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Species-, Sex-, and Cell Type-Specific Effects of Ochratoxin A and B

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The ubiquitous mycotoxin ochratoxin A (OTA) is associated with the development of urothelial tumors and nephropathies in laboratory animals and in humans with stark species and sex differences with respect to susceptibility in disease development. The mechanism of action remains unknown. OTA-mediated disruptions in normal cell-cycle control could be a major constituent of the mechanisms underlying both its carcinogenic and nephropathy-inducing activities. Assessment of OTA's toxic effects (sum of antiproliferative, apoptotic, and necrotic effects) in rat and porcine continuous cell lines and in primary cells from humans and pigs of both sexes, have displayed a similar sex- and species-sensitivity rank order to that observed in previous *in vivo* experiments. Furthermore, these toxic effects were observed at nM concentrations in the presence of serum *in vitro,* thus closely mimicking the *in vivo* situation. These effects were reversible in all cell types except in human primary epithelial cells of both sexes and did not appear to be primarily dependent on the amount of OTA taken up. Indeed, fibroblasts (NRK-49F) were insensitive to OTA-mediated cell cycle inhibition in spite of accumulating comparable amounts of OTA. The results presented here support the continued use of primary renal epithelial cells for the investigation of the mechanism of OTA-induced carcinogenesis and nephropathy and provide an as-yet preliminary data set that supports the existence of a causal relationship between OTA exposure and human nephropathy.

Key Words: **ochratoxin A; nephropathy; renal cancer; uptake; cytostasis; toxicity.**

The mycotoxin ochratoxin A (OTA) is produced as a secondary metabolite by certain *Penicillium* and *Aspergillus* fungal species and is a common contaminant of human foodstuffs and animal feeds. Improper storage conditions of grain, coffee, dried fruits, etc. and their products can result in contamination levels of 0.1–3.8 μg/kg (Speijers and van Egmond, 1993; Studer-Rohr *et al.,* 1995; Wolff *et al.,* 2000). Average daily human intake of approximately 1.2 ng/kg leads to plasma levels of 0.5 ng/kg although this concentration may be considerably higher based on regional and individual dietary habits (Kuiper-Goodman and Scott, 1989; Studer-Rohr *et al.,* 2000). OTA is thought to be associated with 2 human renal diseases, namely Balkan endemic nephropathy (BEN) and urothelial tumors, both endemic to Balkan states (Radic *et al.,* 1997; Radonic and Radosevic, 1992).

Currently available data show large species- and sex differences in susceptibility towards OTA-induced nephrotoxicity and carcinogenicity. Chronic studies (210 μg/kg bw/day for 2 years) in rats have demonstrated a clear causal relationship between OTA exposure and the induction of renal cell tumors (RCT), with 60% of male rats developing carcinomas of cortical origin. This RCT was coupled with a distinct pathology of the *pars recta* of the proximal tubule. Female rats were found to be much less susceptible to OTA-mediated toxicity, displaying milder P3 pathology and only a 6% tumor incidence (Boorman, 1989; Boorman *et al.,* 1992; Castegnaro *et al.,* 1998; Rásonyi *et al.,* 1999). More pronounced sex differences were observed in the carcinogenic response of mice with 28% of male mice treated with 4800 μg/kg bw for 2 years presenting with RCT, while females were completely refractive (Bendele *et al.,* 1985; Grosse *et al.,* 1997). The mechanism(s) underlying these differences however, remain unknown. OTA toxicity at μM concentrations has been suggested to be due to DNA strand breaks, sister chromatid exchanges, DNA adduct formation, or reactive oxygen species (Galtier, 1991; Rahimtula and Chong, 1991). Other *in vitro* studies have been carried out either in the presence of heat inactivated serum (Creppy *et al.,* 1995), in the absence of serum (Bondy and Armstrong 1998; Föllmann *et al.,* 1995; Gekle *et al.,* 1995) or more recently, at nM concentrations, under serum-free conditions in previously transformed cells (Gekle *et al.,* 1998b, 2000). If the conditions of the *in vitro* system are to closely mimic the *in vivo* situation, investigation and characterization of the mechanisms involved in the acute toxicity of OTA *in vitro* should also include serum. However, the effects of nM concentrations of OTA (dietary relevant blood levels) in primary cell cultures and in the presence of serum have until now, not been investigated.

