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Structures of Covalent Adducts between DNA and Ochratoxin A: A New Factor in Debate about Genotoxicity and Human Risk Assessment

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The potent renal carcinogenicity of ochratoxin A (OTA) in rats, principally in the male, raises questions about mechanism. Chromatographic evidence of DNA adducts after 32P-postlabeling analysis contrasts with experimental attempts to demonstrate the absence of OTA in such adducts. Proffered schemes for alternative epigenetic mechanisms in OTA carcinogenicity remain unsatisfying, while structural data substantiating DNA-OTA adducts has also been lacking. We report refined 32P-postlabeling methodology revealing one principal adduct isolated in small amounts from the kidneys of all five Fischer and five Dark Agouti rats to which OTA had been given on four consecutive days. We also describe structural data for the principal adduct from OTA/DNA interaction in vitro and its subsequent preparative isolation by the postlabeling methodology (as C-C8 OTA 3’dGMP), essentially creating an ochratoxin B–guanine adduct. Reasoning for the unsuitability of experimental protocols in published evidence claiming nongenotoxicity of OTA is given. In vivo exposure of renal DNA to cycles of adduction with OTA, necessarily protracted for carcinogenesis to occur, can reasonably explain an occasional focal neoplasm from which metastasizing carcinoma could develop.

Introduction

Whether the nephrotoxic mycotoxin ochratoxin A (OTA) exerts its potent nephrocarcinogenicity in male rats by direct interaction with DNA has long been a matter of debate. First positive claims came from the application of 32P-postlabeling methodology to DNA isolated from mice given OTA (1). Autoradiographic evidence of radiolabeled compounds separated after contact transfer on two-dimensional chromatograms was proposed as showing that OTA had made covalent bonds with nucleotides in vivo. Subsequently, postlabeling methodology has consistently detected adduct spots on chromatograms which were attributed to exposure of rats, mice, and pigs to OTA in laboratories in Strasbourg, Bordeaux, Lyon (IARC) and Toulouse. Little or no evidence for this conclusion was experienced in other laboratories (e.g., see ref 2), thus fueling the current reluctance to accept positive postlabeling evidence as an indicator of genotoxicity of OTA (3). Academic debate about whether or not OTA conforms to the current definition of a genotoxin might have rested as a minor unresolved esoteric controversy if it were not for concerns that experimental carcinogenicity in male rats and mice pointed to the possibility that OTA could be a human carcinogen (4–6). Clear demonstration that a chemical carcinogen is a genotoxin influences legislative attitude, whereby a 10-fold reduced human risk differential may be applied when assessing the extent of exposure to a nongenotoxic carcinogen rather than to a carcinogen concerning which adducts of defined structure are known. Food and drinks industries with commercial interests in the significance of natural and largely unavoidable traces of OTA in some agricultural commodities, and a need to comply with statutory limits, will naturally prefer that OTA is shown not to be a genotoxin.

In recent years, several publications consistently claiming the nonexistence of direct OTA/DNA adducts have been noted (summarized in ref 7); concurrently, several other publications have claimed the opposite (summarized in refs 8 and 9). However, there has been little critical appraisal of the experimental material used in publications claiming that DNA/OTA covalent adducts do not occur. Meanwhile, we have addressed the difficult challenge of obtaining structural data relating to rat kidney DNA adducts. Demonstration of the structure of a principal DNA/OTA adduct, prepared in vitro from calf thymus DNA and isolated preparatively via postlabeling methodology, here extends proof of covalent binding of OTA on guanine beyond the quite compelling cochromatographic implication described in ref 10.

Materials and Methods

Rats and OTA. Ten 8-week old males of the Fischer and Dark Agouti strains (B & K Universal Ltd., Grimston, Hull, UK) were ~220 and 180 g, respectively, and were maintained on standard (14.4% protein) diet (SDS Services, Witham, Essex, UK).

Design of OTA dosing strategy was based on previous experience (11) of short-term administration with only mild clinical effects and best incidence of renal DNA adducts achieved through daily oral gavage. OTA (77 mg) was dissolved in ethanol (1.2 mL) and
the solution made up to 20 mL with 3% w/v NaHCO₃ in water. Then 0.4 mL (1.5 mg OTA) was given from this solution to each of seven rats, at the same time of day by oral gavage on three consecutive days. The daily dose was therefore 8.3 and 6.8 mg/kg b.w. for Dark Agouti and Fischer rats, respectively. Controls were given 0.4 mL of vehicle (5% ethanol in 3% aqueous NaHCO₃). Twenty-four hours after the last dose, four treated rats from each strain and all controls were euthanized, and blood was withdrawn from the heart. The kidneys were excised, separated into quarters, flash frozen in liquid N₂, transferred into separate Eppendorf tubes, and stored at −20 °C. Blood was centrifuged immediately and plasma separated for quantitative OTA analysis at the Central Science Laboratory, York, UK. The mean weights of the remaining three OTA-treated Fischer and Dark Agouti rats were 310.7 ± 12.1 g and 261.7 ± 10 g, respectively, 5 weeks after the first OTA dose. After 3 months (370 ± 17.3 g and 278.3 ± 4.7 g, respectively), the rats were euthanized and the blood collected as before. A narrow central transverse section was removed from each kidney for H & E-stained histology and the remaining tissues flash frozen in liquid N₂ and stored at −20 °C pending ³²P-postlabeling analysis.

