Induction of Unscheduled DNA Synthesis in Primary Human Urothelial Cells by the Mycotoxin Ochratoxin A

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Ochratoxin A (OTA) is a widespread contaminant in human staple food. Exposure of humans to this mycotoxin is a matter of concern because OTA is a known rodent carcinogen. As the urothelium is one target tissue of this mycotoxin, primary cultured human urothelial cells (HUC) from adults and children were used to analyze the induction of unscheduled DNA synthesis (UDS) by OTA. HUC were isolated from the ureters or renal pelvis of two nephrectomized adults and of two children with ureteropelvic junction stenosis and cultured under serum-free conditions. After a confluency of 70–80% was reached, cell proliferation was suppressed by arginine-deficient medium (ADM), and UDS was assessed autoradiographically by [³H]-thymidine incorporation upon exposure to OTA (10–2000 nM), ethyl methanesulfonate (EMS, 5 mM, positive control), or dimethyl sulfoxide (DMSO, 0.2%, solvent control). In control cultures the level of UDS was low. Exposure to EMS resulted in an induction of UDS (2- to 5-fold compared to control), thus allowing the sensitive detection of repair resulting from induction of DNA lesions in all four specimens, and demonstrating that repair of EMS-induced DNA lesions can take place under the chosen culture conditions. In two HUC cultures derived from adults, a significant induction of UDS was observed in the concentration range of 50–500 nM OTA. The highest fraction of cells in repair (CIR) was found at 50 nM OTA for the HUC from the older male (50% CIR). The maximum response in the other specimens from the adult female and the 7-year-old boy were seen at OTA concentrations of 500 and 250 nM, respectively. In contrast to all other specimens, no significant induction of UDS by OTA was found in the HUC cultures derived from an infant’s urothelium. Signs of cytotoxicity were observed above 500 nM OTA in all cultures. The varying susceptibility toward OTA observed in vitro may hint at varying predispositions of individuals in vivo.

Key Words: ochratoxin A; DNA repair; human urothelium; primary culture.

The mycotoxin ochratoxin A, a metabolite of Aspergillus and Penicillium species, is found worldwide as a contaminant in human staple food (Speijers and van Egmond, 1993). Thus the human population, adults as well as children and even infants, is likely to be exposed to OTA.

OTA has a long half-life and is very slowly cleared from the body (Schlatter et al., 1996). A considerable fraction of OTA and its metabolites is excreted via urine (Li et al., 1997).

It is of concern for scientists and regulatory authorities that OTA is apparently involved in the etiology of a kidney disease known as Balkan Endemic Nephropathy (BEN), as well as in the occurrence of urinary tract tumors in patients with BEN (Castegnaro et al., 1990; Petkova-Bocharova and Castegnaro, 1991; Sostaric and Vukelic, 1991). Although evidence for carcinogenicity of OTA in humans is still inadequate, IARC has classified OTA as a possible human carcinogen (group 2B) based on sufficient evidence for carcinogenicity in animal studies (Bendele et al., 1985, Boorman, 1989; IARC, 1993).

The carcinogenicity of OTA in rats was recently confirmed and sex- and strain-specific differences in the induction of renal and bladder tumors were observed (Castegnaro et al., 1998).

In recent years, evidence has accumulated that OTA is a genotoxic carcinogen (Dirheimer, 1998). However, the mechanism that results in DNA-damage has not been fully resolved; it is an open question whether metabolism is required in the manifestation of adverse OTA effects in target tissues such as kidney and urothelium. On the other hand, OTA yielded negative results in the classical Ames assay (Bartsch et al., 1980; Bendele et al., 1985a; Kuczuk et al., 1978; Wehner et al., 1978), the mouse lymphoma assay (Bendele et al., 1985b), and in a modified Ames assay with rat hepatocytes (Bendele et al., 1985a). Equivocal results were obtained in the UDS assay with mouse hepatocytes (Mori et al., 1984) and in a modified Ames assay (Henning et al., 1991). By means of postlabelling methods, DNA adducts have been found in urinary bladder of mice treated in vivo with OTA (Obrecht-Pflumio et al., 1996; Pföhl-Leszkoowicz et al., 1993a). Moreover, OTA has been shown to induce sister chromatid exchanges in primary cultures of porcine urinary bladder epithelial cells (Föllmann et al., 1995) and the formation of micronuclei in ovine seminal vesicle cells (Degen et al., 1997). Recently, OTA was found to induce DNA repair in rat hepatocytes and in urinary bladder epithelial cells...
from pigs (Dörrenhaus and Föllmann, 1997) and in human urothelium (Flieger et al., 1998) as a further indication for its genotoxicity.

