

Linked projects:

- 1 **Mycotoxin-Prevention Cluster: Prevention of Mycotoxins Entering the Human and Animal Foodchain**, coordinator Prof. Naresh Magan, n.magan@cranfield.ac.uk, www.mycotoxin-prevention.com
- 1 **Risk Assessment and Integrated Ochratoxin A Management in Grapes and Wine**, coordinator Prof. Paola Battilani, paola.battilani@pc.unicatt.it, www.ochra-wine.com
- 1 **Risk Assessment of Biological Control Agents**, coordinator Dr. Tariq M. Butt, t.butt@swansea.ac.uk, www.swan.ac.uk/biosci/rafbca

Final project meeting:

26. September 2004

in association with the **European Society of Toxicologic Pathology (ESTP) Congress 2004**

An Integration of Mechanistic Investigation and Morphological Evaluation

Joint Speciality Symposium on Renal Toxicology and Toxicologic Pathology

September 26 – October 1, 2004
Lindau/Bodensee, Germany

www.eurotoxpathorg
www.toxicology.org

Participants:

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www.uni-wuerzburg.de/toxikologie/EU-OTA/OchratoxinA.html

5th RTD Framework Programme

1998-2002



The European Commission

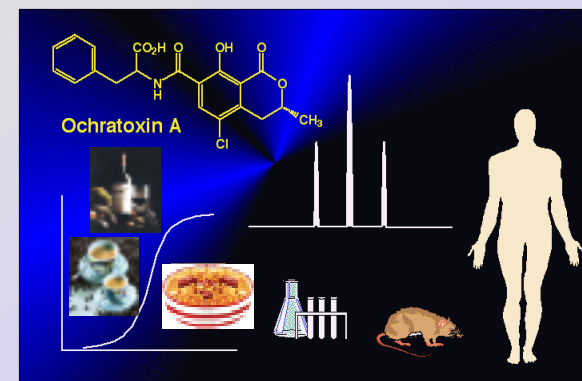
Community Research

Quality of Life and Management of Living Resources

Mechanisms of ochratoxin A induced carcinogenicity as a basis for an improved risk assessment

Ochratoxin A-risk assessment

Project No.: QLK1-2001-01614



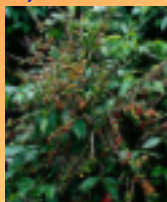
Project Progress Report
(October 2001 – September 2003)

This project is supported by the European Commission
Quality of Life and Management of Living Resources
Fifth Framework Programme



Ochratoxin A is a mycotoxin which contaminates a variety of human food. In experimental animals, ochratoxin A is nephrotoxic and induces tumors in the kidney, but also in other organs of rodents. In humans, exposure to high levels of ochratoxin A in diet has been linked with chronic renal disease (Balkan endemic nephropathy, interstitial nephritis) and an increased incidence of urinary tract tumors.

Objectives: To elucidate mechanisms of ochratoxin A tumorigenicity, this project intends to quantitate relevant biochemical endpoints in the toxicity and tumorigenicity of ochratoxin A. Individual projects address the mechanisms of DNA-damage induced by ochratoxin A and the time course of ochratoxin A induced changes on the bio chemical, cellular and morphological level during tumorigenesis.



In addition to providing information on mechanisms of tumor induction by ochratoxin A, the project will also give relevant information on general mechanisms of tumor induction, promotion and progression in the kidney. The major focus is to obtain information on the dose-response curve for ochratoxin A-induced toxic effects and the relevance of observations made in animals at high dose for human risk assessment.

Results and Milestones:

Accelerator mass spectrometry studies on DNA-binding performed by partner 1 has been completed and do not show a covalent binding of ^{14}C from ochratoxin A to liver and kidney DNA from rats treated *in vivo*. The stability of potential adducts and the role of DNA-isolation procedures was investigated. The results show that adduct recovery from a positive control is not different when the two procedures are compared. Analytical methods to detect ochratoxin A metabolites were developed. In samples from ochratoxin A treated these metabolites could not be detected. Both ochratoxin A and ochratoxin B treated animals induced cell proliferation in the kidney, only limited nephrotoxicity and some DNA-damage in the kidney after ochratoxin A treatment., but OTB was much less toxic than OTA due to rapid biotransformation.

The work performed by partner 2 has established procedures to analyse biomarkers of oxidative stress and showed an induction of the inducible nitric oxide synthase (iNOS), however, the stress-response was inconsistent with regard to the different parameters determined and a possible delay in the response *in vivo* is indicated. The liver of animals treated for 7 and 12 months with OTA showed a significant induction of 4-HNE adduct formation. Results from a DNA-array experiment will be upcoming in the 3rd reporting period.

In the laboratory of partner 3, groups of rats was exposed to ochratoxin A in the diet and samples from animals sacrificed at different time-points were evaluated. P3 provided radiolabeled ochratoxin A for AC/MS studies and has produced large amounts of ochratoxin A. Samples were taken at 11 months in the main group in which treated animals received 100 micrograms OTA daily in feed. Body weight of the treated group closely matched (95%) that of controls. No gross abnormalities were seen. Microscopically, there was only a more prominent karyocytomegaly in renal proximal tubule epithelia than was seen at earlier stages.

