OPINION OF THE SCIENTIFIC PANEL ON CONTAMINANTS IN THE FOOD CHAIN ON A REQUEST FROM THE COMMISSION RELATED TO OCHRATOXIN A IN FOOD

Question N° EFSA-Q-2005-154

Adopted on 4 April 2006

SUMMARY

Ochratoxin A (OTA) is a mycotoxin produced by several fungal species of the genera *Penicillium* and *Aspergillus*. Contamination of food commodities, including cereals and cereal products, pulses, coffee, beer, grape juice, dry vine fruits and wine as well as cacao products, nuts and spices, has been reported from all over the world. In addition, contamination of animal feeds with OTA may result in the presence of residues in edible offal and blood serum, whereas the OTA contamination in meat, milk and eggs is negligible. Despite efforts to reduce the amount of this mycotoxin in foods as consumed, a certain degree of contamination seems unavoidable at present.

Some early epidemiological data had suggested that OTA might be involved in the pathogenesis of distinct renal diseases and otherwise rare tumours of the kidneys in certain endemic regions of the Balkan Peninsula. However, these epidemiological data are incomplete and do not justify the classification of OTA as a human renal carcinogen. OTA has been found to be a potent renal toxin in all of the animal species tested. It induces a typical karyomegaly and a progressive nephropathy. The extent of renal injury is dose-dependent, but also associated with the duration of exposure, as OTA accumulates in renal tissue. Previous National Toxicology Program (NTP) studies in United States showed that OTA can induce renal tumours in rodents at high dosages.

Recent scientific evidence indicates that the site-specific renal toxicity as well as the DNA damage and genotoxic effects of OTA, measured in various *in vivo* and *in vitro* studies, are most likely attributable to cellular oxidative damage. Furthermore, advanced chemical analytical procedures failed to demonstrate the existence of specific OTA-DNA adducts. Considering the lack of evidence for the existence of OTA-DNA adducts, the Panel used a threshold-based approach in its risk assessment of OTA. On the basis of the lowest observed adverse effect level (LOAEL) of 8 µg/kg body weight (b.w.) per day for

early markers of renal toxicity in pigs (the most sensitive animal species), and applying a composite uncertainty factor of 450 for the uncertainties in the extrapolation of experimental data derived from animals to humans as well as for intra-species variability, a Tolerable Weekly Intake (TWI) of 120 ng/kg b.w. was derived for OTA.

Recent analyses of the dietary exposure of adult European consumers to OTA revealed that at present the weekly exposure ranges from 15 to 60 ng OTA per kg bodyweight per week, including high consumers of foods containing OTA. This rate of exposure is below the TWI value of 120 ng/kg b.w. as derived by the Panel. However, as current EFSA consumption databases do not include infants and children, the CONTAM Panel concluded that more data would be needed to assess exposure rates of this segment of consumers, taking into account their dietary preferences.

KEY WORDS

Ochratoxin A, Balkan Endemic Nephropathy, renal toxicity, karyomegaly, renal tumours, food, risk assessment

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BACKGROUND

Ochratoxin A (OTA) is a mycotoxin produced by several fungi (*Penicillium* and *Aspergillus* species), and occurs naturally in a variety of plant products such as cereals, coffee beans, beans, pulses and dried fruit all over the world. It has been detected also in products such as coffee, wine, beer and grape juice. It occurs in kidney, liver and blood from farm animals by transfer from animal feed (EFSA, 2004).

OTA has been previously evaluated by the Scientific Committee for Food (SCF) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The conclusions of these evaluations are summarised below.

Commission Regulation (EC) No 466/2001 of 8 March 2001 setting maximum levels for certain contaminants in foodstuffs[1] as amended by Commission Regulation (EC) 123/2005 of 26 January 2005[2] establishes maximum levels for OTA in cereals and derived cereal products, dried vine fruit, roasted coffee, soluble coffee, wine, grape juice, baby foods and processed cereal-based foods for infants and young children. The legislation provides that the Commission shall review, based on an up-to-date risk assessment on OTA performed by EFSA and taking into account the prevention measures applied to reduce the OTA content, the provisions as regards OTA by 30 June 2006 at the latest.

Scientific Committee for Food

The SCF expressed on 23 September 1994 an opinion on aflatoxins, OTA and patulin (EC, 1996) in food. The Committee concluded that OTA is a potent nephrotoxic agent, a carcinogen and that it has genotoxic properties The Committee provisionally supported the conclusion that an "acceptable safe level of daily exposure would fall in the range of a few ng/kg b.w. per day". It proposed furthermore to reconsider its opinion in the light of new information.

The SCF reviewed the toxicology on OTA and concluded in its opinion on OTA in food on 17 September 1998 (EC, 1998a) that there is an increasing concern about potential genotoxicity of OTA and its mechanism of action as a carcinogen. The Committee was aware that further studies were on-going to elucidate the mechanisms involved in OTA carcinogenicity. Therefore the Committee considered it would be prudent to reduce exposure to OTA as much as possible, ensuring that exposures are towards the lower end

[1] Official Journal, L 77, 16.3.2001, p. 1
[2] Official Journal, L 25, 28.1.2005, p. 3

of the range of tolerable daily intakes of 1.2-14 ng/kg b.w. per day which have been estimated by other bodies, e.g. below 5 ng/kg b.w. per day.

Joint FAO/WHO Expert Committee on Food Additives (JECFA)

OTA was also evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) at its thirty-seventh meeting in 1991 (FAO/WHO, 1991) and a provisional tolerable weekly intake (PTWI) of 112 ng/kg b.w. was established.

OTA was re-evaluated by JECFA at its forty-fourth meeting in 1995 (FAO/WHO, 1996) and the PTWI was reconfirmed, rounding it to 100 ng/kg body weight and the request for further studies on OTA was re-iterated.

The JECFA considered at its fifty-sixth meeting in February 2001 several new studies that had become available since its last evaluation of OTA (FAO/WHO, 2001). JECFA concluded that the mechanism by which OTA causes carcinogenicity is unknown although both genotoxic and non-genotoxic modes of action have been proposed. The JECFA recommended that studies should be conducted to clarify the mechanism by which OTA induces nephrotoxicity and carcinogenicity and noted that studies to resolve these issues are in progress. JECFA retained the previously established PTWI of 100 ng/kg body weight per week, pending the results of these studies.

Research on the mechanisms of OTA induced carcinogenicity

The European Commission funded in the 5th RTD Framework Programme a study on the mechanisms of OTA induced carcinogenicity as a basis for an improved risk assessment (OTA-Risk Assessment – QLK1-2001-01614).

TERMS OF REFERENCE

In accordance with Art. 29 (1) (a) of Regulation (EC) No 178/2002 the Commission asks EFSA to review the opinion of the Scientific Committee on Food of 17 September 1998 on OTA in food in the light of

- results of toxicological studies published since that time, in particular the results of an EU-research project, that have became available on the mechanisms of OTA induced carcinogenicity;
- recent analytical results on the occurrence of OTA in food and exposure assessments;

- any other scientific information of relevance for the assessment of the risks of the presence of OTA for human health.

EFSA is also requested to address in its opinion the exposure of vulnerable groups of the population as well as specific groups of consumers who are exposed to higher levels of OTA than average as the consequence of their dietary habits.

Assessment

1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by several fungal species in the Penicillium and Aspergillus genera, primarily *Penicillium verrucosum, Aspergillus ochraceus* and *Aspergilli* of the section *Nigri*, especially *A. carbonarius*. *A. carbonarius* has been identified as the key species responsible for OTA contamination of grapes, wine and vine fruits. *A. ochraceus* can infect cereals, coffee, cocoa and edible nuts. However, although commonly claimed, adequate confirmation of the occurrence of ochratoxin-producing isolates of *Aspergillus* from cereals has never been reported in the literature. *P. verrucosum* is a particularly important source of contamination in grain in cooler regions of Northern Europe (Olsen *et al.,* 2003). Invasion with ochratoxin-producing fungal species has been reported worldwide and subsequently OTA has been detected in cereal products, pulses, coffee, beer, grape juice, raisins and wine, as well as in cocoa products, nuts and spices. Contamination of animal feeds results in the presence of residues in edible tissues and meat products, with higher concentrations occurring in certain local specialities such as blood puddings and sausages prepared with pig blood serum.

OTA is a stable compound that is not destroyed by common food preparation procedures, as temperatures above 250ºC are required for several minutes to reduce the toxin concentration (Boudra *et al.*, 1995). Thus, raw and processed food commodities can be contaminated with OTA.

OTA comprises a dihydrocoumarin moiety linked to a molecule of L-β-phenylalanine via an amide bond as shown in Figure 1. The systematic chemical nomenclature for OTA is (R)-N-[(5chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-yl)-carbonyl]-L-phenylalanine (CAS No. 303-47-9).

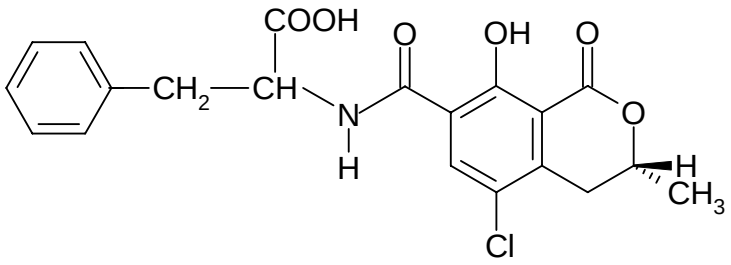

Figure 1. Chemical structure of ochratoxin A.

Ochratoxin A in food and feed is sometimes accompanied by the non-chlorinated analogue, ochratoxin B (WHO-IPCS, 1990) (see Figure 2). The systematic chemical name for ochratoxin B is N-[(8-hydroxy-3-methyl-1-oxo-3,4-dihydro--1H-isochromen-7-yl)carbonyl]-(R)-L-phenylalanine (CAS No. 4825-86-9).

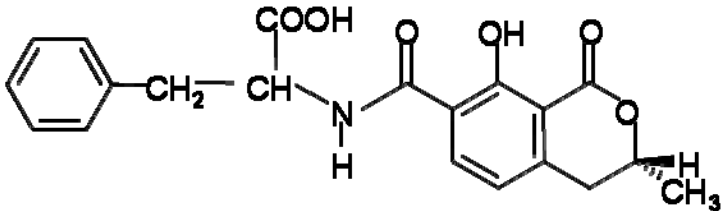

Figure 2. Chemical structure of ochratoxin B.

Although ochratoxin B may have co-existed in some of the naturally contaminated materials tested for toxicity in animal studies, the concentrations are generally low and it appears to be much less toxic than ochratoxin A *in vivo* (Mally *et al.*, 2005a).

Exposure to ochratoxin A has been associated with distinct renal diseases endemic in the Balkans, referred to as Balkan Endemic Nephropathy (BEN) and Urinary Tract Tumours (UTT). However, convincing epidemiological data linking exposure rates to the incidence of these diseases are lacking as yet, despite the fact that various publications describe increased OTA blood levels in the population of endemic villages. In consideration of these reports, The International Agency for Research on Cancer (IARC) evaluated ochratoxin A in 1993, and classified it as possibly carcinogenic to humans (group 2B), based on sufficient evidence for carcinogenicity in animal studies and inadequate evidence in humans (IARC, 1993).

OTA was last evaluated by the Scientific Committee on Food (SCF) in 1998 when it concluded that OTA possesses carcinogenic, nephrotoxic, teratogenic, immunotoxic and possibly neurotoxic properties. At that time the Committee expressed concern about the

potential genotoxicity of OTA and its mechanism of action as a carcinogen (EC, 1998a). It considered that daily exposures should be below 5 ng/kg b.w. Since that time, JECFA has carried out a further evaluation of OTA (FAO/WHO, 2001) and concluded that the previously established PTWI of 100 ng/kg b.w. per week should be retained pending the results of further studies on the mechanisms of nephrotoxicity and carcinogenicity. This PTWI had been based on signs of nephrotoxicity in pigs, the most sensitive species for this effect. The JECFA recommended a further review in 2004 and noted that the large safety factor applied to the NOEL for nephrotoxicity in pigs corresponded to a factor of 1300 applied to the NOEL for carcinogenicity in male rats (21 µg/kg b.w. per day), the most sensitive species and sex for this toxicological end-point. In fact the dose described as a NOEL for nephrotoxicity in a chronic pig study was reported to be a LOEL in a sub-chronic study.