In the study presented here the genotoxic potential of OTA was investigated in primary cultured human urothelial cells (HUC) derived from ureters or renal pelves of two nephrectomized adults and two children with ureteropelvic junction stenosis by analyzing the induction of DNA repair.

MATERIALS AND METHODS

Materials. Ochratoxin A was purchased in crystalline form from Aldrich (Steinheim, Germany). Culture media, antibiotics, and antimitotics were from Gibco (Eggenstein, Germany). All other medium supplements and EMS were from Sigma (Deisenhofen, Germany), with the exception of insulin and collagen type I, which were purchased from Serva (Darmstadt, Germany). Dimethyl sulfoxide was from Merck (Darmstadt, Germany), and tritiated thymidine was from Amersham (Braunschweig, Germany).

Culture dishes were from Becton and Dickinson (Heidelberg, Germany) and ThermaNox™ coverslips were from Nunc (Wiesbaden, Germany). NTB2 film emulsion was purchased from Integra Biosciences (Fernwald, Germany) and Agêfix fixative and Kodak D-19 developer were obtained from local photo shops.

Culture of human urothelial cells. Specimens of about 0.5 cm² from ureters or renal pelvis of two nephrectomized adults (specimen I, male, 66 years old; specimen II, female, 26 years old) and of two children (specimen III, male, 7 years old; specimen IV, male, 9 months old) with ureteropelvic junction stenosis were obtained from the surgery departments of local hospitals. The medical indications leading to nephrectomy in the case of the adult patients were renal cell carcinoma (specimen I) and hydronephrosis (specimen II). No malignancy was detected by the pathologists. At the laboratory the epithelial mucosa of the specimens was washed with sterile phosphate-buffered saline solution (PBS) to remove blood cells. Human urothelial cells were carefully scraped from the underlying muscle layer with a bone curette under sterile conditions. The isolated urothelial cells were transferred into serum-free Ham’s F-12 medium supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin (1.25 µg/ml), human transferrin (5 µg/ml), bovine insulin (10 µg/ml), epidermal growth factor (20 ng/ml), nonessential amino acids (0.1 mM), and glucose (2.7 mg/ml). Cells were washed twice by centrifugation (50 × g, 5 min). Viability ranged between 30% and 40% according to trypan blue exclusion (Neubauer chamber). Aliquots of 0.5 ml containing 5 × 10⁴ viable cells were distributed on ThermaNox™ coverslips (Ø 13 mm) in 24-well plates, coated with collagen type I (5 µl/coverslip). The cells were cultured under serum-free conditions in a humidified incubator at 37°C and 5% CO₂ atmosphere. The culture medium was changed twice a week. After 9–10 days, HUC reached a confluency of about 75% of the expansion area of the coverslip, which is sufficient for the performance of the UDS experiments. In preceeding experiments, epithelial origin and differentiation had been demonstrated by positive immunohistochemical staining for cytokeratines 7, 8, 19, and 20 (Flieger et al., 1997 and unpublished results).

Detection of unscheduled DNA synthesis. According to Williams (1977), unscheduled DNA synthesis can be assessed by autoradiography by measuring the incorporation of tritiated thymidine into the nuclear DNA of nondividing cells. Thus, cell proliferation and associated replicative DNA synthesis have to be suppressed. The use of hydroxyurea, as employed in several other studies with proliferating cells, was rejected because hydroxyurea can interfere with cell proliferation and replicative DNA synthesis. This is a prerequisite to detect unscheduled DNA synthesis.