Partner 4 has incubated ochratoxin A with different enzymes in the presence of DNA-oligomers and assessed DNA modifications. Preliminary results indicate that OTA induces break in the oligomers. Polymeric nucleosides were incubated in presence of pig kidney microsomes and DNA modifications were indicated with adenine and guanine. Cross link were also formed in poly dG-dC. Pig liver and kidney microsomes form several OTA-metabolites. In pigs, OTA -modulation of the expression of COX1, COX2 and 5-LIPOX is different in genders. COX1 was inhibited in male cortex, an over-expression is observed in females. Expression of COX2 is only modulated in male kidney (cortex and medulla) where an over-expression is observed in cortex. Expression of 5-LIPOX was only modulated in males by OTA. No basal expression of 5-LIPOX was observed in male cortex, whereas high expression is observed in cortex and medulla of female independently of OTA treatment. CYP 2C9 is not expressed in cortex in both sexes. OTA induced slightly the expression of this CYP in cortex. In medulla, CYP 2C9 is expressed, but is not modulated by OTA.

P5: Cytotoxicity experiments showed dose- and time-dependent cytotoxicity between 8 and 80 μM of OTA and human cell lines were more sensitive than rodent cells; within the rodent cells the liver epithelial cells were most resistant. Moreover, a cell system (V79 cells in the presence of kidney S9) which showed a significant increases in mutation frequency by OTA, was identified. The mutagenic effect occurred only in a narrow range. Results from HPRT mutation assays in V79 cells in the absence of metabolic activation and results from the TK assay in LY5178 mouse lymphoma cells suggest that OTA is mutagenic by oxidative damage. OTA-induced HPRT mutant clones were expanded to mass culture, RNAs were isolated and the sequencing of cDNAs is under progress to identify the type of mutations and DNA-damage. Experiments are in progress where the plasmid DNA is treated with OTA and Fpg-sensitive sites were not detected after 30 min incubation of the plasmid with OTA doses up to 1mM.

P6: Exposure of rats to OTA by feed for a period of time 6 months (mean serum OTA 7 $\mu\text{g}/\text{ml}$) resulted in slight genetic damage in some tissues and organs. Increases in the "tail moment values" of "comets" observed in the liver and bone marrow reflect the induction of primary DNA lesions. The presence of micronuclei and chromosomal aberrations in bone marrow erythrocytes indicate that "primary DNA lesions" have been processed by DNA repair activities, thus generating chromosomal damage. *In vitro* studies show that OTA does not induce chromosomal aberrations and SCE's, but an increase in the frequencies of both endoreduplicated and polyploid cells. Since OTA *in vitro* does not show any cytogenetic activity we evaluated the possibility whether the induction of endoreduplication could have been caused by a possible activity of OTA on the cell cycle. Endoreduplication is not induced via inhibition of microtubuli and can be caused by interference of OTA with cell cycle progression. Metabolically competent cell lines were also treated with OTA and did not show an increase in the frequency of micronuclei indicating no role of bioactivation. Bone marrow, spleen, liver, kidney and whole blood of rats chronically treated for approximately 15 month with OTA in food only showed marked increases in DNA breakage in kidney. The effect was amplified by Fpg indicating unrepaired oxidative damage which is not converted into permanent cytogenetic damage.

P7: In serum-free aggregating brain cell cultures, nanomolar concentrations of ochratoxin A decreased the levels of the two T3-dependent parameters cyclic nucleotide phosphohydrolase (CNP), an oligodendroglia-specific enzyme, and glial fibrillary acidic protein (GFAP), an astrocyte-specific intermediate filament protein. Increasing the levels of T3 during prolonged OTA exposure showed partial protection. Similar results were obtained in experiments with shorter exposure times. However, increased T3 also somewhat raised the basal levels of CNP activity and GFAP content.

In subsequent studies, OTA interference with T3-dependent gene expression was examined by real-time RT-PCR using the Taqman system. Expression of several genes were examined after treatment of early aggregate cultures (DIV 5) with OTA for 4 days and for 9 days.

The effects of ochratoxin A on the expression of one specifically T3-regulated gene have been tested and OTA increased Na^+ , K^+ -ATPase mRNA level in cultured liver cells and kidney cells. The protection by T3 of the effects of ochratoxin A has been tested in aggregating brain cell cultures. The experiments at the protein level did not allow to distinguish between a potential protection by T3 of OTA-effects or a simple stimulatory effect of the increased concentration of T3. In aggregates treated with ochratoxin A in the presence of increased levels of T3 a slight attenuation in the decrease in GFAP mRNA, without increasing GFAP mRNA was seen. It had no effect on ochratoxin A-induced decrease in MBP mRNA, but increased MBP mRNA by itself. No inhibition by ochratoxin A of T3-mediated transcription of MBP has been found.

In summary, the experiments are progressing as planned and changes to the technical annex were made to follow up some of the observations made during the first 2 years. The major discrepancy in the results between the partners when using different techniques are the conclusions regarding covalent binding of OTA to DNA. Consistent with the results from other groups using similar approaches and the absence of mutagenicity of OTA as seen in this project, radioactivity from labelled OTA was not found bound to DNA. Postlabelling by P4 suggests the formation of DNA-modifications in kidney after OTA-administration. The results of the postlabelling exercise show that differences in the DNA-extraction procedures do not account for the different results using radiolabelled OTA and the postlabelling. This point will be addressed again in the 3rd year and P4 will attempt to isolate the DNA-modifications indicated for structure elucidation.

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