All kidney samples were coded for blind analysis for adducts, to avoid suggestion of any analytical bias, and transferred personally by air in dry ice between London and Toulouse.

**DNA Extraction and Postlabeling Separation of DNA Adducts. Materials.** The following enzymes [proteinase K (used as received), RNase A, RNase T1 (boiled 10 min at 100 °C to destroy DNases), and micrococcal nuclease (dialyzed against deionized water)] were purchased from Sigma (Saint Quentin Fallavier, France); spleen phosphodiesterase (centrifuged before use) was from Calbiochem (VWR, France), and nuclease P1 (NP1) (20 mg/mL) and T4 polynucleotide kinase were from Roche diagnostics (Meylan, France). T4 polynucleotide kinase [specific activity 6000 Ci/mmol] was added and the mixture incubated at 37 °C for 45 min. The reaction was stopped by the addition of 3 mL of Tris base 500 mM. The DNA adducts were labeled as follows: to the nuclease P1 digest, 5 µL of the reaction mixture containing 2 µL of bicine buffer [bicine (800 µM), diithiothreitol (400 mM), MgCl₂ (400 mM), and spermidine (400 mM)] adjusted to pH 9.8 with NaOH] and 10 U of polynucleotide kinase T4, and 100 µCi of [³²P]ATP (specific activity 6000 Ci/mmol) was added and the mixture incubated at 37 °C for 45 min. Normal nucleotides, pyrophosphate, and excess ATP were removed by chromatography on PEI cellulose TLC plates (D1) in 3 M NaH₂PO₄ buffer, pH 5.7, overnight. The origin (2 cm) areas containing labeled adducted nucleotides were cut out and transferred to another PEI cellulose TLC plate, which was run (D2) in 4.8 M lithium formate and 7.7 M urea, pH 3.5, for 3 h. A further (D3) migration was performed after turning the plate 90° anticlockwise in 0.6 M NaH₂PO₄ and 5.95 M urea, pH 6.4, for 3 h. Finally, the chromatogram was washed in the same direction in 1.7 M NaH₂PO₄, pH 6, for 2 h (D4). Adduct profiles were analyzed qualitatively and semiquantitatively by autoradiography of the plates, carried out at −80 °C for 48 h in the presence of an intensifying screen, using a radio-analytical system of image analysis (AMBIS, Lablogic). In parallel to the adduct analysis, a series of controls were performed to verify the labeling efficiency of adducts and the efficiency of dephosphorylation of the nuclease P1. Refinement of adduct separation by use of a 3 M phosphate buffer, instead of the former 2.3 M buffer, in the D1 migration, and subsequent transfer of only 2 cm of the origin, is shown in Figure 1. Steps in the refined procedure to resolve two of our synthetic model adducts are illustrated in Figure 2.

**Synthesis of OTA-dG and OTA-3′-dGMP.** A sample of the OTA-dG nucleoside adduct was prepared from the photoreaction of OTA in the presence of excess dG, as outlined previously (12, 13). A reaction mixture (1 mL total volume) of 500 µM OTA and 40 mol equiv of dG in a mixture of 25 vol. % DMSO and 75 vol. % 100 mM aqueous phosphate buffer (pH 7.4) was irradiated at 350 nm for 15 min using a Rayonet Chamber Reactor, Model RPR-200. The OTA-dG nucleoside adduct was isolated using an Agilent 1200 series HPLC equipped with an autosampler, auto-collector, and diode array detector, and a mobile phase consisting of 40 mM ammonium formate (pH 6.1)/acetonitrile and a semi-preparative Phenomenex Kromasil C8 column (10 mm × 250 mm) at a flow rate of 5 mL/min. The isolated sample of OTA-dG had MS and UV–vis characteristics identical to those previously reported (12, 13).

The protocol for the photochemical synthesis of OTA-3′-dGMP was the same as that outlined previously (10). A reaction mixture (1 mL total volume) of 100 mM OTA and 50 mol equiv of 3′-dGMP in 100 mM phosphate buffer (pH 7.4) was irradiated for 2
min at 350 nm. The isolated OTA-3′-dGMP adduct had MS and UV-vis characteristics identical to those reported previously (10) and was used as a postlabeling standard.