Control over cell growth and division is mediated through a carefully orchestrated balance of signals and checkpoints. Loss of this cell cycle control appears inherent to the etiology of various cancers. Furthermore, disruption in normal cell replication control could also be important in the etiology of the nephropathies associated with OTA exposure, for example either via a cytotoxic and/or cytostatic action on epithelial

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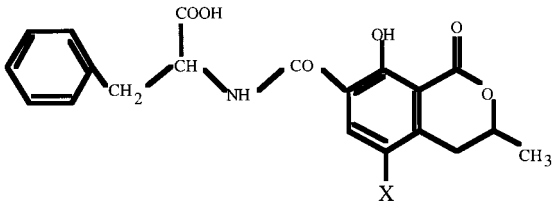

Ochratoxin A: X = Cl; R1 = H
Ochratoxin B: X = H; R1 = H

FIG. 1. Structural formulae of OTA and OTB.

cells, thus allowing enhanced compensatory fibroblast proliferation and hence renal fibrosis. Such a mechanism has been hypothesized earlier in the case of the increased incidences of chronic interstitial nephropathy in Tunisia, which are associated with high blood levels of OTA (Maaroufi *et al.*, 1995b; Simon *et al.*, 1996). The involvement of such an OTA-specific mechanism in the etiology of chronic interstitial nephropathy could be elucidated by the use of a range of relevant cell models of epithelial and fibroblast origin.

Thus, the aims of this study were to characterize the toxic effects (sum of antiproliferative, apoptotic, and necrotic effects) of relatively low concentrations of OTA in cell lines and in primary cells from both laboratory species and humans, in the presence of serum with a view to investigating their suitability for future studies into the mechanism(s) of OTA-mediated toxicity. Primarily, cells of epithelial origin were tested, however, the effects in the NRK-49F cell line (Normal Rat Kidney Fibroblast) was also examined to investigate the theory of epithelial cell replacement leading to the observed nephropathies as outlined above. For direct comparison with OTA, the toxic effects of ochratoxin B (OTB), a significantly less-toxic structural analogue of OTA, differing only by the lack of a chloride group on the isocumarin moiety (Fig. 1) was also investigated.

MATERIALS AND METHODS

Materials. Cell culture media and antibiotics were purchased from Gibco (Eggenstein, Germany). Collagen was obtained from Sigma (Deisenhofen, Germany) and FCS was supplied by Biochrom (Berlin, Germany). Tissue culture plastics were from Greiner (Solingen, Germany). [^{3}H]-OTA (sp. act. 14.8 Ci/mmol, purity $\geq$ 99%,) was purchased from Moravek Biochemicals (La Brea, CA). Scintillation cocktail was from Beckman (Munich, Germany). Ochratoxins A and B were kindly provided by M. Stack, FDA, Washington, DC. All other materials were of the highest grade commercially available.

Cell culture and isolation of primary cells. LLC-PK1 (ECACC No. 86121112), NRK-52E (DSMZ No. ACC 199), and NRK-42F (DSMZ No. ACC 172) cells were maintained under standard conditions in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS and 2000 U/l penicillin and 2 mg/l streptomycin in a humidified atmosphere at 37°C, 5% CO_2. The LLC-PK1 epithelial cell line was derived from renal cortex tissue from a juvenile Hampshire pig (Hull *et al.*, 1976) and the NRK-52E and NRK-49F cell lines were cloned from a mixed culture of normal Osborne-Mendel rat renal cortex cells (DeLarco and Todaro, 1978).

Primary porcine cortical renal epithelial cells (PKC) from German hybrid pigs (local slaughterhouse) and primary cortical human renal epithelial cells (HKC) from human biopsy material (K. Hochberg, Klinikum Konstanz, Konstanz, Germany) were prepared essentially according to Pollock (Pollock and Field, 1993). Briefly, cells were isolated by 30 min digestion in collagenase (1 mg/ml) at 37°C and dissociated by passing through nylon meshes. Following collection by centrifugation and resuspension in MEM/Dval/10% FCS/ to suppress fibroblast growth, cells were assessed for viability by standard trypan blue exclusion and seeded at a density of 2×10^4/ml in Primaria cell culture material with MEM/Dval/10% FCS/2000 U/l penicillin and 2 mg/l streptomycin sulfate and incubated in a humidified atmosphere at 37°C, 5% CO_2. Following 7 days in culture, the medium was replaced by DMEM/F12/10% FCS and then changed every 48 h until confluency was reached. Epithelial character was determined as described previously by us (Dietrich *et al.*, 2001). These cells were designated passage 0. Routine passaging was carried out using 0.025% trypsin/EDTA in PBS. All experiments involving primary cells were carried out using individual cell preparations generated from at least 3 different donors to ensure reproducibility and relevance of results. Primary cells of passage 1–2 and cell lines of passage < 20 only were used for experiments. Unless otherwise stated, cells were seeded at a density of 2×10^4 cells/ml and allowed a 24-h recovery before exposure.

Determination of toxic effects. Toxic effects (sum of antiproliferative, apoptotic, and necrotic effects) were assessed by use of a standard nuclei-counting procedure. Briefly, freshly trypsinized cells were seeded into multiwell plates. Sterile stock solutions of OTA and OTB were prepared in 7% sodium bicarbonate and serially diluted to yield final exposure concentrations of 1 nM–10 μM (OTA) and 10 nM–100 μM (OTB). Vehicle concentration never exceeded 1%. Following 48-h exposure to OTA or OTB nuclei counting was performed in duplicate by replacing the medium with 500 μl of crystal violet (0.2%, w/v) in citric acid (0.1 M). The cells were incubated with this nuclei-releasing and -staining solution for 90 min at 37°C. Stained nuclei were then counted using a haemocytometer. Results were expressed as percentage control $\pm$ SD ($n \geq 3$) and significant differences ($^*p < 0.05$; $^{**}p < 0.01$) calculated using ANOVA and Dunnett's post test. This method allows determination of cell numbers in a given population but does not however enable conclusions to be drawn on how the differences between treated or control cell numbers arise. Reductions in cell numbers following treatment could arise due to maintained proliferation coupled with increased apoptosis and/or necrosis, or via a cytostatic action. Therefore, although hereafter, the effects of OTA and OTB are referred to as "toxic," it should be noted that the actions of OTA and OTB could be of a cytotoxic or cytostatic nature.