The EFSA Scientific Panel on Contaminants in the Food Chain evaluated the consequences of the presence of OTA in animal feed in 2004 (EFSA, 2004) The Panel concurred with earlier evaluations that OTA is a potent nephrotoxin and that it is carcinogenic in rodents, stating at the same time that the mechanism of the renal carcinogenicity remained unresolved. It noted that, in rodents, tumours are generally only observed at dose levels in excess of nephrotoxic doses. The minimum nephrotoxic dose in male rats (karyomegaly of proximal tubule cells) was 15 µg/kg b.w. per day while renal tumours arose at doses of 70 µg/kg b.w. per day when given to the animals for two years. However, at that time, a genotoxic mechanism could not unequivocally be excluded. The Panel noted that pigs are generally considered to be the most sensitive farm animal species to the nephrotoxicity of OTA and that a dietary concentration of 0.2 mg/kg feed (equivalent to 8 µg/kg b.w.) was considered to be the LOAEL.

Since these evaluations, an EC research project devoted to the mechanism of carcinogenicity of OTA has been concluded[3] and the outcomes reviewed at a subsequent ILSI Workshop in 2005 (Hazel and Walker, 2005).

Following the evaluations of the toxicity of OTA, Commission Regulations have been promulgated introducing upper limits for OTA concentrations in various commodities (cereals, cereal products, dried vine fruit, coffee, wine, grape juice, baby foods and dietary foods for special medical purposes)[4]. The current exposure assessment was

[3] Ochratoxin A - risk assessment. Project No.: QLK1-2001-01614. Project homepage at URL: http://www.uni-wuerzburg.de/toxikologie/EU-OTA/OchratoxinA.html.

[4] Commission Regulation EC No. 466/2001 of 8 March 2001. OJ L 77, 16.3.2001, p. 1. and Commission Regulation EC No. 123/2005 of 26 January 2005 (amending EC No.466/2001 as regards ochratoxin A). OJ L 23, 28.1.2005, p. 3.

conducted in the years following the implementation of these regulations, and hence may reflect the impact of these precautionary measures.

2. Occurrence of ochratoxin A in food: sampling and chemical analysis

2.1. Sampling techniques and reliability of surveillance data

The random nature of fungal contamination of raw materials (such as cereals, fruit and coffee) and thus the uneven distribution of subsequent OTA contamination means that sampling is a major issue. It is very difficult to obtain representative samples particularly of cereals, which are handled in bulk amounts (Gilbert, 1996). Statistically-based sampling plans for mycotoxins are available, generally involving taking of multiple sub-samples (say 100 or more) from a bulk lot of several tonnes (say 10-15 tonnes) comprising a total sample weight of 20-30 kg (Whitaker, 2006). This single sample then needs to be thoroughly ground to a fine powder (cereal or coffee) or minced (dried fruit) and slurried, then mixed for a reasonable period to obtain homogeneity prior to sub-sampling for analysis (Spanjer *et al.*, 2006). This process is labour intensive, involves pilot scale processing equipment and is therefore costly to undertake.

As cereals and other potentially contaminated commodities are subsequently processed into final food products, there is some mixing and homogenisation during food processing. Sampling of retail products is therefore less problematic, although again large numbers of individual samples (e.g. biscuits, cake) need to be bulked together, which is not always possible in retail surveys. For some beverages like wine, beer and coffee as consumed, sampling in terms of taking large sample sizes is unnecessary and here representative data are best obtained through analysis of large numbers of geographically varied samples. Data on OTA in animal products does not raise sampling issues *per se* but again reasonably large data sets are needed to obtain a representative picture. In general, literature data on the occurrence of OTA frequently fails to supply adequate detail as to the selection of samples, and one needs to guard against skewed data if targeted samples e.g. only suspect samples were analysed.

These shortcomings, evident in previous surveys, are of less importance in the evaluation of current contamination rates, since an extensive database on the occurrence of OTA in the major food categories has become available, stimulated by the SCOOP exercise compiling data from various EU member states (see section 3).

2.2. Analytical methods

The analysis of OTA in most foodstuffs is relatively straightforward and reliable results generally are obtained as evidenced by the results from proficiency testing. Samples need to be acid- or alkali-extracted from the foodstuffs, with alkaline extraction from most matrices showing generally better recoveries (Senyuva *et al.*, 2005). Conventionally liquid/liquid extraction and solid phase clean-up have been used prior to HPLC determination with fluorescence detection. However, over the past 10 years most laboratories have tended to move towards using antibody-based affinity column clean-up which is relatively simple to carry-out and provides sample extracts generally free of interferences. Good methods are available for dried fruit (MacDonald *et al.*, 1999), beer (Legarda and Burdaspal, 1998), coffee (Leoni *et al.*, 2000; Stegen *et al.*, 1997), dried figs (Senyuva *et al.*, 2005), milk (Valenta and Goll, 1996) and wine (Zimmerli and Dick, 1996). Roasted coffee tends to be the most problematic of foodstuffs to analyse for OTA and in some methods an additional clean-up step is advocated prior to the affinity column stage (Entwistle *et al.*, 2001). Similar methodology has been used successfully to analyse blood and urine for OTA, when this biomarker approach has been employed for exposure assessment (Gilbert *et al.,* 2001). There are CEN standards for determining OTA in cereals, roasted coffee, wine and beer with limits of quantification (LOQ) ranging from 1.3 ng/g for barley (Entwistle *et al.*, 2000) and coffee (Entwistle *et al.*, 2001) to 5 pg/mL for wine (Zimmerli and Dick, 1996). Particular attention was paid to validating a method for OTA in baby food at an LOD and LOQ of 0.05 and 0.22 ng/g respectively, the additional sensitivity being obtained by extracting larger sample sizes and enhancing fluorescence detection by using post-column ammoniation (Burdaspal *et al.*, 2001). Whereas affinity column clean-up for confirmation of results is not essential, verification of results can be undertaken by methylation of OTA followed by the observation of the shift in retention time, or by LC/MS (Senyuva *et al.*, 2005).

3. Assessment of human exposure

As mentioned above, the most recent international exposure assessments were performed respectively by the SCF (EC, 1998a) and the JECFA (FAO/WHO, 2001). The SCF estimated that the mean dietary intake ranged from 0.7 to 4.6 ng/kg b.w. per day. By combining the average contamination levels with the 95th percentile of food consumption, the JECFA estimated a dietary exposure of approximately 90 ng/kg b.w. per week corresponding to about 13 ng/kg b.w. per day. Exposure seems to be associated predominantly with the consumption of contaminated plant-derived products, and only to a minor extent to foods of animal origin (EFSA, 2004). However, it cannot be excluded that regular consumption of certain regional specialities, such as Swedish blood pudding containing substantial amounts of pig blood (Thuvander *et al.*, 2001a), contributes

considerably to the level of exposure, especially in children, in which the relatively lower body weight as compared to adults, results in a higher exposure per kg bodyweight.

3.1. Methodology of exposure assessment

Previous exposure assessments have been based on a deterministic approach (point estimate) or on the estimation of the empirical probability for consumers to exceed the toxicological reference value. In both cases, various levels of consumption were combined with the mean concentrations of OTA in the relevant food categories. With this opinion an update of these data is presented, using the same methodology as in previous risk assessments, but integrating more recent and accurate data for the average level of occurrence with mean and high levels of consumption of certain food categories.

In detail, the current update considers seven food categories representing the main contributors to OTA exposure, identified by the SCOOP report and by JECFA. These categories are cereals and cereal products, wine, beer, grape juice, brewed coffee, cocoa and cocoa products as well as pork meat. Other food categories analysed for OTA, including pulses and fruit juices (others than grape juice) are not considered in this assessment because of their low reported levels of OTA contamination (0.1 and 0.01 µg/kg respectively) (EC, 2002). Similarly, it was noted in the SCOOP task reports that none of the 41 samples of poultry meat analysed reached the LOD for OTA. Dried fruits and spices are not considered because of their low rate of consumption of 2.9 and 0.5 grams per day, respectively (WHO-GEMS/Food, 2003). Data on green coffee and cacao beans are not considered as they are not consumed as such, and because dietary exposure of products thereof is already considered with the corresponding processed products.

3.2. Occurrence data

Concerning the occurrence of OTA, the JECFA compiled about 10,000 data points from the seven food categories mentioned above. Those data included results from the first EU scientific co-operation, SCOOP task No. 3.2.2, (EC, 1995), but not the data from the European SCOOP task No. 3.2.7. (EC, 2002) that was published in January 2002. In the latter 18,599 analytical results from 13 EU Member States were compiled. Average concentrations from this most recent SCOOP task are compiled in Table 1 together with the levels reported in the JECFA report. In general, there is no apparent difference between the SCOOP 2002 data and the JECFA data, except in the case of cocoa (0.24 and 0.55 µg/kg in the most recent SCOOP report and the JECFA, respectively). For this food category, the results from SCOOP are considered to be more reliable due to the larger number of samples analysed (n=547).

In addition, new information published in the scientific literature since 2002 was considered in the present update. For example, in a recent submission of the Swedish Food Administration (Möller, 2005, personal communication) 192 analytical results on the occurrence of OTA in cereals and cereal products are presented, with an average level of 0.12 µg/kg. This level is about one half of that reported by JECFA and SCOOP. For wine, 5 surveys were published including about 550 analyses (Pietri *et al.,* 2001; Stefanaki *et al.,* 2003; Soufleros *et al.*, 2003; Shephard *et al.,* 2003; Blesa *et al.,* 2004). Average contamination ranged between 0.2 and 0.4 µg/L and the highest values reported were below 4 µg/L. No systematic differences between countries could be found. These results are consistent with both the JECFA and the SCOOP task. For beer, the reported average contamination level was 0.033 µg/L with a maximum level of 0.18 µg/L (Tangni *et al.,* 2002). Again, the occurrence of OTA in beer is similar to that reported in the JECFA and the SCOOP reports. Other publications were also reviewed, but due to the limited number of samples analysed, they were not considered to be of use to estimate the average level of contamination. Moreover, in all these publications, the analytical results were in the range of international reports. Consequently, the SCOOP report was used for the assessment of the exposure, because it provides the largest and most recent dataset.

It should be noted that the average concentration was derived from a large number of samples of food available on the entire EU market. This implies that in certain countries or regions due to climate or storage conditions the mean value for food contamination could be higher. Finally, it should be mentioned that the SCOOP reports collected only data related to the occurrence of OTA. Comparable surveys for the presence of OTB in foods are lacking.

Table 1. Average OTA contamination levels of relevant food category and number of samples included calculating the average.

	JECFA (FAO/WHO, 2001)		SCOOP (EC, 2002)	
	n	mean (µg/kg)	n	mean (µg/kg)
Cereals and products	1538	0.20 (a)	5180	0.29
Beer	975	0.02	496	0.03
Wine	1828	0.32	1470	0.36
Grape juice	87	0.39	146	0.55
Cocoa	171	0.55	547	0.24
Pork (edible offal)	3603	0.17	1860	0.20
Roasted coffee (b)	2085	0.62	1184	0.72
Instant coffee (c)	767	0.76	-	-

a) average contamination level of 1.02 µg/kg was reported by JECFA for raw cereals.
b) ground coffee expressed as dry matter
c) expressed as dry matter

3.3. Food consumption data

For the present purpose, the draft EFSA Concise Food Consumption database compiling consumption data for about 20 major food categories was used. A draft template for the format of the database can be found in the appendix of the opinion on exposure assessment of the EFSA´s Scientific Committee (EFSA, 2005). This database is currently under construction and contains at present only data from three countries, i.e. Italy, France and Sweden. It includes consumptions by adults for the entire population, and for consumers only, expressed in grams per person and per day. A consumer of a food category is an individual consuming this food at least once during the survey duration.

