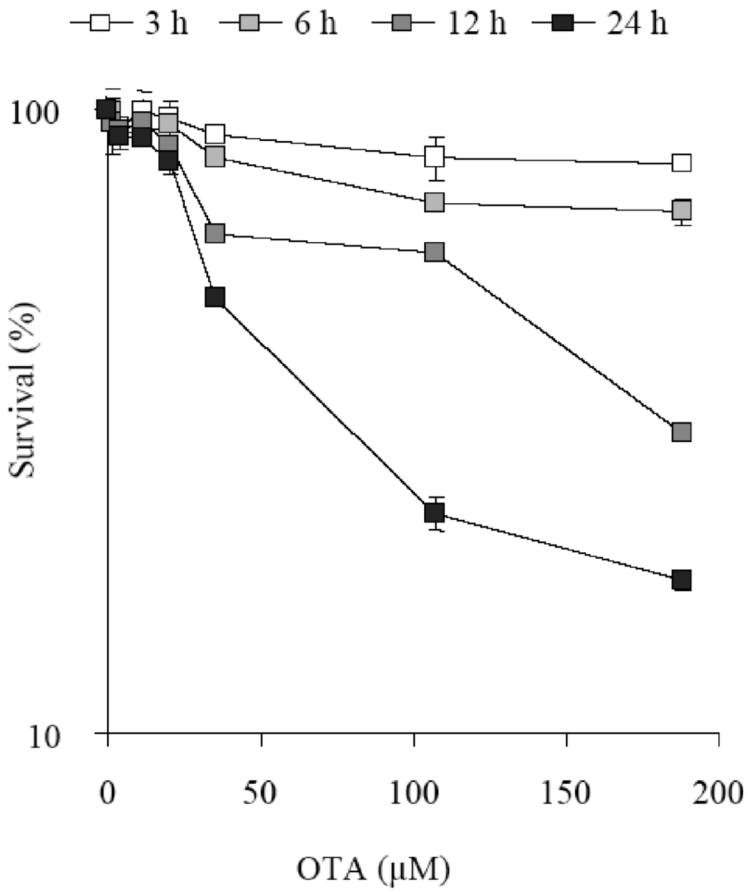

Figure 1.
Cytotoxicity induced by OTA in V79 cells following 24 h of exposure at different doses. Cells were enumerated by cell counting. OTA surviving cells are expressed as percentage of the surviving untreated cells. Bars indicate SD.

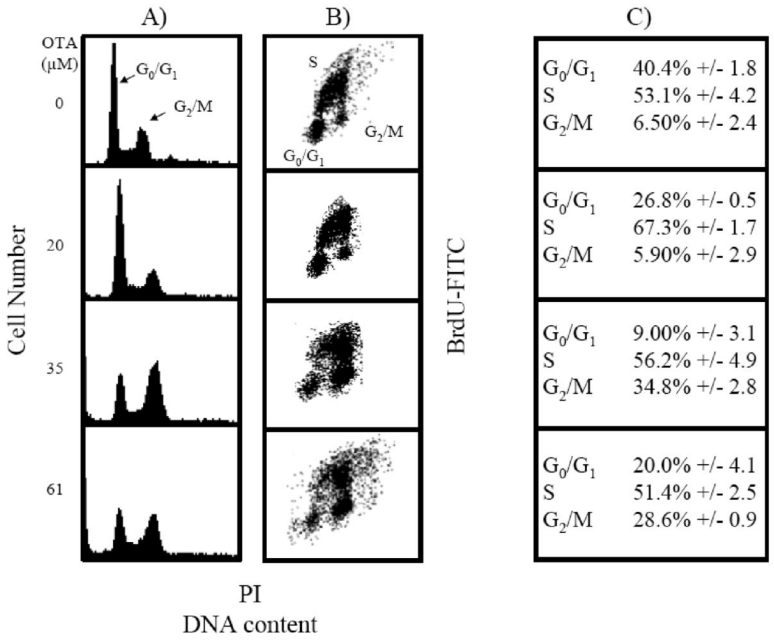

Figure 2.