Reversal of toxic effects. Cells were seeded in 25 cm^2 tissue culture flasks and exposed to 10 μM OTA or 80 μM OTB for 48 h (the respective concentrations above which, no further decrease in cell number could be observed in concentration/response assays). Medium was then removed and the cells washed 3 times in phosphate-buffered saline. One control and 1 treated flask were trypsinized and cell number determined using a haemocytometer. In the remaining flasks, full fresh medium was added and cell number determined 24 and 48 h subsequent to OTA/OTB removal. Results were expressed as the percentage of the respective 48-h control value $\pm$ SD ($n \geq 3$). Significant differences ($^*p < 0.05$; $^{**}p < 0.01$) were determined using the Tukey-Kramer multiple comparisons test.

Measurement of [^{3}H]-OTA uptake. OTA uptake was measured by addition of 1 μCi [^{3}H]-OTA per well to the culture medium. This concentration was selected as it had been demonstrated to have no effect in concentration-response experiments and also to be representative of normal serum OTA concentrations. At the indicated time points (10, 20, 30, 40, and 60 min), medium was removed and the cells were washed 3 times with ice-cold PBS to remove free [^{3}H]-OTA and terminate uptake. Cells were then solubilized using 2% SDS, acidified and mixed with Beckman Ready Safe scintillation cocktail. [^{3}H]-OTA uptake was determined by liquid scintillation counting (Beckman LS 6500 liquid scintillation counter). Results are expressed as dpm/cell $\pm$ SD ($n \geq 3$). A previous study, using the same source and specific activity of ^{3}H-OTA has previously determined the degradation rate of ^{3}H-OTA generated by tritium exchange, to be less than 2% over 21 days at 37°C (Studer-Rohr *et*