Table 2. Number of consumers (a) of the seven food categories (b) in the survey in the three countries investigated: Data reflect regional differences in consumption patterns.

	EFSA code	France	Italy	Sweden
Total number of subjects		1875	1425	1214
Cereal products	1	1870	1425	1214
Cocoa	2	1856	1345	114
Coffee	8	1788	1250	1184
Beer	9A	380	432	798
Wine	9B	1175	882	536
Offal	10A	308	148	586
Fruit juices	7A	966	538	715

a) Table 2 provides the total number of subjects for each survey and the number of consumers for each food category on which the high percentiles are based.

b) Consumption of fruit juices, hot beverages and confectionery was matched in the applied model calculations with at the OTA concentrations in grape juice, coffee and chocolate products. This may lead to an overestimate of the number of consumers per category and to an overestimate of average levels of exposure in the total population. However, it probably does not lead to an overestimate of the rate of exposure of high consumers, since in most cases high consumers of a single food commodity are not high consumers of other food commodities within the same category. For example, the high consumers of grape juice do not consume large quantities of other fruit juices at the same time.

c) Assuming a coefficient of 18 to convert "solid coffee" in liquid coffee.

The individual consumption of cereals and cereal products, confectionery (taken as a representative for cocoa and cocoa products, see note b in Table 2), liquid hot beverages (taken as a representative for coffee, see note b in Table 2), beer, wine, edible offal (mainly liver and kidney) and fruit juices (taken as a representative consumption for grape juice, see note b in Table 2), were taken from the national food consumption survey of Italy (Turrini *et al.*, 2001), France (Volatier, 2000) and Sweden (Becker and Pearson, 2002). Those surveys were based on 7-days records of individual subjects (Table 2 and 3).

Table 3. Consumption (grams per day per person) of seven food categories in the three selected countries: France, Italy and Sweden.

	Mean consumption			97.5th percentile			99th percentile		
	France	Italy	Sweden	France	Italy	Sweden	France	Italy	Sweden
	Total population (g/day)			Consumers only (g/day)			Consumers only (g/day)		
Cereals and cereal products	218	270	292	501	483	568	601	525	718
Sugar and confectionery (a)	38	20	28	116	72	93	135	98	111
Hot beverages, (a, b)	277	118	592	970	381	1309	1172	481	1663
Beer	30	34	140	797	487	714	1049	693	972
Wine	107	91	39	652	531	257	868	700	352
Edible offal	3	3	6	47	75	43	60	79	48
Fruit juices (a)	46	17	87	314	216	486	429	299	596

a) Consumption of fruit juices, hot beverages and confectionery was matched in the applied model calculations with at the OTA concentrations in grape juice, coffee and chocolate products. This may lead to an overestimate of the number of consumers per category and to an overestimate of average levels of exposure in the total population. However, it probably does not lead to an overestimate of the rate of exposure of high consumers, since in most cases high consumers of a single food commodity are not high consumers of other food commodities within the same category. For example, the high consumers of grape juice do not consume large quantities of other fruit juices at the same time.

b) Assuming a coefficient of 18 to convert "solid coffee" in liquid coffee.

3.4. Assessment of the exposure

Firstly, the contribution to the dietary exposure for each category was estimated using the mean concentrations of OTA in the individual food categories from the most recent SCOOP report, and the mean and high consumption data from the draft EFSA food consumption database (Table 4).

Table 4. Dietary exposure to OTA in mean consumers (average consumption in the total population) and high consumers (97.5th percentile consumption in consumers- only) from the relevant food categories using the draft EFSA concise database.

	EFSA Code	Concentration OTA	Average dietary exposure			97.5th percentile exposure		
	(a)	(b)	France	Italy	Sweden	France	Italy	Sweden
		µg/kg	(ng/day/person)			(ng/day/person)		
Cereals and cereal products	1	0.29	63	78	85	145	140	165
Sugar and confectionery (c)	2	0.24	9	5	7	28	17	22
Hot beverages (c)	8	0.72 (c)	11	5	24	39	15	52
Beer	9A	0.028	1	1	4	22	14	20
Wine	9B	0.36	39	33	14	235	191	93
Edible offal	10A	0.2	1	1	1	9	15	9
Fruit juices (c)	7A	0.55	25	9	48	173	119	267

a) EFSA opinion on exposure assessment (EFSA, 2005)

b) Mean concentrations from SCOOP 3.2.7 (EC, 2002)

c) Consumption of fruit juices, hot beverages and confectionery was matched in the applied model calculations with at the OTA concentrations in grape juice, coffee and chocolate products. This may lead to an overestimate of the number of consumers per category and to an overestimate of average levels of exposure in the total population. However, it probably does not lead to an overestimate of the rate of exposure of high consumers, since in most cases high consumers of a single food commodity are not high consumers of other food commodities within the same category. For example, the high consumers of grape juice do not consume large quantities of other fruit juices at the same time.

As a second step, the total exposure for an "average consumer" was estimated using the average dietary exposure for all food categories. Results, expressed in ng/kg b.w. per day, and assuming a body weight of 60 kg, are summarized in table 5. According to these data, the dietary exposure of the average adult consumer varies between 2 and 3 ng/kg b.w. per day corresponding to about 15 to 20 ng/kg b.w. per week. This result is consistent with the previous assessment of the SCF (0.7-4.6 ng/kg b.w. per day).

Table 5. Dietary exposure of an "average consumer" (60 kg body weight).

	EFSA Code (a)	Number of samples	Concentration (b)	Average dietary exposure		
				France	Italy	Sweden
			OTA µg/kg	(ng/kg b.w. per day)		
Cereals and cereal products	1	5180	0.29	1.05	1.31	1.41
Sugar and confectioneries (c)	2	547	0.24	0.15	0.08	0.11
Hot beverages (c, d)	8	1184	0.72 (a)	0.18	0.08	0.39
Beer	9A	496	0.028	0.01	0.02	0.07
Wine	9B	1470	0.36	0.64	0.55	0.23
Edible offal	10A	1860	0.2	0.01	0.01	0.02
Fruit juices (c)	7A	146	0.55	0.42	0.16	0.80
TOTAL				2.5	2.2	3.0

a) EFSA opinion on exposure assessment (EFSA, 2005)

b) Mean concentrations from SCOOP 3.2.7 (EC, 2002)

c) Consumption of fruit juices, hot beverages and confectionery was matched in the applied model calculations with at the OTA concentrations in grape juice, coffee and chocolate products. This may lead to an overestimate of the number of consumers per category and to an overestimate of average levels of exposure in the total population. However, it probably does not lead to an overestimate of the rate of exposure of high consumers, since in most cases high consumers of a single food commodity are not high consumers of other food commodities within the same category. For example, the high consumers of grape juice do not consume large quantities of other fruit juices at the same time.

d) Assuming a coefficient of 18 to convert "solid coffee" in liquid coffee.

The third step in the applied calculations (derived from the conclusions of the EU Scientific Cooperation related to the dietary exposure to food additives (EC, 1998b) as mentioned above) consists of the application of model diets that simulate the exposure of high consumers using exposure data at the 97.5th percentile for consumers-only of the two main contributing food categories (assuming at the same time a mean dietary exposure from the other food categories).

With this approach, three country-specific scenarios could be described, considering alternatively cereals and wine (as main contributors in Italy), wine and fruit juice (the main contributors in France) and cereals and fruit juice (the main contributors in Sweden). These scenarios are presented in Table 6.

Table 6. Model diets for high consumers based on three scenarios assuming that the total dietary exposure is represented by the sum of the exposure at the 97.5th percentile associated with the two food categories that had been identified as main contributors to exposure and the average exposure from other food categories.

	EFSA Code (a)	Number of samples	Concentration (b)	Scenario 1 France	Scenario 2 Italy	Scenario 3 Sweden
			OTA µg/kg	OTA ng/kg b.w. per day		
Cereals and cereal products	1	5180	0.29	1.05	2.33	2.75
Sugar and confectionery (c)	2	547	0.24	0.15	0.08	0.11
Hot beverages (c, d)	8	1184	0.72 (a)	0.18	0.08	0.39
Beer	9A	496	0.03	0.01	0.02	0.07
Wine	9B	1470	0.36	3.92	3.18	0.23
Edible offal	10A	1860	0.20	0.01	0.01	0.02
Fruit juices (c)	7A	146	0.55	2.88	0.16	4.45
TOTAL				**8.2**	**5.9**	**8.0**

a) EFSA opinion on exposure assessment (EFSA, 2005)

b) Mean concentrations from SCOOP 3.2.7 (EC, 2002)

c) Consumption of fruit juices, hot beverages and confectionery was matched in the applied model calculations with at the OTA concentrations in grape juice, coffee and chocolate products. This may lead to an overestimate of the number of consumers per category and to an overestimate of average levels of exposure in the total population. However, it probably does not lead to an overestimate of the rate of exposure of high consumers, since in most cases high consumers of a single food commodity are not high consumers of other food commodities within the same category. For example, the high consumers of grape juice do not consume large quantities of other fruit juices at the same time.

d) Assuming a coefficient of 18 to convert "solid coffee" in liquid coffee.

These data indicate that the dietary exposure for high consumers ranges from 6 to 8 ng/kg b.w. per day corresponding to about 40 to 60 ng/kg b.w. per week.

It should be mentioned that the current EFSA Food Consumption Database does not allow an accurate assessment of the dietary exposure of children. The Panel noted that even considering the fact that children generally do not consume wine, beer and coffee, they might experience a higher exposure to OTA from other foods contaminated with OTA due to the relatively lower body weight of children. Moreover dietary preferences of children, as well as the exposure via breast milk are often neglected in the overall

exposure assessment. Children may be high consumers of certain local specialities (such as Swedish blood pudding) resulting in an exceptionally rate of exposure as well.

In conclusion, the use of more recent and accurate data (individual food consumption data from three EU countries and more than 15,000 analytical results) suggested that the exposure of high consumers varies between 40 and 60 ng OTA per kg b.w. per week, which is lower than the exposure rate considered in the previous JECFA assessment (90 ng/kg b.w./week). The presented estimates of the current exposure of the adult European population are still considered to be conservative, since broad food categories were used in these calculations. Nevertheless, it has to be reiterated that in some cases, children and distinct groups of consumers preferring locally produced food or distinct food specialties may experience higher exposure rates.

4. Toxicokinetics of OTA

Following oral ingestion, OTA is rapidly absorbed and reaches the systemic circulation, where it is extensively bound to plasma proteins. In animal studies the extent of absorption varies between 40% in chickens and 66% in pigs (Galtier *et al.*, 1981). Following absorption, OTA is bound readily to serum albumin and other macromolecules (Galtier *et al.*, 1981; Hult and Fuchs, 1986). The unbound fraction is as low as 0.02 % in humans, indicating an extent of protein binding of 99.98% (Hagelberg *et al.*, 1989). In most animal species, the kinetic behaviour of OTA has been described as a two compartment open model, although recent data on the accumulation in kidneys suggest that these models are too simple and data should be re-analysed using multi-compartment models. In many species, including monkeys and humans, the major route of excretion is renal elimination, whereas in rodents biliary excretion seems to prevail. Biliary excretion and entero-hepatic re-circulation of OTA-glucuronides may account for the inter-individual and interspecies variability of kinetic parameters observed in the various kinetic studies. The elimination half-lives of OTA in Wistar rats and pigs were reported to be 5 and 6 days, respectively (for review see Dietrich *et al.*, 2005). OTA is hydroxylated in the liver only to a minor extent resulting in the R- and S-epimers of 4-OH-OTA. In some animal species also 10-OH-OTA production has been described. Recent data indicate the formation of pentose and hexose conjugates of OTA, which could also be detected in the urine of OTA-treated rats (Gross-Steinmeyer *et al.*, 2002; Zepnik *et al.*, 2003). In addition to hepatic metabolism, bacterial metabolism in the gastrointestinal tract yields the cleavage product, ochratoxin α, which can be absorbed from the lower gastro-intestinal tract.