Proliferation of HUC was inhibited by replacing the standard medium and culturing the cells for 96 h in ADM with supplements described for the standard medium, but without epidermal growth factor and nonessential amino acids. During the last 24 h of arginine deprivation, HUC were incubated with OTA (final concentration 10–2000 nM) or EMS (final concentration 5 mM, positive control) and [³H]-thymidine (370 KBq/ml, stock solution 3.11 TBq/mmol). For each specimen, control values were determined adding only [³H]-thymidine but not OTA or EMS to the culture medium and in solvent controls containing [³H]-thymidine and dimethyl sulfoxide (DMSO, final concentration 0.2% in the culture medium).

For the autoradiography, the coverslip cultures were washed five times with PBS to remove nonincorporated [³H]-thymidine, followed by an incubation (7 min) with 1% sodium citrate solution to swell the nuclei. Afterwards the cells were fixed for 15 min in ethanol:acetic acid (3:1), rinsed with ethanol, and air-dried overnight. The coverslips were pasted cell-side up on microscope slides. Following standard protocols (Swierenga et al., 1991), the slides were coated with Kodak NTB2 photo emulsion diluted 1:1 with distilled, deionized water in the dark and exposed at −20°C for 7 days, followed by development and fixation (Kodak D-19 developer, Agfa fixative) and staining with hematoxylin-eosin.

Analysis of autoradiography. Autoradiograms were analyzed using a Leica Quantimet 520 image analyzing system interfaced to a microscope with a video camera: Net nuclear grains (NNG) were calculated by counting the number of silver grains above the nucleus (nuclear grains) and subtracting the highest number of silver grains found in one equivalent area of the cytoplasm adjacent to the nucleus of each cell (background grains). Nuclear grains, background grains, and the resulting net nuclear grains (each with standard deviation) are shown in Table 1.

Because no large database exists on UDS response for HUC’s, cells that are not in S-phase with NNG > 5 were defined as “cells in repair” (CIR) according to Williams (1977). This value was adopted as an arbitrary cutoff in this study and to facilitate comparison of our results with those from a previous study with rat hepatocytes and porcine urinary bladder epithelial cells (Dörrenhaus and Föllmann, 1997).

The percentage of CIR was determined by counting 50 cells per slide and concentration. Because only small specimens of ureter were available and the yield of viable cells was rather low, at the beginning of the experiments only one slide was available per concentration. Cells showing staining artifacts or morphologic signs of cytotoxicity such as cell rupture or isolated nuclei (Mitchell and Mirsalis, 1984; Swierenga et al., 1991) were excluded from scoring.

Data were statistically analyzed by Fisher’s exact test (Bünning and Trenkler, 1978) to detect significant elevations of CIR in the treated cultures versus solvent controls. This information is included in Figures 1–4.

RESULTS

After human urothelial cells had been cultured in arginine-deficient Ham’s F-12 medium for 96 h, less than 1% of cells were in S-phase, which indicates a sufficient suppression of cell proliferation and replicative DNA synthesis. This is a prerequisite to detect unscheduled DNA synthesis.

The data of cytoplasmic and nuclear grain counts, including net nuclear grains, are presented in Table 1. Addition of EMS (5 mM, positive control) induced DNA repair in HUC cultures of all four specimens, indicating that the arginine-deprived cells were able to undergo UDS. The UDS response to EMS was 40% CIR in specimen I, 46% CIR in specimen II, 30% in specimen III, and 58% in specimen IV (Figs. 1–4).
These responses to EMS correspond with a 2.2-, 4.6-, 5-, and 4.8-fold increase compared to the control values (Table 2); in untreated HUC and in solvent controls, 18%, 10%, 6%, and 12% CIR were measured.