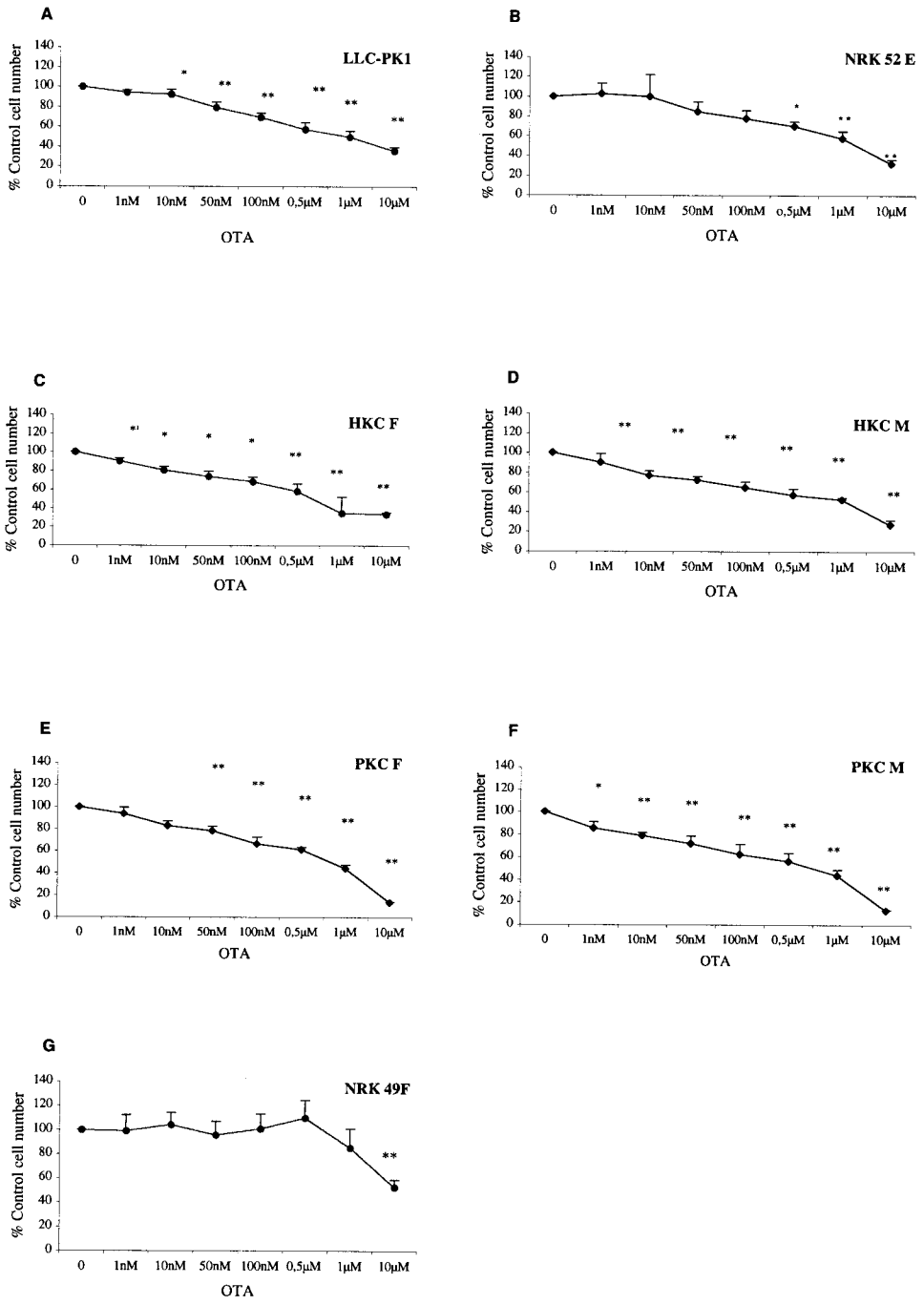

FIG. 2. (A–G) Toxic action of OTA in 7 cell types. Values are expressed as percentage control cell number and represent the mean ± SD. Those significantly different from the control (ANOVA and Dunnett's post test) are indicated, $*p < 0.05$; $**p < 0.01$. The 10 nM value does not appear significant using a Dunnett′s post test carried out on the entire data set. However, if control, 1 nM, and 10 nM OTA are compared, a significant difference is noted.

al., 2000). Thus, the random labelling of OTA is stable under the conditions of the uptake studies carried out here.

RESULTS

Concentration-Response Curves

Following 48-h exposure, OTA was observed to cause a concentration dependent reduction in cell numbers in all cell types tested except for the NRK-49F cell line (compared to their respective 48-h controls (Figs. 2A–2G). Established cell lines were noted to be considerably less sensitive to the effects of OTA than primary cells (Table 2). First significant effects were noted at 50, 500, 10, 10, 10, and 1 nM OTA for LLC-PK1, NRK-52E, HKC(f), HKC(m), PKC(f), and PKC(m), respectively, yielding a species/sex sensitivity rank order of pig male > pig female = human male/female >LLC-PK1 (pig) >NRK-52E (rat epithelial). A similar sensitivity rank order is apparent when the EC_{50}s are estimated from the concentration-

TABLE 1
Toxic Action of OTB in 7 Cell Types

	Cell type						
OTB conc.	LLC-PK1	NRK-52E	HKC(f)	HKC(m)	PKC(f)	PKC(m)	NRK-49F
10 nM	99.5 ± 2.4	97.6 ± 8.9	95.7 ± 6.6	87.9 ± 7.4	86.7 ± 6.6	97.9 ± 6.0	93.2 ± 8.8
100 nM	89.4 ± 5.9	89.9 ± 9.4	86.2 ± 4.6	78.6 ± 11.1	85.3 ± 13.7	87.7 ± 1.9**	93.2 ± 8.8
500 nM	88.0 ± 4.9**	84.6 ± 8.9*	81.1 ± 8.4	62.7 ± 4.9**	77.4 ± 10.0	76.5 ± 2.9**	92.7 ± 15.2
1 μM	78.4 ± 4.9**	79.3 ± 2.5**	77.8 ± 11.2*	54.8 ± 0.7**	71.6 ± 5.3*	71.0 ± 8.1**	86.4 ± 19.7
10 μM	72.0 ± 3.2**	61.4 ± 3.8**	57.7 ± 8.3**	34.6 ± 7.2**	46.4 ± 11.4**	41.0 ± 2.3**	68.1 ± 22.7
60 μM	56.4 ± 0.8**	25.3 ± 6.9**	41.0 ± 9.2**	27.8 ± 13.0**	29.4 ± 19.2**	24.9 ± 4.7**	31.5 ± 4.0**
100 μM	26.5 ± 1.6**	13.2 ± 2.9**	38.8 ± 11.1**	28.5 ± 17.3**	22.8 ± 18.2**	16.1 ± 5.7**	21.3 ± 5.4**

Note. Values are expressed as percentage control cell number following 48-h exposure and represent the mean ± SD. Those significantly different from the control (ANOVA and Dunnett's post test) are indicated.

*$p < 0.05$.

**$p < 0.01$.

response curves, except that the differences between the primary cell types are not as obvious. Interestingly, OTA did not affect cell numbers of the NRK-49F cell line. The first observable significant effects in NRK-49F cells were noted at a concentration of 10 μM (Fig. 2G). An EC_{50} was not reached within the concentration range tested.