Mechanisms involved in renal cellular uptake

Previous data from *in vivo* experiments had indicated that OTA accumulates in the kidneys. Subsequently it had been suggested that the marked differences in the relative sensitivity of individual species and sexes towards OTA might be due to variations in the transport mechanisms and cellular uptake in renal cells. *In vitro* data with cell cultures indicate that OTA is a substrate for the family of organic anion transporter proteins. In humans, the best characterized of these transporters is the SLC22A (formerly OAT1) family that has a wide substrate specificity. The rodent equivalents of the human proteins include the members of the slc22a (formerly OATS) grouping (Russel *et al.,* 2002). Which specific transporters are responsible for OTA transport remains a matter of debate. Some workers have reported that transport occurs solely via the *para*-aminohippurate transport pathway, whereas others postulate that uptake occurs via passive diffusion, and/or non-specific binding and carrier-mediated processes (Dietrich *et al.,* 2005).

Support for the hypothesis of an involvement of transporters in the observed sex- and species-differences in OTA-mediated toxicity is also to be found in the observations of Buist and Klaassen (Buist, 2002; Buist and Klaassen, 2003) who demonstrated large sex-, age- and species-dependent variations in the expression levels of a number of organic anion transporters (Dietrich *et al.,* 2005).

As mentioned above, OTA is also a substrate for transporters of the ATP-dependent transporter family that excrete compounds out of the cells (Schrickx *et al.,* 2005). These transporters are assumed to play a role in the excretion of OTA-glucuronides by liver cells and in the kidneys. The fact that OTA is a substrate for various efflux transporters seems also to explain the typical excretion with milk, as observed in (lactating) females (Jonker *et al.,* 2005, Skaug *et al.,* 2001).

Finally, renal excretion and renal accumulation and hence toxicity may be influenced by other kidney diseases that impair renal functions. Various studies have demonstrated that the blood levels of OTA are higher in patients with chronic renal insufficiency treated with dialysis (for review see Fuchs and Peraica, 2005).

4.1. Toxicokinetics of OTA in primates

Because nonhuman primates are more likely to resemble humans than any other animal species, the toxicokinetics of OTA were investigated in vervet monkeys *Cercopithecus aethiops* (Stander *et al.*, 2001). Three female monkeys were treated intravenously with

OTA at doses of 0.8, 1.5 and 2 mg/kg b.w., respectively. Blood and urine samples were collected over a period of 21 days. Plasma and urine extracts were analysed by high-performance liquid chromatography followed by either fluorescence or negative ion electrospray ionization mass spectrometric detection. The obtained results suggest that the clearance of OTA from plasma followed a two-compartment model. The elimination half-life of OTA in the monkeys was determined to be 19-21 days and the average total body clearance was 0.22 mL/hour per kg b.w. The mean apparent volume of distribution of the central compartment and the peripheral compartment were of similar value (59 mL/kg).

4.2. Toxicokinetics of OTA in humans

The toxicokinetic profile of OTA was studied in one human volunteer following ingestion of 395 ng ^{3}H-labelled OTA (3.8 μCi) (Studer-Rohr *et al.*, 2000). A two-compartment open model was found to describe the *in vivo* data. This two-compartment model consisted of a fast elimination and distribution phase ($t_{1/2}$ about 20 hours) followed by a slow elimination phase (renal clearance about 0.11 mL/min) and a calculated plasma half-life of 35 days.

In addition, the intra-individual fluctuation of plasma levels of OTA was investigated in eight volunteers over a period of two months. The concentrations determined ranged between 0.2 and 0.9 ng OTA/mL plasma. The plasma levels in some individuals remained nearly constant over time, while others varied considerably (e.g. increase of 0.4 ng/mL within three days, decrease of 0.3 ng/mL within 5 days) during the observation period. The authors calculated a renal clearance varying between 0.093 and 0.109 mL/min (approximately 0.13 L/day) indicating that even a non-regular exposure (consumption of contaminated foods once a week or once a month) can result in persistent blood levels.

The major analyte in blood serum is the parent compound, and only small concentrations of OTA metabolites and/or conjugates could be measured (Studer-Rohr *et al.*, 2000). In contrast, analysis of urine samples indicated that only about 50% of the radioactivity in the urine was parent OTA, suggesting the presence of OTA metabolites (particularly ochratoxin α) and/or OTA glucuronic acid conjugates.

In conclusion, in all species studied to date (rodents, pigs, rabbits, fish, birds), OTA kinetics has been characterized by a open two-compartment model. In mammals, toxicokinetic studies have generally revealed a slow plasma clearance and subsequently a long half-life. Limited data indicate that the longest elimination half-lives has to be

expected in humans (35 days based on one individual) and non-human primates (19 to 21 days). These species differences seem to be attributable largely to differences in the degree of serum protein binding and its effect on renal clearance, as well as the rate of conjugation and extent of entero-hepatic re-circulation.

4.3. Internal dose and biomarkers of exposure in humans

4.3.1. Blood analyses

In recognition of the long serum half-life and the renal elimination of OTA, various studies present serum/plasma and/or urine analyses as markers of exposure. A summary of previous findings on the occurrence of OTA in blood samples of healthy persons is presented in the JECFA report (FAO/WHO, 2001). The high variability in the prevalence of positive samples may arise from differences in analytical sensitivity as well as difference in exposure rates. Comparing all reported data (n=3,717) blood concentrations were found to range between 0.1 and 10 ng/mL (with an exceptional maximum of 160 ng/mL). Recent surveys (see Table 7) confirm these previous findings.

For example, in Scandinavia, blood levels of OTA were determined in 406 blood donors (206 from Oslo, Norway, and 200 from Visby on the island of Gotland, Sweden), using an HPLC method (Thuvander *et al.*, 2001b). In connection with the blood collection, the test persons were asked to fill in a food questionnaire, with the aim of obtaining individual dietary information relevant to OTA exposure. The mean plasma level of OTA was 0.18 ng/mL in Oslo and slightly higher (0.21 ng/mL; p=0.046) in Visby. Consumption of several foods, including cereal products, wine, beer and pork, were to a minor degree related to high plasma levels of OTA. The strongest correlation between consumption patterns and blood levels was observed for women, corresponding to the consumption of beer or medium brown bread.

In Italy, serum levels of OTA were measured in a group of 138 healthy adults, living in the area of Florence (Palli *et al.*, 1999). OTA levels ranged between 0.12 and 2.84 ng/mL. Mean levels were significantly higher in men than in women (0.64 versus 0.50 ng/mL) and a strong correlation was found with the season in which blood samples were obtained, with summer values being higher than those taken in autumn.

In the Croatian cities of Osijek, Rijeka, Split, Varazdin and Zagreb, human blood samples were collected randomly from blood donors in June, September and December 1997, and March 1998 (Peraica *et al.*, 2001). OTA was measured in all samples (n = 983) using an HPLC technique with fluorescent detection. Samples containing OTA above the detection

limit (0.2 ng/mL of plasma) were found in populations from all Croatian cities at all collecting periods. The highest frequency (59%) of samples containing OTA above the detection limit and the highest mean concentration (0.39 ng/mL) were found in June. Both the frequency and the mean concentrations were lowest in all samples taken in December (36% and 0.19 ng OTA/mL, respectively). Osijek was the city with the highest frequency of OTA-positive samples (81%) and the highest mean OTA concentration (0.56 ng/mL).

In Morocco, samples from 309 healthy volunteers (213 males, 96 females) were analysed (Filali *et al.*, 2002). The analyses revealed that 60% of the human plasma sampled were positive for OTA (61.5% in the male and 56% in the female population), and an average concentration of 0.29 ng/mL (0.31 ng/mL in males, 0.26 ng/mL in females). The highest concentration found was 6.59 ng/mL. The authors concluded that, although the Moroccan population is exposed to OTA, the plasma levels were lower than those previously reported in some North African countries.

In Lebanon, exposure of populations to OTA was evaluated (Assaf *et al.*, 2004). Plasma samples from healthy individuals were collected from the different regions of Lebanon. OTA was detectable in 33% of tested plasma samples (n =250) with concentrations ranging between 0.1 to 0.87 ng/mL. No sex and age differences in plasma concentrations were found. The frequencies of OTA-positive plasma samples obtained in the South of Lebanon and in the Bekaa valley (50 and 47%, respectively) were significantly higher than plasma samples obtained in the Beirut/Mount Lebanon region (19%).

A comparison of these data with previous findings shows a tendency that in recent years the OTA blood serum levels in the human population seem to decline (FAO/WHO, 2001). Moreover, extreme peak levels are no longer reported. These developments can be attributed most likely to an increased awareness of the potential adverse health effects of mycotoxins, and to the preventive measures (establishing of maximal permissible levels in food commodities) taken by various countries, including the community regulations to be applied in all EU member states.

Table 7. Recent studies on the occurrence of OTA in blood samples of healthy humans.

Country	Collecting period	Number of positive samples (%)	Detection limit (ng/mL)	Mean plasma concentration (ng/mL)	Reference
Italy	1995 - 1996	134/138 (97)	0.1	0.56	Palli *et al.*, 1999
Croatia	1997	148/249 (59)	0.2	0.39	Peraica *et al.*, 1999
Croatia	1997-1998	468/983 (48)	0.2	0.30	Peraica *et al.*, 2001
Norway, Sweden	1997 - 1998	393/393 (100)	0.01	0.18 – 0.21	Thuvander *et al.*, 2001b
Morocco	2000	185/309 (60)	0.1	0.29	Filali *et al.*, 2002
Lebanon	2001-2002	82/250 (33)	0.1	0.17	Assaf *et al.*, 2004

In conclusion, in consideration of the large data set on human blood analyses presented by JECFA (FAO/WHO, 2001) and some recent analytical data, a tendency for a decline in the plasma concentrations in recent years can be noticed.

4.3.2. Urine analysis

Determination of OTA in human blood has been used as an indicator of human exposure to this mycotoxin from the diet (Breitholtz *et al.*, 1991; Zimmerli and Dick, 1995; Ruprich and Ostry, 1993; Maaroufi *et al.*, 1995). However, assessment has always been based on assumptions and extrapolations concerning OTA bioavailability and clearance rates based on animal data (Breitholtz *et al.*, 1991; Schlatter *et al.*, 1996). Analysis of OTA in human blood samples is compromised by the long half-life of the toxin, and frequent dietary exposure will result in a steady state concentration (Table 7). This is confirmed by a recent total diet study with 50 individuals, in which composite duplicate diet samples and plasma and urine samples were analysed over a period of 30 days. The method applied, using affinity column methodology achieved a sensitivity of 0.001 ng/g in composite foods, 0.1 ng/mL in plasma and 0.01 ng/mL in urine (Gilbert *et al.*, 2001). Using these sensitive techniques, a significant correlation was found between OTA consumption and OTA concentrations in urine (Gilbert *et al.*, 2001). Hence the latter method seems to be a suitable biomarker to estimate exposure OTA.

In Hungary, the OTA content of urine samples from 88 healthy humans living at five settlements in three counties was determined by immuno affinity column cleanup and high-performance liquid chromatography (Fazekas *et al.*, 2005). OTA was detected in 61% of the samples in an average concentration of 0.013 ng/mL (range: 0.006-0.065

ng/mL). The OTA concentration of samples from Heves County was significantly higher than that of samples from Hajdu-Bihar and Somogy counties.