Photoreaction of OTA with DNA. A reaction mixture (1 mL total volume) of 500 µM OTA and 100 µg of calf thymus DNA (repurified) in a mixture of 10 vol. % DMSO and 90 vol. % 100 mM aqueous phosphate buffer (pH 7.0) was irradiated at 350 nm for 13 min using a Rayonet Chamber Reactor, Model RPR-200. The sample was then divided into four 250 µL fractions, and 1 mL of 100% ethyl alcohol was added to each fraction in microcentrifuge tubes. After incubation at −20 °C for 10 h, samples were centrifuged for 15 min at 12000 rpm and 6 °C in an AccuSpin centrifuge. The DNA pellet was washed with 70% ethanol, then dried under vacuum and sent to the Pfohl-Leszkowicz laboratory for 32P-postlabeling analysis and preparative isolation of two prominent adducts.

Preparative Isolation of Adducts from the Photoreaction of OTA and DNA. The protocol for postlabeling was exactly the same as that for rat DNA, except that unlabeled ATP was used instead of that radiolabeled with 32P. For postlabeling of 10 µg of DNA, 20 pmol of ATP was used. After postlabeling, a preparative chromatogram was prepared in which typically (Figure 3) were applied radioactive spots of our standard C-C8 adduct, products of the calf thymus DNA−OTA interaction postlabeled with 32P ATP, our standard O-C8, and a mixture of C-C8 and O-C8 adducts. In the central region of the plate, a stripe of products of the calf thymus DNA−OTA interaction only with unlabeled ATP was applied. Chromatography in the D1 direction was run with NaH2PO4 (2.3 M, pH 5.7) for 16 h. The plate was washed twice by soaking in ultrapure water, dried, and autoradiographed. Cellulose in the central area corresponding to spots of interest was scraped off, the cellulose suspended in methanol, shaken, centrifuged, the solution, and the extraction cycle repeated. Residual cellulose was then similarly extracted with triethanolamine. Combined extracts were evaporated to dryness in vacuo. The preparative eluates from spots 1 and 2 (Figure 3) were sent, blind as samples 1 and 2, respectively, for LC-MS analysis.

Chemicals for HPLC/MS Analysis of Adducts. OTA from Aspergillus ochraceus was purchased from Sigma-Aldrich Co. (Dorset, UK), formic acid was purchased from Perbio Science (Northumberland, UK), HPLC grade acetonitrile was purchased from Fisher Scientific (Loughborough, UK), and HPLC grade water was produced by an Elga Maxima Ultra Pure Water system (Buckinghamshire, UK). All solvents and chemical standards were of the highest grade commercially available. Authentic standards of C-C8 OTA 3′-dGMP (Figure 4) and O-C8 OTA 3′-dGMP (Figure 5), and OTA−DNA samples were synthesized in Guelph.

Buffers, Reagents, and Standards. A 100 µg/mL MS standard of OTA in 50:50 acetonitrile/water was prepared by diluting a 10 µL of a 1 mg/mL solution of OTA in water with 990 µL of 50:50 acetonitrile/water. A 0.1% formic acid solution was prepared by diluting 1 µL of concentrated formic acid with 999 µL of water.

Method Development of MS Analysis of Model Synthetic Adducts in Preparation for the Analysis of Products of in Vitro OTA−DNA Interaction. Authentic synthetic standards of model OTA−guanine compounds, C-C8 OTA 3’-dGMP, and O-C8 OTA 3’-dGMP were used to develop a suitable HPLC/MS method.

Figure 2. Autoradiographic illustration of steps in chromatographic resolution of two of our model synthetic adducts after 32P-postlabeling, showing discrete separation resolved from stray radioactivity after a second D4 step. Transition from D1-D2 was by contact transfer of the radioactive region after the D1 step.

Figure 3. Annotated autoradiograph of a model chromatogram (D1 separation) illustrating the preparative isolation of samples 1 and 2 used for structural analysis. Lane 1 is synthetic C-C8dG-OTA processed via standard 32P-postlabeling. Lanes 2 and 3 are products of calf thymus DNA/OTA photolysis processed via standard 32P-postlabeling to guide preparative excision of unlabeled compounds from the central region. Lane 4 is synthetic O-C8dG-OTA also processed via 32P-postlabeling. It was necessary to avoid 32P contamination of samples for LC-MS.
and then reconditioned at 5% B for 10 min. and 95% B in 15 min. The column was kept at 95% B for 5 min. The samples were separated using a gradient elution between 5% (solvent A) and acetonitrile (solvent B) were used as mobile phases. Thermo Fisher Scientific, Waltham, USA); 0.1% formic acid MS performance. HPLC/MSn analysis was performed on an Agilent column (Thermo Hypersil Gold 5 µm, 4.6 mm i.d. x 250 mm, Thermo Fisher Scientific, Waltham, USA); 0.1% formic acid (solvent A) and acetonitrile (solvent B) were used as mobile phases. The samples were separated using a gradient elution between 5% and 95% B in 15 min. The column was kept at 95% B for 5 min and then reconditioned at 5% B for 10 min.