Treatment with OTA also led to a significant induction of UDS in HUC cultures derived from the first three specimens (Figs. 1–3), but not in those obtained from an infant (Fig. 4).

In the HUC cultures of the adults, OTA in a concentration range between 50 and 250 nM (specimen I) and 10 and 500 nM (specimen II) led to clear increases in DNA repair (Figs. 1 and 2), with maximum responses in specimen I at 50 nM OTA and in specimen II at 500 nM OTA. Compared to the corresponding solvent controls, the percentage of CIR increased 2.8- and 4.4-fold, respectively (Table 2).

At OTA concentrations ≥750 nM, DNA repair decreased (Figs. 1 and 2) and morphologic signs of cytotoxicity were observed, e.g., pycnotic cells, lysed nuclei, or nuclei not surrounded by cytoplasm (typical features described by Mitchell and Miersalis, 1984).

As the chosen OTA concentrations were based on concentrations necessary to induce UDS in porcine urinary bladder epithelial cells (Dörrenhaus and Föllmann, 1997), and because of the small amount of tissue available in this study, we did not investigate the effect of OTA concentrations below 50 nM in HUC cultures from adults. Thus, the data presented here probably reflect only the upper part of the dose-response curve.

In HUC of a 7-year-old boy, the effects of OTA were studied in a concentration range between 10 and 1500 nM OTA (Fig. 3). A significant induction of UDS compared to solvent controls was found at 250 and 500 nM OTA; above 500 nM OTA cytotoxicity appeared as well. The observed percentage of CIR was lower in these HUC cultures (30%) than those of adults

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### TABLE 1

<table>
<thead>
<tr>
<th>Specimen 1</th>
<th>Specimen 2</th>
<th>Specimen 3</th>
<th>Specimen 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>N ± SD</td>
<td>C ± SD</td>
<td>NNG ± SD</td>
<td>N ± SD</td>
</tr>
<tr>
<td>Solvent control</td>
<td>7.6 ± 3.5</td>
<td>4.4 ± 1.9</td>
<td>3.2 ± 3.4</td>
</tr>
<tr>
<td>Medium control</td>
<td>7.0 ± 3.4</td>
<td>2.4 ± 1.2</td>
<td>4.5 ± 3.4</td>
</tr>
<tr>
<td>0.025 μM OTA</td>
<td>12.5 ± 8.8</td>
<td>2.6 ± 1.1</td>
<td>9.9 ± 8.6</td>
</tr>
<tr>
<td>0.050 μM OTA</td>
<td>12.2 ± 9.5</td>
<td>2.4 ± 1.0</td>
<td>9.8 ± 9.2</td>
</tr>
<tr>
<td>0.100 μM OTA</td>
<td>11.8 ± 8.9</td>
<td>3.7 ± 1.8</td>
<td>8.2 ± 8.3</td>
</tr>
<tr>
<td>0.250 μM OTA</td>
<td>9.7 ± 6.3</td>
<td>3.0 ± 1.3</td>
<td>6.7 ± 6.3</td>
</tr>
<tr>
<td>0.500 μM OTA</td>
<td>6.5 ± 3.2</td>
<td>2.2 ± 1.0</td>
<td>4.3 ± 3.3</td>
</tr>
<tr>
<td>1.000 μM OTA</td>
<td>2.5 ± 1.3</td>
<td>1.4 ± 0.9</td>
<td>1.1 ± 1.3</td>
</tr>
<tr>
<td>2.000 μM OTA</td>
<td>1.16 ± 1.2</td>
<td>1.4 ± 1.5</td>
<td>7.2 ± 7.2</td>
</tr>
</tbody>
</table>

*Note.* C, mean number of the highest cytoplasmic grains; EMS, ethyl methanesulfonate; N, mean number of nuclear grains; NNG, mean of net nuclear grains; OTA, ochratoxin A; SD, standard deviation. Solvent control: DMSO. Medium control: ADM (arginine-deficient medium).
(50% and 45%, respectively, Figs. 1–3), but the relative increases induced in both OTA and EMS were similar (5.3- and 5-fold, respectively), due to the lower background levels of CIR in the solvent controls (Table 2).