4.3.3. Analysis of human milk

In humans (as well as in monogastric animals) OTA is excreted in breast milk. Recent evidence suggests that this excretion is supported by BCRP (breast cancer related protein), a member of the ATP-dependent efflux transporter family, known to be responsible for the excretion of various xenobiotics into milk (Jonker *et al.*, 2005; Schrickx *et al.*, 2005). Previous data from Italy had already indicated a high prevalence of OTA in human milk samples with concentration ranging between 1.2 and 6.6 ng/L milk (Micco *et al.*, 1991). More recently, a second study has been carried out involving 231 lactating women randomly selected in seven hospitals in Lombardy (Turconi *et al.*, 2004). Women's milk samples on the third or fourth day after delivery were tested to determine OTA levels. OTA was detected in 198 samples (85.7%) at an average value of 6.01 ng/L.

Data from milk samples collected from 80 Norwegian women indicated that 21% of human milk samples contained OTA in the range 10-182 ng/L. (Skaug *et al.,* 2001). The contamination of the milk was unrelated to smoking, age, parity, and anthropometric data other than body weight.

Analysis of human milk samples hence may serve as an additional marker of exposure. At the same time, OTA levels in human milk indicate the exposure of new-born infants, which needs to be considered in the overall risk characterisation.

4.3.4. Biomarkers of OTA toxicity in humans

Although experimental evidence (*in vitro*) indicated that OTA can induce micronuclei in human lymphocytes, the concentrations required to induce significant effects were almost a factor of 1000 higher than the levels measurable *in vivo* in human blood samples (Donmes-Altuntas *et al.*, 2003). The same applies to the *in situ* analysis of DNA damage by means of the Comet assay, which has been found to be a typical (but non-specific) marker of OTA effects in *in vitro* assays.

Analysis of urine samples for markers of proximal tubule damage associated with exposure to OTA is also considered to be non-specific. Although proteinuria and leakage of tubular enzymes have been described in controlled experiments with OTA, these

parameters cannot be used in epidemiological investigation as comparable changes can be induced by a variety of other nephrotoxic compounds, including commonly used drugs such as non-steroidal anti-inflammatory drugs (Dietrich, 2005).

In contrast, genetic analysis of patients from areas, in which Balkan Nephropathy is endemic, revealed typical cytogenetic changes such as chromosomal abnormalities (on position 3q25) (Toncheva and Dimitrov, 1996) and other loci of chromosome 3 (Toncheva *et al.,* 2002). However, no correlation between these findings and OTA has been established yet.

5. Toxicity of OTA

The toxicity of OTA has been extensively reviewed by the JECFA (FAO/WHO, 2001) where further details are available. Hence only the most relevant and recent findings are summarised below.

5.1. Renal toxicity

The kidney is considered to be the major target organ for OTA effects and OTA has induced renal toxicity in all mammalian species tested. Short-term studies in mice, rats, dogs, and pigs have shown a dose- and time-dependent development of progressive nephropathy. Significant sex- and species-differences have been observed in the sensitivity to the nephrotoxic action of OTA.

5.1.1 Studies in rats

In rats, the effects of OTA on renal function and morphology are indicated by increased relative kidney weight, urine volume, blood urea nitrogen, urinary glucose, proteinuria, and impaired urinary transport of organic substances. Renal lesions were histologically characterised by karyomegaly (large kidney epithelial cells with giant polyploid nuclei and prominent nucleoli), necrosis of tubular cells, and thickening of tubular basement membranes. The target site is specific, being the straight segment of the proximal tubule S3 in the outer stripe of the outer medulla. Male rats were found to be more sensitive than females (Munro *et al.,* 1974; Berndt and Hayes, 1979). In the study by Munro et al. (1974) groups of 15 weanling Wistar rats of each sex were fed on semi-purified diets containing OTA at concentrations equivalent to 0, 15, 75, or 370 µg/kg b.w. per day for

90 days. After 90 days, eight animals from each group were sacrificed, and the remaining rats were fed the control diet for an additional 90 days. In all groups, dose-related changes, involving karyomegaly and increased eosinophilia in cells of the proximal convoluted tubules, were seen in the kidneys after 90 days of treatment. In the high dose group the lesions persisted for an additional 90 days during which the animals received a control diet. In the low dose group the changes were mild and reversible. From these results, for Wistar rats a (sensitive) LOAEL of 15 µg/kg b.w. per day was derived.

The most comprehensive studies on OTA toxicity in rats have been performed within the US National Toxicology Program (US-NTP, 1989). In these studies, groups of 80 male and female Fischer 344/N rats were given OTA by gavage in maize oil at a dose of 0, 21, 70, or 210 µg/kg b.w. per day, 5 days/week for up to 103 weeks. The studies confirmed the specific site of renal injury and the sex differences in susceptibility. Renal lesions consisted of contraction and disorganization of the normal linear pattern of the S3 tubules due to marked development of karyomegaly and cytomegaly. The overall NOAEL derived from these studies was 21 µg/kg b.w. per day for 5 days/week, equivalent to 15 µg/kg b.w. per day (US-NTP, 1989; FAO/WHO, 2001).

Dortant *et al.* (2001) reported that old female rats (aged 27-30 months) are more sensitive to OTA than young adult rats (aged 12 weeks) regarding the induction of tubular karyomegaly and vacuolation/necrosis.

Petrik *et al.* (2003) showed that administration of 120 µg OTA/kg b.w. per day to Wistar rats, for 10, 30 or 60 days, produced oxidative stress and dose/time related apoptosis in both, proximal and distal epithelial kidney cells. OTA concentrations in the kidneys were proportional to the time of exposure, and amounted to 547, 753 and 930 ng OTA/g kidney tissue after 10, 30 and 60 days, respectively. Similarly, Domijan *et al.* (2004) reported that OTA administration to rats (0.25, 0.50 or 1.00 mg of OTA/kg b.w., i.p. three times a week for four weeks) produced time- and dose-related increased apoptosis in the kidneys. In a recent study, OTA was administered by gavage to groups of three male F344 rats at dose levels of 0, 0.25, 0.5, 1, and 2 mg/kg b.w. per day for 2 weeks. The typical pathological changes, including apoptosis were seen in the kidneys at all dose levels, with a clear increase in severity with higher doses. A dose-dependent increase in the expression of proliferating cell nuclear antigen (PCNA) was observed in kidneys, but not in livers of treated animals. The most prominent change in the composition of urine induced by OTA consisted of a major increase in the excretion of trimethylamine N-oxide (analysed by ^{1}H NMR and principal component analysis). This pattern was said to be unique and different to the typical changes observed with other proximal tubular toxins (Mally *et al.*, 2005b).

Studies in rats have shown a preventive effect of pre- or co-treatment with melatonin (10-20 mg/kg b.w./day) on parameters of OTA-induced liver and kidney toxicity (Aydin *et al.* 2003) and on OTA-induced oxidative stress, such as changes in liver and kidney glutathione peroxidase, superoxide dismutase and malondialdehyde (Meki and Hussein, 2001; Abdel-Wahhab *et al.*, 2005). In addition, aspartame (L-aspartyl-L-phenylalanine methyl ester; 25 mg/kg b.w. per day) has shown to provide protection against most of the nephrotoxic effects induced by OTA (289 μg/kg b.w. per day) (Baudrimont *et al.,* 2001). Bertelli *et al.* (2005) have reported that flavonoids in red wine may exert a protective effect against OTA nephrotoxicity in rats by limiting oxidative damage as measured by renal lipohydroperoxides, reduced and oxidized glutathione, and renal superoxide dismutase activity.

5.1.2. Studies in pigs

Pigs are generally considered as the animal species most sensitive to the nephrotoxicity of OTA. In a series of experiments, groups of three to six sows were given feed containing OTA at a concentration of 0, 0.2, 1, or 5 mg/kg, equivalent to 0, 8, 40, and 200 μg/kg b.w. per day, for periods of 5 days, 5 or 12-16 weeks, respectively, or up to 2 years. In a 3-month study a dose-related decrease in the renal activity of phosphoenolpyruvate carboxykinase and *gamma*-glutamyl transpeptidase was accompanied by a dose-related decrease in renal function, as indicated by a reduction in the maximal tubular excretion of *para*-aminohippurate and clearance of inulin, and an increase in glucose excretion. Only cytosolic, and not mitochondrial, phosphoenol-pyruvate carboxykinase activity was reduced. Progressive nephropathy but no renal failure was seen in female pigs given feed containing 1 mg OTA/kg (40 μg/kg b.w. per day) for 2 years whereas nephropathy was not observed after 0.2 mg OTA/kg (8 μg/kg b.w. per day) for 2 years. Effects on enzymes and kidney function were not examined in the 2-year study (Krogh and Elling, 1977; Elling, 1979a,b, 1983; Elling *et al.,* 1985; Meisner and Krogh, 1986; Krogh *et al.,* 1988; FAO/WHO, 2001). From these studies in pigs, a Lowest Observed Effect Level (LOEL) for effects on the kidneys (effects on enzymes and function) was established by the JECFA at 8 μg/kg b.w. per day in a 90-day feeding study (FAO/WHO, 2001).

In later studies, groups of 3 male and 3 female pigs were exposed to diets contaminated with strains of *Aspergillus ochraceus* producing OTA and penicillic acid (PA) and containing 90, 130, or 180 μg OTA/kg. Two pigs from each group were examined after 3 months. Microscopic lesions, as well as changes in various haematological and biochemical parameters, were observed in all groups. For an additional 2 months, the OTA levels in the diets were raised to 130, 305, or 790 μg OTA/kg. Histological

examination (2 pigs per group) at the end of the exposure period showed degenerative changes affecting the epithelial cells of the proximal tubules, which predominated at the initial stage, and proliferative changes in the interstitium, which predominated at the later stage of the disease (Stoev *et al.*, 2001). Subsequently, Stoev *et al.* (2002) reported only mild nephropathy in 3 male and 3 female pigs given a diet containing OTA at 800 μg/kg for 1 year. Histological examination showed two types of changes: degenerative changes affecting epithelial cells in some proximal tubules of pigs after 6 months, and proliferative changes in the interstitium which predominated after 1 year of exposure to OTA.

5.1.3. Renal toxicity of ochratoxin B

It is generally assumed that OTB is less toxic than OTA, although only few data are available (Bondy and Armstrong, 1998; Harris and Mantle, 2001). For example, male F344 rats were administered either a single dose of OTB (10 mg/kg b.w.) or repeated doses (2 mg/kg b.w., 5 days/week for 2 weeks). The animals were euthanised 72 hours after the last dosing. Histopathological investigations revealed a slight increase in mitotic figures in proximal tubule cells of animals treated with a single high dose of OTB, but no treatment-related changes were evident in clinical chemistry, in renal function, and histopathology after repeated administration. Excretion of OTB and metabolites in urine and faeces was analysed using both HPLC with fluorescence detection and LC-MS/MS. Ochratoxin beta, which results from cleavage of the peptide bond, was the major metabolite excreted in urine in addition to small amounts of 4-hydroxy-OTB. In total, 19% of the dose administered was recovered as OTB and ochratoxin beta in urine and faeces within 72 hours after a single dose). In contrast to OTA, no tissue-specific retention of OTB was evident after single and repeated administration (Mally *et al.*, 2005a).

Studies suggest that OTA and OTB have a similar potential to induce cytotoxicity *in vitro*, but differ largely in their potential to induce nephrotoxicity in rodents. OTB is more extensively metabolised and more rapidly eliminated than OTA. The lack of specific retention of OTB in the kidneys and the differences in toxicokinetics may therefore provide an explanation for its lower *in vivo* toxicity (Mally *et al.*, 2005a).

In conclusion, this brief summary of studies devoted to the assessment of renal toxicity of OTA indicates that karyomegaly and progressive necrosis of proximal tubule cells are the most typical markers of OTA induced nephrotoxicity. The presented data also indicate that pigs are generally considered as most sensitive animal species. In pigs, following a study with an exposure period to OTA of three months, a LOEL of 8 μg/kg b.w. per day was derived. At this level, measurable, but transient, alterations in renal-specific enzymes

and in the excretory function of the kidneys were observed. According to the few data available for OTB, the derivative is less toxic *in vivo*, and has a lower capacity to accumulate in the kidneys.