OTB also elicited a concentration-dependent decrease in cell numbers in all epithelial cell types tested, however, at concentrations 5–10 times higher than observed for OTA (Table 1). First significant effects were noted at 0.5, 0.5, 1, 0.5, and 1 μM and 100 nM for LLC-PK1, NRK-52E, HKC(f), HKC(m), PKC(f), and PKC(m), respectively (Table 2), yielding a species/sex sensitivity rank order of pig male > LLC-PK1 = NRK-52E = human male = human female > pig female. Cells of type NRK-49F also displayed reduced proliferation following OTB exposure, with a first effect noted at 60 μM (Table 2). When the EC_{50}s were calculated, however, a different sensitivity rank order became apparent: pig female > pig male = human male > human female > NRK-52E > NRK 49F > LLC-PK1.

Reversibility of Toxic Effects

Following 48-h exposure to 10 μM OTA and subsequent replacement of full fresh medium, cell numbers significantly increased within 48 h of OTA removal in the LLC-PK1, NRK-52E, NRK-49F, and PKC(m) cell types (Table 3). In PKC(f) cells, although the observed increase in cell numbers was not significantly increased, a tendency toward renewed cell proliferation was apparent suggesting that longer recovery times may be required for these cells. HKC(m) and HKC(f) cells, however, displayed no tendency towards recovery within the time frame examined.

In contrast to the situation with OTA, only NRK-49F cells appeared to possess the potential to recover from the toxic effects of 80 μM OTB (Table 4). For all other cell types, OTB appeared to have an enduring effect that persisted more than 48 h following substance removal (Table 4) possibly suggesting a different mode of action to that of OTA.

[^{3}-H]-OTA Uptake

As apparent species and/or sex differences could be due to differences in the amount of OTA entering into the cells from the medium, the uptake of [^{3}H]-OTA into the various cell types was determined over time. Uptake remained constant over time for each individual cell type (Figs 3A–3E) suggesting that by 10 min a plateau or an equilibrium level had already been attained. The relatively large standard deviations observed in primary cell types are likely to have

TABLE 2
Summary of OTA- and OTB-Mediated Cytotoxic Effects

	LLCPK1	NRK52E	HKC(f)	HKC(m)	PKC(f)	PKC(m)	NRK49F
First effect OTA	50 nM	0.5 μM	10 nM	10 nM	10 nM	1 nM	10 μM
EC_{50} OTA	1 μM	5 μM	1 μM	0.7 μM	0.7 μM	0.7 μM	nd
First effect OTB	0.5 μM	0.5 μM	0.5 μM	0.5 μM	0.5 μM	100 nM	60 μM
EC_{50} OTB	70 μM	30 μM	30 μM	5 μM	1 μM	5 μM	40 μM

Note. Values represent the mean concentration at which the first significant reduction in cell number could be observed (first effect) and the concentration at which a 50% reduction in cell number was apparent (EC_{50}) following 48-h exposure. nd, not determined.

TABLE 3
Reversal of the Toxic Effect of OTA

Cell type	OTA exposure regimen: 48 h Exp.	48 h Exp. + 24 h free	48 h Exp. + 48 h free	Reversible
LLC-PK1	41.1 ± 8.7	44.1 ± 11.8	101.47.7*	Yes
NRK-52E	29.72	36.02 ± 4.1	56.6 ± 17.4*	Yes
HKC(f)	14.6 ± 1.2	14.0 ± 0.6	21.1 ± 2.18	No
HKC(m)	14.3 ± 2.2	6.6 ± 0.5	23.3 ± 10.0	No
PKC(f)	14.3 ± 2.2	15.9 ± 6.3	23.3 ± 10.0	No
PKC(m)	14.1 ± 4.03	10.5 ± 1.8	19.2 ± 2.0*	No
NRK-49F	71.6 ± 11.4	134.4 ± 37.6	168.6 ± 28.7**	Yes

Note. Values are expressed as mean percentage control cell number ± SD and those significantly different from 48-h exposure values (Tukey-Kramer's multiple comparison test) are indicated.

*$p < 0.05$.

**$p < 0.01$.

arisen from the interindividual variations in the donors. Although no statistically significant differences in OTA uptake were noted between the 7 cell types tested, probably due to these large standard deviations in primary cell types, a distinct rank order can be ascertained. Uptake was similar in all continuous cell lines, including the relatively OTA-insensitive NRK-49F cell line. Uptake was consistently 10 to 15-fold higher in primary cells than in continuous cell lines. HKC(f) cells consistently accumulated higher amounts of OTA per cell than any other cell type. The rank order for OTA uptake was HKC(f) > HKC(m) > PKC(m) > PKC(f) >> NRK-49F = NRK-52E = LLC-PK1. The nature of this uptake (i.e., active via a transporter molecule, facilitated diffusion, or via simple osmosis) is currently under investigation.