For all analytes, the instrument was tuned to give the best possible MS performance. HPLC/MSn analysis was performed on an Agilent 1100 HPLC system (Santa Clara, USA) consisting of a binary pump and column thermostat, coupled to a Waters QToF F Premier mass spectrometer (Manchester, UK) equipped with an electrospray ionization (ESI) source with separate Lockmass interface and operated in negative ion mode. Samples were introduced to the HPLC via a CTC-Analytics PAL autosampler (Zwingen, Switzerland) consisting of a 100 µL loop. The autosampler, HPLC system, and mass spectrometer were controlled using Waters software MassLynx version 4.1 (Manchester, UK). Calibration over the range m/z 50–1000 was carried out using a NaI/CsI calibration standard purchased from Waters. MS tuning was performed using direct infusion of a 10 µg/mL standard solution of OTA, at a flow rate of 10 µL/min, using the integrated instrument syringe pump. The following mass spectrometer conditions were common to all LC/MS experiments performed: capillary voltage, 1.5 kV; sampling cone, 35 V; source temperature, 140 °C; desolvation temperature, 350 °C; cone gas flow, 50 L/h; desolvation gas flow, 400 L/h; and LM and HM resolution, 15 arbitrary units. A 1 µg/mL solution of leucine enkephalin (m/z 554.2615) in 50:50 acetonitrile/water was pumped into the lockmass interface at a rate of 20 µL/min using a dedicated HPLC pump (Shimadzu UK Limited, Manchester, UK). Lockmass data was collected with a scan time of 0.5 at 10 s intervals and averaged over 10 scans. Mass spectral data was collected in MS centroid mode with a scan time of 1 s and interscan time of 0.02 s, over a mass range of m/z 100–1000.

All samples were pretreated with concentrated formic acid (50 µL), vortex mixed, and heated on a heating block at 80 °C for 60 min. The samples were then reduced to dryness under N₂ gas, resuspended in 100 µL of 0.1% formic acid (aqueous), and vortex mixed. Ten microliters of each sample was injected onto a C18 column (Thermo Hypersil Gold 5 µm, 4.6 mm i.d. x 250 mm, Thermo Fisher Scientific, Waltham, USA); 0.1% formic acid (solvent A) and acetonitrile (solvent B) were used as mobile phases. The samples were separated using a gradient elution between 5% and 95% B in 15 min. The column was kept at 95% B for 5 min and then reconditioned at 5% B for 10 min.

**Results**

**Pharmacokinetics in Rats Treated with OTA.** Plasma OTA concentration in the four Fischer rats treated with OTA was 45.7 ± 2.6 µg/mL and in the Dark Agoutis was 36.8 ± 6.4 µg/mL. At first sight, the lower value in the smaller rats given the same amount of OTA appears incongruous. Purely on a body weight basis, the plasma concentration in the Dark Agoutis might have been ~56 µg/mL. Subsequent discovery that Dark Agoutis have much shorter plasma OTA half-life than that of Fischer rats (14) can account for the apparent disparity in accumulation in plasma after the three consecutive doses. However, substantial potential for delivery of OTA via vascular circulation to kidneys in both rat strains was demonstrated. Histology of kidneys from animals euthanized 3 months after giving OTA showed sparse karyomegalgy diffused across the outer stripe of the outer medulla, consistent in our experience with a typical response in renal tubule epithelia to circulating OTA.

**Results of Postlabeling Analysis.** Autoradiographs relating to the kidney of each animal were captured electronically from Toulouse to London for matching to the blind code. No spots were present in samples from control (untreated) rats or from treated rats euthanized 3 months after the administration of OTA. This applied to all replicates of both strains of rats. In contrast, for the kidneys of all OTA-treated rats analyzed after the three consecutive doses, one or two radioactive spots were present in otherwise very clean autoradiographs of chromatograms, indicating the usual ³²P-postlabeling evidence of adducted DNA arising from consistent delivery of OTA to nephron epithelia (Figure 6). Notably, there was close replication of the strain-specific pattern of DNA adducts. In Fischer rats, there was predominantly one adduct, while in Dark Agoutis the same adduct was evident together with a minor component with slightly greater chromatographic mobility. The abundance of the principal adduct across the 10 animals was within the range of 20–70 adducts per 10⁶ nucleotides.