5.2. Neurotoxicity

OTA was shown to be neurotoxic *in vitro,* and *in vivo* in rats at oral doses of 0.12-0.29 mg/kg b.w. per day given for 1-6 weeks, as summarized by JECFA (FAO/WHO, 2001). New data have been published since then and are summarized below.

5.2.1. *In vitro* studies

In rat brain cells, 10-20 nM OTA was shown to increase the expression of genes involved in the brain inflammatory system (mRNA of peroxisome proliferator-activated-receptor, haem oxygenase-1, and inducible nitric oxide synthase) and to reduce the expression of glial fibrillary acidic protein, a constituent of the intermediate filaments in astrocytes (Zurich *et al.*, 2005). In rat embryonic midbrain cells, 0.5 and 1 µg OTA/mL caused a reduction in the number of viable cells, an induction of transcription factors activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB) activation as well as an inhibition of neurite outgrowth at the higher concentration (Hong *et al.*, 2002).

5.2.2. *In vivo* studies

Recent studies showed also neurotoxic effects in mice (Sava *et al.*, 2006), rats (Wangikar *et al.,* 2004b; Dortant *et al.,* 2001) and rabbits (Wangikar *et al.,* 2005). In mice, striatal dopamine was depleted in a dose-dependent manner at single i.p. doses of 3 mg OTA/kg b.w. or higher (up to 6 mg/kg b.w. tested) (Sava *et al.*, 2006). In addition, oxidative stress, oxidative DNA damage, and a transient inhibition of oxidative DNA repair was seen in different brain regions of these mice (cerebellum, cortex, hippocampus, midbrain, caudate/putamen, pons/medulla). In oral teratogenicity studies, brain lesions were seen in foetuses of rats (Wangikar *et al.*, 2004b) and rabbits (Wangikar *et al.*, 2005), when dams were treated during gestation with 500 µg OTA/kg b.w. per day or more (rats) or 50 µg/kg b.w. per day (rabbits), respectively. In another rat study, vacuolation of the white brain matter (cerebellar medulla and ventral parts of the brain stem) was significantly increased in female SPF Wag rats given 70 µg OTA/kg b.w. per day or more by gavage for 28 days (up to 1680 µg OTA/kg b.w. per day tested) (Dortant *et al.*, 2001). In a drinking water study by Delibas *et al.* (2003) with rats receiving 289 µg OTA/kg b.w. per

day for four weeks, reduced hippocampal N-methyl-D-aspartate (NMDA) receptor subunits 2A and 2B concentration were reported.

In conclusion, neurotoxicity associated with OTA exposure was reported at oral doses of 0.05-0.07 mg OTA/kg b.w. per day in rats and rabbits and with 3 mg OTA/kg b.w. per day in mice. Hence, the lowest neurotoxic dose of OTA is about six times higher than the dose leading to minimal renal changes in pigs in the 90-day study, as discussed above.

5.3 Immunotoxicity

The immunotoxicity of OTA was reviewed by JECFA in 2001 (FAO/WHO, 2001). The recent data from *in vitro* and *in vivo* studies, presented after the JEFA evaluation, can be summarized as follows:

5.3.1. *In vitro* studies

In an *in vitro* study performed by Alvarez-Erviti *et al.* (2005) with rat lymphoid cells using concentrations of 0, 0.5, 2, and 20 µM OTA (0, 0.2, 0.8 and 8 mg/L), the cytotoxic activity of natural killer cells was dose-dependently decreased and the cytotoxic T lymphocyte activity was significantly decreased at the lowest concentration only. The bacteriolytic activity of macrophages varied only slightly, and the proliferative response of lymphocytes to concanavalin A and lipopolysaccharide was not affected. In a study by Keblys *et al.* (2004) using very high concentrations, OTA inhibited the mitogen (Con A)-induced lymphocyte proliferation in purified lymphocytes from piglets (IC_{50} 1.3 µmol/L = 0.52 mg/L, which is several orders of magnitude higher than the highest plasma levels found in humans).

5.3.2. *In vivo* studies

In an oral toxicity study conducted according to OECD testing guideline 407, male Wistar rats received 0, 50, 150, or 450 µg OTA/kg b.w. per day by gavage for 28 days (Alvarez *et al.*, 2004). At the end of treatment, several immune function assays were performed. The natural killer cell activity was strongly affected in all treatment groups. The response of splenocytes to sheep red blood cells (SRBC) was decreased in a dose-dependent manner, but effects were not statistically significant. The cytotoxic T-lymphocyte activity was lowered in the 50 µg/kg b.w. per day group only; and the bacteriolytic capacity of macrophages was significantly reduced at 50 and 450 µg/kg b.w. per day. Thymus and spleen did not show significant differences from control.

In another oral 28-day study, young (12 weeks) and older (27-30 weeks) female SPF Wag rats (Dortant *et al.*, 2001) were given 0, 70, 340, or 1680 µg OTA/kg b.w. per day by gavage. Mortality occurred at the highest dose in the older rats. Decreased IgG levels were seen at 340 µg/kg b.w. per day or more in both age groups. In addition, OTA induced a dose-related reduction in the splenic T-cell fraction reaching statistical significance in the highest dose in young rats only.

There are also studies indicating immunotoxic effects in pigs. In a study by Harvey *et al.* (1992), 0 or 2.5 mg OTA/kg feed (about 100 µg/kg b.w. per day, calculated from feed consumption and b.w.) was given to growing pigs for 35 days. In OTA-treated pigs, reduced cutaneous basophilic hypersensitivity was seen after injection of the mitogen phytohaemagglutinin (PHA), as well as diminished delayed hypersensitivity responses, reduced macrophage activity and number of red blood cells phagocytosed, decreased stimulation index (blastogenesis) of lymphocytes to PHA, and decreased interleukin-2 production after stimulation with concanavalin.

In a more recent study by Stoev *et al.* (2000), growing pigs received 0, 1 or 3 mg OTA/kg feed (equivalent to 0, 6 or 200 µg OTA/kg b.w. per day) for up to 3 weeks. Although some immunotoxic effects were seen in the highest dose group, the results are difficult to interpret due to a concurrent, spontaneously occurring salmonellosis that lead to death in all animals of the highest dose group and in some animals of the low dose group.

In conclusion, signs of immunotoxicity were observed in limited studies with rats and pigs following oral doses of 0.05-0.1 mg OTA/kg b.w. per day given for 28-35 days. The lowest immunotoxic dose of OTA was at least six times higher than the dose leading to measurable renal changes in pigs in the 90-day study.

5.4. Reproductive toxicity

No adequate studies on reproductive toxicity of OTA are available, but several studies referring to developmental toxicity are summarized in previous evaluations (FAO/WHO, 1991, 1996, 2001; EC, 1998a) showing that OTA can cross the placenta and that it is embryotoxic and teratogenic in rats and mice. A more recent rat study supports those findings in respect to teratogenicity (Wangikar *et al.*, 2004a,b). In this study, pregnant Wistar rats (10 per group) were given doses of 0, 0.125, 0.25, 0.50 and 0.75 mg OTA/kg b.w. by gavage during days 6-15 of gestation. OTA caused a dose-dependent increase in various skeletal and visceral anomalies in foetuses, being statistically significant in the

two highest dose groups. Major anomalies included skeletal defects in skull, ribs, sternebrae and vertebrae (mainly failure or incomplete ossification), exencephaly, incomplete closure of skull, micrognathia, micromelia, scoliosis, small hind portion, and soft tissue defects such as hydrocephalus, microphthalmia, dilated renal pelvis, hydronephrosis, and cryptorchid testis. In addition, histological examination revealed an increased incidence of lesions in liver, kidney, brain and eyes as well as bile duct proliferation, new bile duct formation, defective ossification of cranial bones, hypoplasia of cerebellum, and defects of the retina.

A teratogenicity study on OTA was conducted in New Zealand White rabbits (5 per group) at oral doses of 0, 0.025, 0.05, 0.10 mg OTA/kg b.w. per day given by gastric intubation from day 6-18 of gestation (Wangikar *et al.*, 2005). In the highest dose group, there was a significant decrease in foetal weight, number of live foetuses and an increased incidence of malformed foetuses (skeletal and visceral anomalies, e.g. knuckling of fetlock, rudimentary tail or agenesis of tail, wavy ribs, hydrocephalus, microphthalmia and agenesis of kidney).

In conclusion, previous evaluations of the JECFA (FAO/WHO, 1991, 1996, 2001), the SCF (EC, 1998a) and recently published data indicate that OTA is teratogenic in mice, rats and rabbits in the dose-range between 0.1-1 mg OTA/kg b.w. per day given orally during gestation. The lowest teratogenic dose of OTA was about twelve times higher than the dose leading to minimal renal changes in pigs in the 90-day study.

5.5. Carcinogenicity

5.5.1. Experimental studies in rodents

As noted in the previous reviews by the SCF (EC, 1998a) and JECFA (FAO/WHO, 2001), OTA produces renal tumours in rats and mice with marked differences in species and sex specificity (US-NTP, 1989). Male animals are more sensitive than females and rats are considerably more sensitive than mice (see Table 8). The dose dependence of the incidence of renal tumours in the male rat found in the NTP study is shown in Table 9.

Table 8. LOELs and NOELs for karyomegaly and carcinogenicity of OTA in male rats and mice (US-NTP, 1989)

Species	Effect	Study duration	LOEL (µg/kg b.w. per day)	NOEL (µg/kg b.w. per day)
Mouse (male)	Kidney tumours	2 years	4,400	130
Rat (male)	Karyomegaly of proximal tubule cells	90 days	15	Not established
	Kidney tumours	2 years	70 [(a)]	21 [(a)]

a) Administered by diet in mouse and by gavage in rat on 5 days per week for 2 years

Table 9. Renal tumours and karyomegaly in male rats exposed to OTA in the NTP study (US-NTP, 1989)

OTA Dose (µg/kg b.w. per day) [(a)]	Adenomas	Carcinomas	Adenomas and carcinomas	Karyomegaly
0	1/50	0/50	1/50	0/50
21	1/50	0/50	1/50	1/50
70	6/51	16/50	20/51	51/51
210	10/50	30/50	36/50	50/50

a) Administered by gavage on 5 days per week for 2 years

Since this NTP study, in which administration was by gavage to rats, only few new data on the carcinogenicity of OTA were presented within the frame of the ochratoxin A risk assessment project[5] following dietary administration of OTA (Mantle *et al.*, 2005). These authors discuss that a statistical comparison of total carcinoma incidence in their study, with the NTP data, suggests that OTA is significantly less potent when given in feed than by oral gavage. However, only one dose level (approx. 300 µg OTA/kg b.w. per day) was used in the study presented by Mantle *et al.* (2005) and no details are presented. Hence taking into account the lack of information on the details of the study, it is not considered suitable as a basis for a quantitative risk assessment.

There are no experimental data on the carcinogenicity of OTA in domestic animal animals although renal toxicity, nephropathy and immune-suppression have been reported in several animal species. This lack of epidemiological data most likely reflects the short life span of farm animals.

[5] Ochratoxin A - risk assessment. Project No.: QLK1-2001-01614. Project homepage available at URL: http://www.uni-wuerzburg.de/toxikologie/EU-OTA/OchratoxinA.html.

5.5.2. Human epidemiological data

Various reports have suggested in the past that ochratoxin A might be involved in the pathogenesis of endemic diseases in the Balkan region, including Balkan Endemic Nephropathy (BEN) and associated urinary tract tumours (UTT). Based on the available data, the International Agency for Research on Cancer (IARC, 1993) classified OTA as possible human carcinogen (group 2B), based on the sufficient evidence for carcinogenicity in animal studies, but inadequate evidence in humans. At the last JECFA review (FAO/WHO, 2001) it had been also concluded that the available data did not provide a basis for calculating the likely carcinogenic potency in humans. This conclusion was taken in consideration of the unknown mechanisms by which ochratoxin A caused carcinogenicity, although both genotoxic and non-genotoxic modes of action had been proposed (see Sections 5.6 and 5.7). It was also stated that the epidemiological data on the Balkan Endemic Nephropathy (BEN) and associated urinary tract tumours (UTT) were inconclusive and hence it would not be excluded that other nephrotoxic agents are involved in the aetiology of BEN. Since 2001, no additional epidemiological data have become available.