None of the tested OTA concentrations led to a significant induction of DNA repair in the cultured urothelial cells of the 9-month-old boy, as analyzed by pairwise comparison of each of the tested OTA concentrations with the solvent control (Fig. 4). Interestingly, OTA concentrations between 25 and 100 nM led to observable increases in CIR, but this effect was not statistically significant. This points to a rather steep dose-response curve. In contrast, EMS led to a pronounced UDS response with 58% CIR, which corresponds to a 4.8-fold increase compared to the solvent control (Table 2).

DISCUSSION

Ochratoxin A has been shown to induce kidney and bladder tumors in rodents (Bendele et al., 1985b; Boorman, 1989; Castegnaro et al., 1998; IARC, 1993). Moreover, OTA exposure of pigs has been associated with porcine nephropathy (Hald, 1991; Krogh et al., 1973), a condition that resembles Balkan endemic nephropathy (BEN) in humans (Krogh et al., 1972, see introduction).

Treatment of porcine urinary bladder epithelial cells with OTA in vitro can result in a dose-dependent increase in sister chromatid exchange and induction of unscheduled DNA synthesis (Dörrenhau et al., 1997a, 1998), but the mechanisms that are responsible for the adverse effects of OTA and/or its metabolites have not been elucidated. DNA adducts have been demonstrated by 32P postlabelling methods in mycotoxin-treated tissues and cells (unpublished results see Dirheimer, 1998; Obrecht-Pflumio et al., 1996; Pfohl-Leszkowicz et al., 1998).

Interestingly, the HUC cultures derived from the infant’s ureter (Fig. 4) did not respond to OTA in the same way as cultures from the other three specimens (with no significant induction of DNA repair upon exposure to OTA), whereas EMS caused a significant increase of UDS similar to that found for the other specimens (Figs. 1–3). This shows that, in principle, the mechanism of DNA repair is present in the infant’s cells. As EMS needs no further metabolism for its genotoxic effect, a possible explanation for the observation that OTA did not induce UDS in these cultures is a lack of or lower expression of drug-metabolizing enzymes due to the developmental status of the infant.

It has been suggested that OTA requires metabolic activation in order to exert genotoxic effects (Henning et al., 1991; Pfohl-Leszkowicz et al., 1993b, 1998), but the mechanisms that are responsible for the adverse effects of OTA and/or its metabolites have not been elucidated. DNA adducts have been demonstrated by 32P postlabelling methods in mycotoxin-treated tissues and cells (unpublished results see Dirheimer, 1998; Obrecht-Pflumio et al., 1996; Pfohl-Leszkowicz et al., 1998).
concentrations found to be effective may influence the response to OTA exposure. Results can be regarded as a first hint that individual factors, such as age, sex, or enzymatic predisposition, might be involved. However, the tested specimens represent only a small part of potential variables. A pilot study with cultures from four different specimens clearly demonstrated that differential responses are able to cause biochemical lesions.

Another important question pertains to the relevance of OTA concentrations found to be effective in vitro for the in vivo situation. In a recent study, the plasma OTA levels in humans resulting from exposure to contaminated food were measured. Based on food occurrence and consumption data, estimates of mean intake based on human urothelial cells compared to porcine cells treated with OTA were made. Exposure to OTA would result in different steady-state plasma levels (in humans approximately 30-times higher than in pigs; Schlatter et al., 1996). OTA is eliminated more rapidly in pigs (t1/2 = 120 h) than in humans. Thus, a given external exposure to OTA would result in different steady-state plasma levels (in humans approximately 30-times higher than in pigs; Hult, 1995).

In addition to the apparently more pronounced response of human urothelial cells compared to porcine cells treated with OTA, the pharmacokinetic properties of this mycotoxin should be taken into account in a risk assessment for this compound. Special thanks to S. Reich for her advice and help with the statistical analysis.

### ACKNOWLEDGMENTS

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