DISCUSSION

OTA has been demonstrated to be a potent renal toxin in a number of species including rats (Boorman, 1989), mice (Bendele *et al.*, 1985), and pigs (Krogh *et al.*, 1974, 1976) following administration or dietary intake. Furthermore, OTA has been implicated as a potential factor in the observed increased incidences of urothelial tumors and nephropathy endemic for some areas of the Balkan states (Hult *et al.*, 1982; Nikolov *et al.*, 1996; Tatu *et al.*, 1998). Despite intensive research at both the epidemiological and the experimental/mechanistic levels, the mode of action of OTA and its causal association to BEN and urothelial tumors remain unclear. OTA serum levels in humans vary considerably, depending on, amongst other influences, culture, socioeconomic situation, and dietary habits (Kuiper-Goodman *et al.*, 1993; Studer-Rohr 1995; Studer-Rohr *et al.*, 2000). Several studies have reported OTA serum levels in BEN areas to vary between 0.9 and 100 nM (Hult *et al.*, 1982; Peraica *et al.*, 1999; Petkova-Bocharova *et al.*, 1988). These levels are 7 to 30-fold higher than those reported for nonendemic areas of the Balkans and for other European and North American countries (Frohlich *et al.*, 1991; Hald, 1991; Nikolov *et al.*, 1996; Peraica *et al.*, 1999). A more recent report on the exposure of various age groups to OTA in food and foodstuffs in Germany (Wolff *et al.*, 2000), based on OTA blood levels and food analyses, suggested that infants and young children face a higher level of exposure than adults. Furthermore, the fact that the elimination half-life of OTA in humans, with approximately 36 days (Studer-Rohr *et al.*, 2000), is considerably longer than noted in other mammalian species (mice, 40 h; rats, 55–120 h [Ballinger *et al.*, 1986; Galtier *et al.*, 1979; Hagelberg *et al.*, 1989]; pigs, 72–120 h [Galtier *et al.*, 1981; Mortensen *et al.*, 1983]; monkeys, 820 h [Kuiper-Goodman and Scott, 1989]), suggests that OTA constitutes a higher risk in humans. The OTA concentrations used for the *in vitro* experiments presented here were comparable to the levels of OTA found in the serum of patients afflicted with BEN or UT in the endemic areas. All cells of renal epithelial origin responded to 48 h exposure to OTA in the low nM range for with a concentration-dependent decrease in cell numbers. This could be due to a cytostatic or cytotoxic effect or indeed to an increase in apoptosis or necrosis. This is in agreement with and an extension of the work of Schwerdt and coworkers who demonstrated OTA to induce apoptosis at nM concentrations in immortalized human (IHKE) cells (Schwerdt *et al.*, 1999) and in dedifferentiated MDCK-C7 continuous cell lines (Gekle *et al.*, 2000). It should be noted however, that these effects were obtained in the absence of serum, in previously transformed cells or following pretreatment with hydroxyurea (Dopp *et al.*, 1999; Dörrenhaus *et al.*, 2000; Dörrenhaus and Föllmann, 1997; Gekle *et al.*, 1998a, 2000). In contrast, the observations of the study presented here were obtained in both cell lines and in primary cells and furthermore, in the presence

TABLE 4
Reversal of Toxic Effects of OTB

Cell type	OTB exposure regimen: 48h Exp	48h Exp. + 24h free	48h Exp. + 48h free	Reversible
LLC-PK1	17.9 ± 7.3	10.5 ± 3.7	17.4 ± 9.1	No
NRK-52E	18.8 ± 2.7	16.5 ± 3.1	16.1 ± 4.0	No
HKC(f)	24.3 ± 8.8	11.3 ± 5.5	17.9 ± 10.6	No
HKC(m)	14.6 ± 0.7	10.8 ± 0.4	6.6 ± 0.3	No
PKC(f)	7.8 ± 1.0	5.4 ± 1.3	6.8 ± 2.2	No
PKC(m)	9.2 ± 2.4	4.0 ± 1.2	7.5 ± 2.7	No
NRK-49F	26.1 ± 1.2	41.0 ± 3.18**	78.1 ± 3.0**	Yes

Note. Values are expressed as mean percentage control cell number ± SD and those significantly different from 48h exposure values (Tukey-Kramer's multiple comparison test) are indicated.

*$p < 0.05$.