5.5.3. Genotoxicity

The genotoxicity of OTA has been reviewed several times (e.g.: IARC, 1993; EC, 1998a; FAO/WHO, 2001). Notwithstanding the very large number of studies so far carried out, the genotoxicity of OTA, and in particular its mode of action, is still a matter of debate.

5.5.3.1. *In vitro* studies

At the time of the last SCF opinion (EC, 1998a) the following evidence was available: OTA was negative in conventional tests at the gene and chromosome level, i.e.: gene mutation assays in *Salmonella typhimurium* and *Escherichia coli* WP2, in mouse lymphoma cells (assay) and chromosome aberration test in Chinese Hamster Ovary (CHO) cells. Controversial results were obtained in assays for unscheduled DNA synthesis (UDS) and sister chromatid exchanges (SCEs). OTA was also negative for induction of mitotic crossing-over in *Saccharomyces cerevisiae* D3.

Induction of gene mutations was reported in *S. typhimurium* TA1535, TA1538 and TA100 strains only after incubation in the presence of conditioned medium derived from OTA–exposed primary rat hepatocytes. In a similar way, hepatocyte-mediated induction of SCEs was reported in human peripheral lymphocytes. Induction of gene mutations (predominantly large deletions) was observed in murine NIH/3T3 cell lines expressing different human P450 enzymes, with a shuttle vector as a tool for mutation analysis. The mutational spectrum was considered compatible with the induction of DNA single-strand breaks and oxidative DNA damage. Induction of micronuclei in ovine seminal vesicle (OSV) cells, lacking mono-oxygenase activity but expressing high levels of prostaglandin H synthase (PGHS) was reported also. The OTA-induced micronuclei were derived by a mixed clastogenic (predominant) and aneuploidizing mode of action. OTA induced DNA single-strand breaks in CHO cells and in kidney, liver and spleen cells from mice and rats (for detailed reference see FAO/WHO, 2001).

More recent studies published since the SCF opinion indicated that OTA induces gene mutations in *S. typhimurium* TA1535 and TA1538 strains following metabolic activation by mouse kidney but not liver microsomes, fortified with arachidonic acid or NADPH as cofactors. These findings led to the conclusion that OTA undergoes bioactivation by both prostaglandin H synthase (PGHS) and CYP450 isoforms in the kidney (Obrecht–Pflumio *et al.*, 1999).

Zepnik *et al.* (2001) could not observe mutagenic effects in *S. typhimurium* TA98 and TA100 strains when using rat liver microsomes and other enzymes for metabolic activation. Gene mutations were also not detected by Föllmann and Lucas (2003) in the *S. typhimurium* TA98, TA100, TA1535, TA1538, TA102 and TA104 strains and in the HPRT assay with Chinese hamster V79 fibroblasts, in the presence and in the absence of rat liver S9 enzyme mix. OTA did not induce gene mutations in *S.typhimurium* TA98 and TA100 after addition of HepG2-derived enzyme homogenate (S9 mix) (Knasmüller *et al.*, 2004). A significant increase of structural chromosome aberrations and SCEs associated with a reduction of the mitotic index was reported in experiments with cultured bovine lymphocytes (Lioi *et al.*, 2004). A clear effect of OTA on cell viability mediated by enhanced apoptosis was also observed. Induction of UDS was reported in rat hepatocytes, porcine urinary bladder epithelial cells, and primary human urothelial cells (Dorrenhaus *et al.*, 2000). Dopp *et al.* (1999) showed that OTA was able to induce micronuclei in cultured Syrian Hamster Embryo (SHE) cells. The results of kinetochore analysis revealed that mainly clastogenic events were involved in OTA genotoxicity. OTA caused a concentration-dependent induction of micronuclei and DNA damage detected by Comet assay in the human hepatoma-derived cell line HepG2 (Knasmüller *et al.*, 2004). By use of DNA–centromeric probes it was found that induction of micronuclei by OTA involves chromosome-breaking effects (55-60%).

OTA has been reported to be able to induce DNA single-strand breaks detectable by the Comet assay in several types of cell lines even in the absence of exogenous metabolic activation (Lebrum and Föllmann, 2002; Ehrlich *et al.*, 2002).

Recent results from Mosesso *et al.*[6] showed that OTA did not induce chromosomal aberrations and SCEs, but an increase of endoreduplicated and polyploid cells in Chinese hamster V79 cells both in the absence and in the presence of rat liver S9 fraction. OTA did not induce chromosomal aberrations and SCEs in human renal (ACHN) and bronchial (W126) cells lines expressing several CYP450 isoenzymes (CYP1A2, 2A6, 2B6, 2C9, 2D6, 2E1 and 3A4) as well as in a Chinese hamster epithelial liver cell line (CHEL) that retains metabolic competence. OTA did not induce micronuclei in cytochalasin B-blocked human lymphocytes, neither in the absence nor in the presence of rat kidney S9 fraction.

Dogliotti *et al.* showed that OTA caused a slight but significant increase in gene mutation frequency in Chinese hamster V79 cells in the absence and in the presence of S9 fraction from rat kidney. The mutagenic effects occurred only in a narrow range of concentrations. The molecular nature of OTA-induced mutations at the HPRT locus revealed a similarity with the spontaneous spectrum with an excess of A>C transversions and possibly large deletions. A similar mutagenic effect was also recorded in the *tk* mouse-lymphoma assay either in the presence or in the absence of S9 kidney fraction. Both events were considered compatible with oxidative-stress induced mutagenesis. Preliminary results of a mutation assay carried out in V79 cells indicated that the mutation frequency in the presence of the anti-oxidant, N-acetyl-L-cysteine (NAC), decreased compared to that observed in the absence of NAC. These findings support the hypothesis that the induction of gene mutations is mediated by oxidative DNA damage.

Ochratoxin B was devoid of genotoxic activity under identical conditions, but caused pronounced inhibition of cell division. Significant dose-dependent increases in the frequency of DNA single-strand breaks measured by Comet assay, and in the micronuclei frequency were observed in primary kidney cells from both male rats and humans of both genders at sub-toxic concentrations (Robbiano *et al.*, 2004).

[6] unpublished results from the Ochratoxin A - risk assessment. Project No.: QLK1-2001-01614. Project homepage at URL: http://www.uni-wuerzburg.de/toxikologie/EU-OTA/OchratoxinA.html.

5.5.3.2. DNA adduct formation

The evidence for formation of specific OTA-DNA adducts remains controversial, despite the increasing number of studies devoted to this issue (for review see Mally and Dekant, 2005, Pfohl-Leszkowicz and Castegnaro, 2005). Radiochemical assays (^{3}H and ^{14}C) did not have the sensitivity to detect OTA-specific adducts in rat kidney DNA after a single dose (1 mg/kg b.w.) or after *in vitro* incubation with microsomal preparations or cultured human and rat hepatocytes (10^{-5}–10^{-7} M) (Gautier *et al.*, 2001; Gross-Steinmeyer *et al.*, 2002). Using the unspecific ^{32}P-postlabelling assay, with nuclease P1-enrichment, TLC analysis showed several spots could be detected in chromatograms of the DNA fraction of liver and kidneys of rats and mice chronically exposed to high levels of OTA (0.4-2.5 mg/kg b.w.) for two years. The largest number of TLC-spots was found in chromatograms of DNA from male DA rats, a strain highly susceptible to OTA kidney carcinogenesis, with fewer spots in females and in Lewis rats. The higher relative adduct level (RAL) in male mouse kidney as compared to the liver and the female kidneys is consistent with the observation that male mice are more susceptible to renal carcinogenesis than are females (Faucet *et al.,* 2004; Pfohl-Leszkowicz and Castegnaro, 2005). The chemical structure of these spots visible in the TLC chromatograms has not yet been identified, and hence there is no indication that the spots represent genuine OTA-DNA adducts.

Recent experiments aimed to identify the chemical structure of potential OTA adduct. In *in vitro* studies, incubation of OTA with DNA or dG in the presence of various activation systems did not reveal any adducts, neither by ^{32}P postlabelling nor by LC-MS/MS (Mally *et al.*, 2004). Subsequently, DNA was isolated from the livers and kidneys of male Fisher rats treated with a single dose of ^{14}C-OTA (0.5 mg/kg b.w.) and analysed by ^{14}C-accelerated mass spectrometry (AMS). No significant difference between control group and treated group could be observed in ^{14}C-activity in isolated DNA from treated animals, and no specific OTA-DNA adducts were detected with AMS (Mally *et al.,* 2004). A concern in the interpretation of these results is that DNA adducts may have been repaired, as DNA was isolated 72 hrs after a single treatment with a relatively low dose, which is in contrast to other studies, in which DNA was isolated within 24 hours after exposure to OTA.

Previously it had been also postulated that OTA-DNA adducts might originate from the reaction of the hydroquinone derived from OTA with cellular DNA. In these experiments adduct formation was studied using ^{3}H-OTA as substrate, and HRP and PGHS as activation system to facilitate the formation of the hydrochinone. DNA adducts were measured by scintillation counting (Gautier *et al*., 2001). Moreover, Faucet *et al.* (2004) showed that OTA-3´-dGMP adducts can be generated by photoirradiation. It was

hypothized that by this reaction an OTA phenoxy radical if formed by oxidative dechlorination, which subsequently metabolised into an OTA hydrochinone (Dai *et al.*, 2004). The chemical structure of the OTA-DNA adducts were not been completely characterized but it was suggested that these represent C-C8 and O-C8 OTA-dGMP. These synthetic adducts appear to co-chromatograph with adducts initially described with the ^{32}P-post labelling technique when kidneys of pig following sub-acute exposure (0.20 mg/kg b.w.) and in rats after chronic exposure (thrice weekly, two years; total dose 100 mg/kg b.w.) were analysed (Faucet *et al.,* 2004; Pfohl-Leszkowicz and Castegnaro, 2005). However, the co-chromatography was only demonstrated with one set of chromatographic conditions and is thus remains to be confirmed. No clear association between exposure and TLC-spots and between TLC-spots and carcinogenicity could be demonstrated in any of the animal experiments.

In conclusion, the data on DNA adduct formation remain controversial, despite the various reports on adducts detectable by ^{32}P-postlabelling techniques under different conditions. It cannot be excluded that these adducts represent non-specific oxidative DNA adducts. As yet, chemical analysis, even with advances methods such as AMS failed to detect DNA adducts that contain ochratoxin A or parts of this molecule.

5.5.3.3. Oxidative DNA damage

***In vitro* studies**

OTA induces oxidative stress in cells, which can result in either lipid peroxidation eventually resulting in etheno-DNA adducts or direct oxidation of DNA in e.g. the 8-position of guanine. Oxidized DNA bases can be determined directly by ED-HPLC or immunological methods. Alternatively, the Comet assay demonstrates the oxidative damage resulting from treatment with OTA following incubation of cells with formamido-pyrimidine glycosylase (Fpg) which is known to convert oxidative DNA damage into strand breaks. OTA has been shown to induce oxidative damage to DNA, as measured by Comet assays in V79 and CV-1 cell lines and in primary rat kidney cells at low, non-cytotoxic concentrations (Kamp *et al.*, 2005a). Moreover, OTA was shown to induce DNA strand-breaks as assessed by comet assay in liver, kidney and spleen of F344 rats given doses of 0, 0.25, 0.50, 1.0, 2.0 mg/kg b.w./day for 2 weeks, (5 days a week) (Mally *et al.*, 2005b). In liver and kidney, the extent of DNA damage was further enhanced in a dose-dependent manner in the presence of Fpg, suggesting the presence of oxidative DNA damage. In the same experiments, DNA damage was also induced by OTB in rat kidney and spleen despite its lower cytotoxicity compared with that of OTA (Mally *et al.,* 2005a).