**MS Analysis.** Preparative isolation of two adducts from in vitro interaction between OTA and DNA, occurring in approximately equal amounts, provided material for LC-MS analysis coded only as samples 1 and 2. An approximate limit of detection of 1 ng of material on column had been determined during method development.

No peaks were identified in sample 1 at mass m/z 713, the expected mass of the C-C8 OTA 3’dGMP adduct. However, a prominent peak was identified in the m/z 517 extracted ion chromatogram at 11.7 min. The mass spectrum under the peak is shown in Figure 7. The base peak ion at m/z 517 corresponded to the loss of the phosphate conjugated deoxyribose sugar moiety from the C-C8 OTA 3’dGMP, which is consistent with hydrolysis due to the addition of formic acid in the sample pretreatment step.

Extracted ion chromatograms of m/z 517 for samples 1 and 2, together with the analysis of the authentic standard of C-C8 OTA 3’dGMP cochromatographed at 11.7 min, confirming the deconjugated aglycone moiety, were present in all three analyses (Figure 8).

The mass spectrum under the peak at 11.7 min in OTA-DNA sample 2 is shown in Figure 9.

The peak at m/z 517 corresponds to the loss of the phosphate conjugated deoxyribose sugar moiety from the C-C8 OTA 3’dGMP as seen for OTA-DNA sample 1. However, additional adduct related ions are also observed in the spectrum. The ion m/z 655 corresponds to the sodium adduct of the C-C8 OTA 3’dGMP which has lost the phosphate conjugate but still has
the deoxyribose sugar moiety intact (Figure 10a). The ion at m/z 815 represents the sodium adduct of the 3′,5′-bisphosphate OTA-dGMP, showing additional phosphate conjugation on the 5′ hydroxyl group of the deoxyribose sugar (Figure 10b).

The structure of the deconjugated aglycone observed in OTA-DNA sample 2 was confirmed by MS/MS analysis of the precursor ion m/z 517 (Figure 11). MS/MS data was generated on the Waters QT of Premier in continuum mode over the mass range m/z 100–600 at a collision energy of 25 eV, a scan time of 1 s, and an interscan time of 0.02 s.

The ions m/z 473 and m/z 429 represent the loss of the carboxylic acid group as CO₂, with a further loss of CO₂ from the lactone moiety, thus confirming the presence of the OTA adduct in OTA-DNA sample 2. Matching of analytical findings to the blind sample code showed sample 1 to be C-C8 OTA 3′dGMP and sample 2 to be its triphosphorylated homologue.

Discussion

The present findings have taken the search for definitive evidence on the structures of adducts, revealed consistently in the Pfohl–Leszkowicz laboratory by ³²P-postlabeling in renal tissue after exposure to OTA, as far as possible with limited research resources. Limited resource of renal tissue precluded preparative isolation for LC-MS of sufficient amounts of either of the two key adducts revealed only by the 2D chromatography of ³²P-postlabeling after contact transfer. At least 1 ng would
be needed on the column and would require \( \sim 100 \) rats. The present finding confirms previous recognition of C-C8-dG-OTA as an in vivo DNA-OTA adduct by cochromatography in a 2D system after contact transfer with an authentic standard (10).

We also show (Figure 12) similarly consistent chromatographic behavior through five sequential steps in two dimensions for C-C8-dG-OTA as a product of DNA-OTA interaction both in vitro and in vivo. However, research effort may better be focused on understanding the genetic changes in rat renal tumors caused by OTA and the mechanism for marked gender difference in sensitivity and pharmacokinetics (14, 15). Approximately \( 10^{18} \) molecules of OTA necessarily pass via a male rat’s kidneys during several months to cause any renal cancer (16).