**$p < 0.01$.

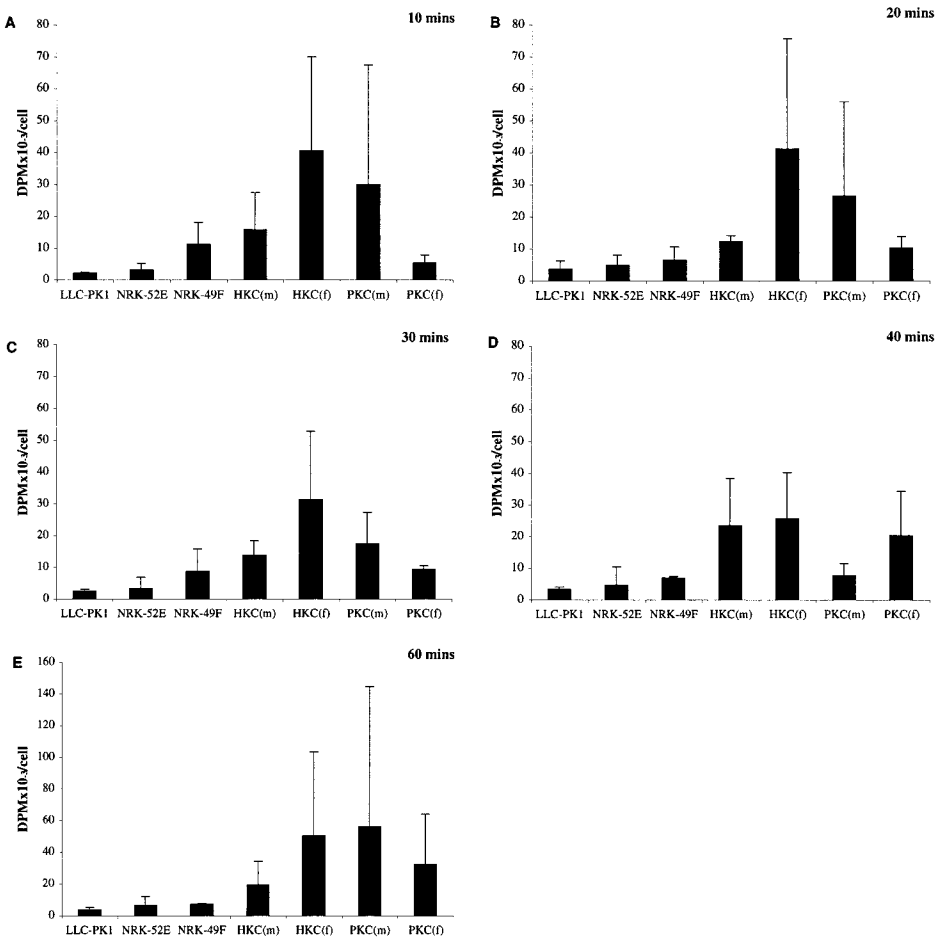

FIG. 3. (A–E) Uptake of [^{3}H]-OTA in 7 cell types. Values represent the mean ± SD.

of serum. Variations in metabolism and detoxification of OTA were not investigated in this study but are unlikely to account for the observed toxicity differences between cell types as the hydrolysis of OTA to OTAα *in vitro* by kidney and liver homogenates has been demonstrated to be insignificant (Madhyastha *et al.,* 1992; Stormer *et al.,* 1983). Previously observed effects on phenylalanine metabolism are also unlikely to be involved in the differences noted in this sudy, as these effects first become apparent following exposure to μM concentrations of OTA (Creppy *et al.,* 1995).

The results obtained in the current study strongly suggest that low nM OTA (but not OTB) concentrations in serum could elicit a "toxic" response in porcine and human primary renal epithelial but not mesenchymal (fibroblastic) cells, whereas rat primary cells and continuous pig epithelial cell lines are less susceptible to OTA-induced effects (Heussner *et al.,* 2000). These observations suggest a succinct nephrotoxic activity of OTA primarily in humans and also in pigs. The effects observed in primary cells in this study were consistent in cells from several donors, suggesting them to represent a generalized phenomenon rather than idiosyncratic actions specific for 1 individual. The latter interpretation is further corroborated by the observation that tissue homogenates from the human renal cortex display a higher binding affinity and capacity for [^{3}H]-OTA than the respective tissue homogenates from rat, mouse, or pig (Dietrich *et al.,* 2000). This specific binding appears unrelated to previously proposed organic transport systems for OTA (Groves *et al.,* 1998, 1999).

Concentration-response curves for OTB versus cell number were in agreement with previously published results, which indicate OTB to be a factor 10–100 less toxic than OTA (Heussner *et al.,* 1998, 2000; Rahimtula *et al.,* 1988; Schwöbel *et al.,* 1999). This supports the suggestion made by Xiao *et al.* (1996) that the toxic effects of OTA are mediated via a strict structure-activity relationship.

The methods employed in this study are simple and do not allow differentiation between inhibition of cell proliferation (cytostasis), apoptosis, and necrosis. Fundamental differences between cell types in their response to OTA exposure can however be delineated. In our laboratory, necrosis has been observed in cultured primary and transformed cells at medium to high OTA concentrations only (Dietrich *et al.,* 2001; Heussner *et al.,* 2000). Apoptosis (Gekle *et al.,* 2000; Seegers, 1994) appears to prevail in the low to medium concentration range