Recently several exocyclic DNA adducts derived from lipid peroxidation (LPO) products have been identified, e.g. ethenobases, formed by reaction of trans-4-hydroxy-2-nonenal with DNA. Another major DNA lesion associated with LPO is the adduct formation by the reaction of malondialdehyde with guanine. Thus, it cannot be excluded that the spots observed by the ^{32}P-postlabelling method are due to these adducts, and hence are not related to OTA itself, but formed via an indirect mechanism. This hypothesis is supported by the observation that treatment of animals with antioxidants prior to OTA exposure significantly reduced the number and intensity of DNA fragments on TLC plates (Pfohl-Leszkowicz *et al.*, 2002). A high level of endogenous DNA damage has been found in human tissues, and this damage has been related to products formed by LPO (Barbin, 2003).

Recent findings provide evidence that OTA affects several cell signalling pathways including the activation of mitogen-activated protein (MAP) kinases, extracellular signal regulated (ERK 1/2) kinases and C-jun amino terminal (JNK 1/2) kinases in renal cells *in vitro* (Schilter *et al*., 2005). Moreover, new toxicogenetic data, based on assessment of the gene expression profile of rats that were exposed to OTA (at concentrations equivalent to approximately 300 µg/kg b.w for a period of up to two years) indicated that OTA exposure resulted in alterations of the expression of genes associated with cellular calcium homeostasis and disruption of pathways regulated by the transcription factors HNF4α (hepatocyte nuclear factor 4 alpha) and Nrf2 (Nuclear factor erythroid 2-related factor 2). Previous data had suggested already that a reduction of HNF4α pathways may be associated with renal carcinogenicity, whereas depletion of Nrf2 –regulated enzymes (among others glutathione) is associated with an impairment of the defence potential of a cell against oxidative stress (Marin-Kuan *et al.*, 2006)

In vivo and *ex vivo* studies

In the SCF opinion (EC, 1998a) it was reported that OTA did not induce SCEs in Chinese hamster bone marrow cells *in vivo*, while it was able to induce DNA single-strand breaks in kidney, liver and spleen of mice and rats. In a recent paper, Mally *et al.*, (2005b) reported that OTA induces DNA single-strand breaks as assessed by Comet assay in a dose-dependent manner in the liver, kidney and spleen of F344 rat (doses: 0, 0.25, 0.50, 1.0, 2.0 mg/kg b.w./day for 2 weeks, 5 days a week). In liver and kidney, the extent of DNA damage was further enhanced in the presence of Fpg, thus suggesting the presence of oxidative DNA damage. Similar DNA damage was induced by ochratoxin B (OTB) despite its lower cytotoxicity compared to that of OTA (Kamp *et al*., 2005a,b). A small, non-significant increase in the incidence of chromosomal aberrations (essentially

chromatid and chromosome-type deletions) was observed in splenocytes from these rats cultured *in vitro* to express the chromosomal damage. These aberrations were considered compatible with oxidative DNA lesions, but are not typical for genotoxic agents that bind covalently to the DNA.

In conclusion, within the limit of sensitivity of the analytical procedures, there is no clear evidence for the formation specific OTA-containing adducts. Exposure to OTA results in the formation of endogenous adducts, some of which may be a consequence of reactive oxygen species, either directly or indirectly through the formation of lipid peroxidation products. Taken together, these data provide suggestive evidence for the role of oxidative stress in renal pathologies associated with OTA.

6. Risk assessment

6.1. Hazard characterisation

In the light of the recent findings relating to hazard characterisation for OTA, the Panel concluded that the most sensitive and pivotal effects of OTA are its effects on the kidneys in rats and pigs. Therefore, in the absence of conclusive evidence that OTA binds to DNA, hazard characterisation should be based on the nephrotoxicity. The data available for a quantitative assessment can be summarized as follows:

- In a two-year gavage study in Fischer 344/N rats the LOAEL for nephropathy was 70 µg OTA/kg b.w. per day, 5 days per week, equivalent to 50 µg/kg b.w. per day whereas the NOAEL was 21 µg OTA/kg b.w. per day, 5 days per week, equivalent to 15 µg/kg b.w. per day. However, in a 90-day study using Wistar rats, the dose level of 15 µg OTA/kg b.w. per day by gavage produced mild and reversible renal changes.
- In the female pig the LOAEL for progressive nephropathy was 40 µg OTA/kg b.w. per day with the diet for 2 years, whereas 8 µg OTA/kg b.w. per day for 2 years was a NOAEL for nephropathy in the pig. However, in a 90-day feeding study in female pigs 8 µg OTA/kg b.w. per day was reported to produce effects on renal enzymes and renal function tests.

On the basis of these studies the Panel concluded that 8 µg OTA/kg b.w. per day is a LOAEL that represents an early marker of renal toxicity in experimental animals (i.e. female pigs) and likely to be close to a NOAEL, as the observed changes in biochemical parameters indicate transient changes in the kidneys.

Various studies had indicated that the extent of renal injury is dose-dependent, but also associated with the duration of exposure, as OTA accumulates in renal tissue. The Panel noted that there were significant differences in the toxicokinetics of OTA between species, particularly with regard to the degree of protein binding. The plasma half life ($t_{½\beta}$) in (one) human was reported to be 35 days, in monkeys approximately 20 days, in Wistar rats 5 days and in pigs about 6 days.

It is prudent to assume that the amount of OTA accumulated in the target organ (the kidney) over time, following daily dietary intake of OTA, will depend on the total body burden obtained at steady state. The body burden at steady state is a function of the daily intake of the compound, its bioavailability (absorption), and its biological half-life in the species in question. Assuming that the oral bioavailability of OTA is similar in humans and pigs, it might then be considered that a daily dose in humans that is 6 times lower than that in pigs will lead to similar body burdens at steady state. Only limited data are available on inter-individual variability in toxicokinetics in humans. Although the half-life was derived from data for a single individual who ingested one single dose slightly higher than the expected daily exposure from the diet, the renal clearance in that subject was similar to the clearances reported in a group of eight subjects studied over a period of 2 months.

In consideration of these findings, the Panel used the LOAEL of 8 μg OTA/kg b.w. per day that represents an early marker of renal toxicity in the pig to derive a tolerable intake of OTA for humans by applying a set of uncertainty factors. To account for interspecies differences (extrapolation from pig to humans) the default factor of 2.5 was used for toxicodynamic differences (WHO-IPCS, 1999) and a factor of 6 for the kinetic differences (half-life) in consideration of the kinetic data mentioned above. For the extrapolation from average humans to potentially sensitive human sub-populations the common uncertainty factor of 10 was used (WHO-IPCS, 1999; WHO, 2005[7]). In addition, a factor of 3 was applied in order to take into account the use of this LOAEL instead of a NOAEL. This additional uncertainty factor is in line with the WHO-IPCS (1999) recommendations stating that when a NOAEL has not been achieved but the LOAEL is of sufficient quality, this LOAEL should form the basis of the risk assessment.

Thus, a composite uncertainty factor of 450 was applied to the LOAEL of 8,000 ng OTA/kg b.w. per day which would result in a tolerable daily intake of approximately 18 ng OTA/kg b.w. per day.

[7] WHO, 2005 ; Harmonization Project No 2 : http://whqlibdoc.who.int/publications/2005/9241546786_eng.pdf

Given the relatively long half-life of OTA in humans, the Panel concluded that a tolerable weekly intake would be more appropriate. In turn a Tolerable Weekly Intake (TWI) of up to 120 ng/kg b.w. was established.

6.2. Risk characterisation

Exposure levels for average adult consumers (2 – 3 ng/kg b.w. per day corresponding to a maximum of 21 ng/kg b.w. per week) and high adult consumers (6 - 8 ng OTA/kg b.w. per day, corresponding to approximately 40 and 60 ng/kg b.w. per week) were established using recent SCOOP data from all member states and the draft EFSA concise data base on food consumption in three representative EU member states. The Panel noted that these estimates, although being considered as conservative due to the broad food categories used in the EFSA concise data base) were below the TWI of 120 ng OTA/kg derived by the Panel. However, it cannot be excluded, that infants and children as well as distinct segments of the population representing high consumers of certain locally produced food specialties experience higher rates of exposure to OTA. Therefore, continued efforts to prevent food contamination with OTA need to be encouraged and a continuing monitoring programme should allow the detection of known and emerging food commodities with high level of contamination.

CONCLUSIONS

- Ochratoxin A (OTA) is produced by several species of the genus *Penicillium* and *Aspergillus* that can contaminate food commodities prior to harvest or more commonly during storage. Many of these mould species produce not only OTA, but also other ochratoxins, including ochratoxin B (OTB). OTB is found rarely and in much lower concentrations in food commodities, and was also less toxic compared to ochratoxin A in recent *in vivo* studies. Cereals, pulses, coffee, wine and grape juice as well as dried fruits and spices have been found to be contaminated frequently with OTA. Human exposure has been confirmed by chemical analysis of biological samples (blood, urine, milk) from randomly selected healthy humans.

- The exposure assessment presented in this opinion combines food analysis data with actual food consumption data, using the draft EFSA "Concise Food Consumption Database" for three EU member states (France, Italy and Sweden). Estimated exposure levels for *average consumers* (2 to 3 ng/kg b.w. per day) and *high consumers* (6 to 8 ng/kg b.w. per day) suggest that the exposure to OTA varies between 15 to 20 ng/kg b.w. per week, and 40 and 60 ng/kg b.w. per week, for low and high consumers, respectively. These estimates are considered as being conservative for the adult EU population as broad food categories were used in the assessment of consumption. However, it cannot be excluded that infants and children as well as distinct segments of the population, representing high consumers of certain locally-produced food specialities, may experience higher rates of exposure.

- Various studies in humans have associated OTA with an endemic kidney disease observed in the Balkans (Balkan Endemic Nephropathy and related Urinary Tract Tumours), but convincing epidemiological evidence associated with OTA exposure is currently lacking.

- OTA is nephrotoxic in all animal species tested, and exerts immunotoxic, neurotoxic and teratogenic effects at higher dose levels. Renal lesions are characterised by karyomegaly and necrosis of tubular cells and thickening of the tubular basement membranes. The observed effects are dose- and time-dependent. There is increasing evidence that renal toxicity caused by OTA is associated with cellular oxidative stress.

- OTA accumulates in the kidneys and experimental data on the toxicity of OTA unambiguously identify the kidney as the most sensitive target organ. Following

long-term exposure it induces kidney and liver tumours in rodents, but only at nephrotoxic doses.

- Studies on the genotoxicity of OTA remained controversial, as conventional mutagenicity testing gave negative results, whereas results obtained with ^{32}P-postlabelling techniques suggested the existence of DNA adducts. Gene mutations or sister chromatid exchange have been demonstrated in some studies in modified test systems using sub-cellular fractions and primary cells as activating systems, while others failed to demonstrate these effects. Increasing evidence suggests that the various genetics effects seen in *in vitro* and *in vivo* studies are compatible with the hypothesis of DNA damage induced by oxidative stress rather than indicating a direct interaction (adduct formation) of OTA with cellular DNA. Furthermore, chemical analysis failed to identify specific DNA adducts of OTA or its metabolites.

- In considering all the toxicological and mechanistic data, the Panel established a Tolerable Weekly Intake (TWI) of 120 ng/kg body weight.

RECOMMENDATIONS

- It is recommended that efforts should continue to reduce OTA-contamination of foods. Monitoring programmes to describe known sources of exposure and to identify potential emerging sources are recommended as the re-evaluation of OTA indicated that infants and children as well as distinct segments of the population, representing high consumers of certain locally-produced food specialities, may have high rates of exposure to OTA.

- There is a need for more data on the relationship between maternal exposure to OTA and the resulting human milk concentrations.

- Adequate studies on reproductive and developmental toxicity are needed.

- There is also a need for more data on species-specific accumulation of OTA in the kidney, particularly the renal tubule.

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