The experimental design described in ref 17 is presumably based on previous OTA toxicokinetics (18) in which a single dose (500 \( \mu \)g/kg body weight) was given by oral gavage. For male rats in that study, assuming that the quoted rat weight (\( \sim 250 \) g) is more reliable than the stated 8 week age (cf. Charles River Web site for Fischer rats), approximately 130 \( \mu \)g of OTA had been given per os in 500 \( \mu \)L of corn oil. Maximum concentration of OTA in blood, measured in plasma 48 h later, had reached \( \sim 1.8 \) \( \mu \)g/mL, corresponding to \( \sim 0.1 \) \( \mu \)g/g measured in kidney from which blood had been flushed by syringing with physiological saline after excision. Thus, in a rat given 130 \( \mu \)g of OTA, less than 0.2% was actually found resident in its kidneys. If blood containing 1.8 \( \mu \)g OTA/mL of the plasma component circulates through the kidney in which blood constitutes 20–25% of tissue (19), OTA concentration in the organ would be \( \sim 0.4 \) \( \mu \)g/g. Since removal of blood from the kidney can only be achieved efficiently, particularly from the peritubular capillaries, by perfusion in situ under normal blood pressure (19), the concentration of OTA in nonvascular tissue within the kidney would have been considerably less than the 0.2% of the given dose recorded in ref 18. The similar dosing of three adult male rats in ref 17, with assumed weight of \( \sim 250 \) g and each rat given \( \sim 130 \) \( \mu \)g \(^{14}\)C-OTA (0.25 mCi/mmol), thus delivered \( \sim 0.08 \) \( \mu \)Ci \(^{14}\)C to each animal. This translates to \( \sim 170,000 \) dpm of \(^{14}\)C, of which only about 50 dpm might have been intracellular in the 300–400 mg of kidney used for the
evidence of successful 32P-postlabeling of parallel material in which, together with failure to have the planned positive control compound the unsatisfactory design of the radiolabeled study, by predictive pharmacokinetic modeling (15). All these factors the DNA above background, about one 14C atom to disintegrate not to have been the case), the total content of radioactivity of Even with very efficient extraction and isolation (which seems unlabeled moiety in the 14C-OTA prepared biosynthetically (21), considering the costly resource investment made elsewhere to produce the 14C-OTA. Optimum concentration of OTA circulat- ing in blood may only be achieved after several weeks of continuous daily intake of the amount of 14C-OTA given in ref 17, as indicated by in vivo rat data (14, 16, 20) and corroborated by predictive pharmacokinetic modeling (15). All these factors compound the unsatisfactory design of the radiolabeled study, which, together with failure to have the planned positive control evidence of successful 32P-postlabeling of parallel material in the Pfohl–Leszkowicz laboratory, fails to justify the claim of nongenotoxicity. The claim was also premature because substitution, of commercial 14C phenylalanine for the corresponding unlabeled moiety in the 14C-OTA prepared biosynthetically (21), by well-established methodology (22–24) was not done.

Experiments described in ref 25 showed integrated findings of concentration of OTA in blood and ochratoxin α (OTalpha) in urine over a 2-week period of 5-day-a-week gavage dosing in vegetable oil. This information provides insight into pharmacokinetics applicable to the NTP study and the renal tumors produced. Clearly, with increased dose, blood OTA concentration increased progressively irrespective of the two nondosing days in the middle and end of the 2-week experimental period. This is consistent with strong binding of OTA to serum albumin and a plasma half-life of about 10 days in the Fischer rat (14). However, the pattern of OTalpha excretion is striking and informative, although no discussion of this finding was made in ref 25. OTalpha in urine indicates metabolism of OTA in nephron epithelia and, at higher OTA doses, this activity is prominent. However, OTalpha excretion falls quickly nearly to zero during the two-day nondosing period, but exactly the same pattern is repeated during the second week. Thus, repeated pulses of OTA dosed by oral gavage are rather quickly subject to metabolism in nephron epithelia. Therefore, waiting for 3 days after a single OTA dose in ref 17 can be seen as counterproductive in an experiment designed to find evidence of DNA/OTA adducts in whole kidney.

The general lack of DNA adduct detection in several publications (2, 17, 25, 26) could be explained by several drawbacks in the methodologies used. Indeed, the experimental conditions and the methodologies used in these papers are not optimal and did not reproduce those described by the others. Notably, there is no quality verification of the DNA used for ACMS analysis or 32P-postlabeling in the key publication (17) used to underpin much of the current claims that OTA is not a genotoxin. For some in vivo experiments, a low dose (0.2–1 mg/kg b.w.) was used for a short exposure time, 24 h; and for ACMS detection, the conditions were 0.5 mg/kg b.w. for 72 h. It has long been stated clearly that most of the adducts at a low (0.6 mg/kg b.w.) dose or a medium (1.2 mg/kg b.w.) dose become undetectable by 72 h (1, 27). Thus, the conditions were not optimal for adduct recognition. Moreover, the ACMS measurement had been performed without piloting authentic samples for comparison, and they used a Turbo Ionspray source and heated the samples to 400 °C. Most of the DNA adducts and OTA-GSH will probably decompose at such high temperatures. For generation of OTA-DNA adduct standards, the photoreaction was carried out with OTA at 500 mM and dG at 20 mM, conditions that are at an exceedingly high concentration and do not match the conditions reported in ref 12 for successful isolation and characterization of the adduct standards. Also, it appears that no purification of the photoreaction was carried out and, at best, the solution would have been a mixture of C-C8 and O-C8 adducts.