Cell cycle and DNA synthesis analysis of V79 cells following 24 h of exposure to OTA. Cell cycle plots: (A) distribution of the PI fluorescence (DNA content) *vs* cell number and (B) distribution of the PI fluorescence *vs* the green fluorescence of the FITC anti-BrdUrd staining (DNA synthesis). The distribution of the cell population in the different cell cycle phases is indicated. Experiments were repeated at least two times.

Table 1

OTA-Induced HPRT Mutation Frequency in V79 Cells after 24 h of Exposure

OTA (μM)	survival (%)	MF[a]
	experiment 1[b]	
0	100	1
7	87	12
11	100	8
20	81	19
35	48	39
EMS[c]	78	1495
	experiment 2[d]	
0	100	3
11	94	12
20	77	38
35	48	35
EMS[c]	40	1116
	experiment 3[b]	
0	100	5
20	56	47
46	31	
108	34	15
251	25	29
EMS[c]	48	2024

[a] MF, mutation frequency per 10^6 cells.

[b] Expression time, 6 days.

[d] 10 mM EMS.

[c] Expression time, 9 days.

Table 2

OTA-Induced HPRT Mutation Frequency in V79 Cells after 3 h of Exposure in the Presence of S9 Kidney Metabolic Fraction

-S9[a]			+S9[a]		
OTA (μM)	**survival (%)**	**MF[b]**	**OTA (μM)**	**survival (%)**	**MF[b]**
0	100	5	0	100	36
35	91	41	35	91	32
80	63	43	80	73	43
187	69	12	187	75	15
438	83	17	438	75	15
EMS[c]	62	1583	DMBA[d]	88	72

[a] Expression time, 6 days.

[b] MF, mutation frequency per 10^6 cells.

[c] 10 mM EMS.

[d] 10 μg/mL DMBA.

Table 3

OTA-Induced TK Mutation Frequency in LY5178 Mouse Lymphoma Cells after 3 h of Exposure in the Presence and in the Absence of S9 Kidney Metabolic Fraction[a]

-S9				+S9			
OTA (μM)	**RTG[b]**	**MF[c]**		**OTA (μM)**	**RTG[b]**	**MF[c]**	
0	100	135		0	100	133	
3	46	162	NS	3	21	230	*
81	21	196	NS	81	22	207	*
188	18	202	*	188	17	237	**
438	20	234	**	438	20	184	NS
MMS[d]	74	871		2-AA[e]	29	1248	

[a] NS, not statistically significant.

[*] Statistically significant at $p < 0.05$.

[**] Statistically significant at $p < 0.01$. The test for linear trend was significant ($p < 0.01$) in the absence of the S9 fraction.

[b] RTG, relative total growth.

[c] MF, mutation frequency per 10^6 cells.

[d] 10 μg/mL MMS.

[e] 30 μg/mL 2-AA (2-aminoanthracene).

Table 4

Type and Position of OTA-Induced Mutations in the HPRT Gene of V79 Cells

type of mutation	number	position
total mutants analyzed	21	
base substitutions	10	
G·C > A·T	4	508, 508, 599, 599
A·T > G·C	1	415
G·C > C·G	2	190
A·T > C·G	3	563
others	11	no cDNA

Table 5

Type and Position of Spontaneous Mutations in the HPRT Gene of V79 Cells

type of mutation	position	number	number[a]
total mutants analyzed		18	76
base substitutions		7	64
G·C > A·T	410	5	9
A·T > G·C			12
G·C > T·A			12
G·C > C·G			13
A·T > T·A			1
A·T > C·G	122	2	16
frame shifts	67 (-T)	4	5
deletions	Ex 4-5	2	13
insertions	463-482	2	
multiple	Ex 4-5, 463-482	2	
duplications			3
others	no mutation	1	1
splice			33

[a] From ref 50.