while cytostasis and/or cellular dedifferentiation appears to predominate at very low concentrations (Heussner *et al.*, 2000). This interpretation is supported by the fact that pancreatic epithelial cells exposed to 2,3-dimethoxy-1,4-naphtoquinone demonstrated similar dose-dependent influence on cell growth, apoptosis, and necrosis (Dypbukt *et al.*, 1994). Indeed, nanomolar OTA concentrations appear to have a cytostatic effect in NRK-52E, LLC-PK1, and PKC(m) cell types. This cytostatic effect of OTA appears to be located to the G2 phase of the cell cycle (Dreger *et al.*, 2000). A G2/M specific cell cycle arrest is also suggested by the marked appearance of giant nuclei in P3 epithelial cells of rats (Maaroufi *et al.*, 1999; Rásonyi *et al.*, 1999) and pigs (Krogh *et al.*, 1976) treated with moderate OTA concentrations *in vivo* and in BEN patients with urothelial tumors (Godin *et al.*, 1997; Maaroufi *et al.*, 1995a,b). Such a G2/M specific block would most likely occur subsequent to the inhibition or over-expression of a particular protein or gene ("molecular switch"), which needs to be either resynthesized or degraded, before the cells can begin to proliferate. This hypothesis is supported by the observation that with the exception of primary human cells from both sexes, the effects of OTA exposure were reversible in all cells, albeit coupled with a distinct lag-phase of 48 h (a significant increase in cell numbers was observed 48 h following OTA removal). The nature of the molecular "switch" mentioned above remains to be elucidated and deserves further attention. Possible candidates could be various members of the CDK family and their associated cyclins, particularly A and B, which are known to regulate G2/M transition, possibly in association with components of the PKC family that have also been shown to be closely associated with G2/M transition in vascular epithelial cells (Kosaka *et al.*, 1996).

As the differences observed in the OTA-mediated inhibition of proliferation could be due to differences in the amount of OTA incorporated into the cells, this was investigated using [^{3}H]-OTA. Uptake remained relatively constant throughout the assay period, indicating the attainment of a plateau level within 10 min. Similar findings have been reported by Schwerdt and coworkers (Schwerdt *et al.*, 1998) and by Tsuda *et al*. (1999). No statistically significant differences between the cell types were observed for [^{3}H]-OTA uptake. Differences, however, were apparent between the uptake into continuous cell lines and primary cells from both pigs and humans. More OTA-sensitive cell types (primary human and porcine) consistently accumulated more radiolabeled OTA. The only exception to this was the NRK-49F cell line. These fibroblasts accumulated similar concentrations of OTA as their epithelial counterparts the NRK-52E cell line, but were not as susceptible to OTA-mediated effects as the epithelial cells. Thus, the decisive factor in cellular response to OTA exposure does not appear to be solely the amount of OTA gaining access to the cells, but how individual cell types process their respective OTA load. Conceivably NRK-49F cells and possibly fibroblasts in general, may possess a protein capable of neutralizing the effects of OTA or conversely, do not express the "molecular switch" apparently involved in OTA-mediated toxicity in epithelial cells. The latter observations lend support to the theory that BEN could be caused by a cytostatic/cytotoxic effect of OTA predominantly in epithelial cells, while fibroblasts are more refractive. In consequence this would lead to increased/maintained fibroblast proliferation coupled with reduced epithelial cell proliferation and/or epithelial cell death and hence the gradual replacement of healthy with fibrotic tissue and the characteristic onset of nephropathy. The observation that primary human cells from both sexes did not resume cell growth following OTA exposure strengthens the likelihood of the epithelial replacement by fibroblastic tissue and stresses further a causal involvement of OTA in the etiology of BEN and karyomegalic interstitial nephropathy.

In contrast, none of the cell types resumed proliferation following 48 h exposure to OTB suggesting that OTA and OTB, although almost identical in structure (see Figure 1), have some fundamental difference in their mechanism of action (Xiao *et al.*, 1996).

The fact that higher (high nM to low μM) concentrations or longer exposure times (96 h) to OTA does not further reduce cell numbers (Dreger *et al.*, 2000) suggests the presence of a subpopulation of cells resistant to OTA. Selection of such a subpopulation has previously been described by Achanzar and coworkers in prostate epithelial cells exposed to cadmium (Achanzar *et al.*, 2000). These authors suggested that cadmium selected for apoptotic-defective prostate epithelial cells via direct or indirect effects on the expression of c-*myc*, p53, PCNA, and c-*jun*. Selection of an apoptotic-defective subpopulation was considered one of the primary steps involved in cadmium-induced tumor formation. If indeed low nM OTA concentrations, as used in the experiments discussed here and present in the serum of the populations in the areas endemic for BEN and UT, are capable of selecting apoptotic-defective epithelial cells, this subpopulation could be the long-sought link between OTA exposure and observed tumor formation. The latter hypothesis is currently under investigation in our laboratory using molecular methods.

In conclusion, the results presented are as yet, preliminary in nature, however, they provide support for the selection of primary renal epithelial cells rather than continuous/transformed cell lines mechanistic investigations on OTA-induced carcinogenesis and nephropathies. Furthermore, the data indicate ochratoxins to be toxic *in vitro* at dietary relevant concentrations and in the presence of serum

Future directions should include an analysis of the cell cycle events leading to cytostasis and cell differentiation/dedifferentiation, together with the expression levels of such factors as c-*myc*, p53, c-*jun* and PCNA, the CDK family and their associated cyclins and components of the PKC family following exposure to both OTA and OTB.

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