Conditions for optimizing DNA extraction are not correct in ref 17. Mainly for the purification, they used a Qiagen column, a technique shown in three interlaboratory studies on postlabeling to induce a loss of DNA adduct (EU project 5 V0448 Comparison and validation of 32P-postlabeling method; EU project BEQALM; EU project OTA Risk assessment QKL1-2001-0614). The purity of the DNA was also not good enough, probably being contaminated by RNA and protein, which would have decreased postlabeling efficiency (28). Figure 13 shows comparison of the UV spectrum of DNA prepared in the Wurzburg laboratory contemporaneously with the studies of ref 17 and markedly contaminated with RNA with that from which the adduct data in Figure 6 was obtained. Other spectra showed contamination with protein. The chromatographic conditions of separation were also not adequate for OTA-DNA adduction. The chromatographic pH conditions for 32P-postlabeling analysis, with D1 at pH 6.8 and D3 at pH 3.5, do not conform to standard published procedures, where D1 is run at a pH value below 6.0, and D3 is run at pH ∼6.4 (10). Indeed, in ref 17, the separation conditions described were those for polycyclic aromatic hydrocarbons (PAH). PAH conditions were too strong for OTA and, even for the aristolochic acid positive control, the pattern was not optimal. The authors speculate that the DNA...
adduct observed is due to the cytotoxicity of oxygen reactive species leading for example to 8-oxoguanine. This modification can, however, not be detected with the postlabeling method using nuclease P1 enrichment, as it detects only bulky lipophilic adducts and not hydroxylated hydrophilic nucleotides. In the same way, etheno bases cannot be detected by this technique (29). Recently, we explained how an oxidative pathway leads to the covalent OTA-DNA adduct via biotransformation of OTA into the quinone derivative OTHQ (10, 30, 31).

If histopathological change is a general indicator of renal cells that are significant active or passive sites of OTA uptake and may be sites of persistent genetic change necessary for tumorigenesis, the outer stripe of the outer medulla is ideally the preferred tissue for finding adducts because prominent aneuploid karyomegaly in response to some mycotoxins is generally located in and around this region (4, 32–34). Even this region is not absolutely defined; packing of nephron P3 segments does not rigidly conform to a sharp line of demarcation in renal tissue, particularly concerning those from peripheral glomeruli. Recognition of precise anatomical transition in segment structure and function from P2 to P3 in a single nephron cannot easily be made in histology sections. There is no doubt that OTA can cause genetic damage both in vitro and in vivo. Demonstration of this (25) in the kidney of rats dosed for a short period has been complemented by data from cells in tissue culture (33); similar demonstration was also made (Mosesso, unpublished data) in kidneys at the 12-month stage of the lifetime experiment described in ref 36.

More recent analysis by isotopic dilution to determine whether OTA forms DNA adducts in vivo has also concluded that it does not (37). However, as with the findings in ref 17, data from sophisticated spectrometric methodology can only be as reliable as is the quality of the material analyzed. The use of kidney from other protocols (25, 37), not specifically designed to optimize the recognition of very small amounts of adducted nucleotide, is disappointing. Kidneys from rats given the higher OTA dose (25) were obtained 3 days after the last OTA dose and from animals with marked polyuria, which indicated some renal dysfunction. It is unreasonable to assume efficient delivery of OTA to proximal tubule epithelia if there is overt evidence of renal dysfunction. We calculate that circulating OTA concentration in blood plasma can account for all measured OTA in kidneys so that no renal parenchyma accumulation of OTA could be perceived.

Material sourced from a 90-day study (38) for the study of ref 37 had plasma OTA concentration for the highest dose group, designed to mimic that of the NTP study (4), only at a mean value of 3 µg/mL (with rather large standard deviation). In any case, such animals would probably not have had a sufficient period of OTA exposure for renal tumorigenesis to have been put in place (16), and there was no evidence that plasma OTA concentration had reached a steady state. This is an important consideration since covalent binding of OTA to DNA could relate only rather specifically to renal tumorigenicity. Adducts can occur without implication that they constitute an actual carcinogenic risk if the toxin insult is below threshold (39). DNA adducts were recognized by 32P-postlabeling in our studies on kidneys of rats with plasma OTA concentration stabilized around 8 µg/mL (19), which was typical of that associated with long-term renal tumorigenesis in male Fischer rats (36). The rats were at the 12 month stage from a large cohort continuing for life on dietary OTA and causing renal cancer (36), but at that time, it was not realized that further exposure to OTA might be unnecessary for tumorigenesis because no one had yet explored latency for this toxin. This emphasizes that sensitive 32P-postlabeling analysis should have been applied to the kidney tissues selected for the study of ref 37 to give assurance that spectrometric methodologies could be used with confidence to assess whether adducts were present. Otherwise, acquisition of negative evidence from protocols that have not been challenged concerning their fitness for purpose is an extremely weak basis for constructing a putative mechanism in carcinogenesis.

It has recently been pointed out that the work presented in ref 37 was flawed because the limit of detection (LOD) (10 fmol dG-OTA, corresponding to 3.5 adducts per 106 nucleotides) was obtained from DNA hydrolysates (500 µg DNA equivalent) and not from the actual rat kidney sample for which the negative results were presented (40). This argument stemmed from the fact that a high amount of sample spiking (1250 fmol) with an isotopically labeled dG-OTA adduct of 96–98% isotopic purity was used, and the nonisotopically labeled dG-OTA adduct impurity was observed in the DNA hydrolysates but not in the rat kidney sample. Clearly, the LOD for the DNA hydrolysates versus the rat kidney sample were not the same. DNA purification from the rat tissue sample is more complicated, requiring extra steps, and interfering species could dramatically change the LOD. It was also pointed out that in the rat kidney sample a peak for the dG-OTA adduct following 90 days of incubation was actually observed in the ref 37 study using the m/z 633 → 429 fragmentation transition in MS/MS detection. This apparent peak was not attributed to the dG-OTA adduct because an adduct peak was not observed using the transition m/z 633 → 517. In response to the criticism, the authors argued (41) that their method of quantification was sound, but they avoided discussing whether they were justified in their assumption that the LOD from the DNA hydrolysates could serve as an accurate LOD for the rat kidney sample. They also point
out that the absence of a signal at transition $m/z$ 633 → 517 is a strong argument against the presence of dG-OTA in the sample, even though an adduct peak was observed using the $m/z$ 633 → 429 transition. However, the $m/z$ 633 → 429 transition is more intense than the $m/z$ 633 → 517 (20% greater), and here a clear adduct peak above background was observed that comigrates with the adduct standard. Given that the $m/z$ 633 → 429 transition provides a more intense adduct signal and showed positive evidence for dG-OTA in rat kidneys (for OTA-treated rat at a dose of 210 μg/kg b.w. for 90 days), we can argue that the findings from the ref 37 study are in agreement with our positive evidence for dG-OTA presented here.

However, a genotoxicity mechanism operating in animals in which renal tumors will form and in which genetic damage has been demonstrated will evoke a relatively simple concept. The concept turns on a reasonable probability of occasional persistent genetic change related to tumorigenesis residing in perhaps just one nucleus in the ~30,000 highly differentiated nephrons (~) in just one of a rat’s kidneys. Such could certainly be expected after a lifetime of continual bombardment of rat tubular epithelial cells with OTA molecules, as in the principal lifetime carcinogenicity studies to date (4, 6, 36). It is not unreasonable that other recent studies have shown that lifetime exposure is not mandatory (16) but that less than half a lifetime or even as little as 6 months of continuous toxic insult during the first half of an ~2-year rat lifespan would also be sufficient to determine that renal tumorigenesis could arise in some individuals. Experimental OTA tumorigenesis seems to be highly focal in rat kidneys, according to observations on about 50 tumorous kidneys throughout recent studies in London. Bilateral OTA tumorigenesis seems, in our experience in the rat, to be dose-related, and this would be consistent with the statistical probability of causing one focus of the necessary genetic damage in each kidney of an animal.

All experimental dose–response data for OTA’s renal carcinogenicity, combined and plotted in the format of ref 43, makes a compelling case for OTA being a thresholded carcinogen in the male rat (16). There should be no philosophical conflict that the mycotoxin can also make covalent adducts with DNA and thereby, after many months of exposure to toxin molecules passing through renal epithelium, occasionally cause the particular combination of persistent DNA change that programs an occasional renal cell toward tumorigenesis, discovered late in life. Recently, it has been demonstrated (44) that genotoxic thresholds can exist for some genotoxins. Thus, determining the lowest dose that does not increase background mutation rate is essential and represents an important new aspect of genotoxicology. We recently showed that continuous treatment of Dark Agouti rats with feed contaminated with 50 ng OTA/kg for 4 weeks led to the formation of DNA adduct (20, 28); this dosage is 8-fold less than that having no adverse clinical effect during lifetime exposure (16).

The current findings relate solely to defining the fundamental genotoxicity of OTA as revealed by 32P-postlabeling and the structure determination of the principal adduct to demonstrate direct covalent binding of OTA to DNA. Further understanding of the precise mutations involved in rat renal tumorigenesis in response to OTA will assist evidence-based application of animal model data to human risk assessment.

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