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Mycotoxins Affecting the Kidney

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INTRODUCTION

Although their existence and, indeed, some of their effects on human and animal health have been known for some time, the first mycotoxin was positively identified and chemically characterized in the early 1960s (Nesbit et al., 1962). Mycotoxins have been linked to one of the "ten plagues of Egypt" as described in both the Ipuwer papyrus and the Bible. In recent times, numerous additions have been made to the ever-growing list of mycotoxins and their analogues. This list currently encompasses more than 300 substances which have been isolated and chemically characterized from pure cultures (Steyn, 1995).

WHAT ARE MYCOTOXINS? A BRIEF OVERVIEW

Mycotoxins are secondary metabolites produced by a variety of mould and fungi species. While their actual function in moulds and fungi has not been definitively identified, one possibility is that they enable competition with other microorganisms for nutrients and space. For example, it has been suggested that production of ochratoxin A and citrinin by *Aspergillus* and *Penicillium* species may interfere with the

uptake of iron in competing microorganisms (Stormer and Hoiby, 1996). A further suggestion is that the production of mycotoxins could contribute to the generation of favorable germination conditions for fungal spores. These theories remain to be clarified.

Of the 300 substances identified to date, approximately 20 may be found with disturbing regularity in foodstuffs and animal fodder (Steyn, 1995). The majority of these are produced by moulds of the *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, and *Claviceps* species and have been implicated in biological effects as diverse as mutagenicity, teratogenicity, neurotoxicity, immunotoxicity, to name but a few, in both animals and humans. In contrast, some mycotoxins have also been suggested to have beneficial activities, displaying antitumor, antimicrobial, and cytotoxic effects.

Many crops that are used for the production of both animal and human foodstuffs harbor fungi which, given the correct conditions of humidity and temperature, produce mycotoxins, either in the field or during suboptimal storage. Thus, mycotoxin contamination is a particular problem in moist, warm climates. Contaminated grain, which is harvested and milled, finds its way into both animal and human food supplies and, due to their heat stability, mycotoxins are not destroyed by industrial processing or domestic cooking. Whereas in former times dietary intake of mycotoxins depended on several factors, including culture, socioeconomic grouping, and local climatic conditions (Studer-Rohr et al., 2000), nowadays, due to the worldwide availability of practically every crop, the importance of geographical factors has diminished, making the complete avoidance of mycotoxin consumption practically impossible.

Structurally, mycotoxins form a diverse group of organic compounds of low molecular weight, which vary from simple compounds with a carbon chain length of four (e.g., moniliformin), to complex substances consisting of several ring structures (e.g., phomopsin) (Culvenor et al., 1989). It is likely that the myriad of biological effects reported for mycotoxins can probably be attributed to this structural diversity, at least in part (Figure 21.1).

THE KIDNEY AS A TARGET ORGAN

As a prerequisite for its role as an organ of excretion, reabsorption, and general homeostasis, the kidney has an extensive bloodflow, receiving

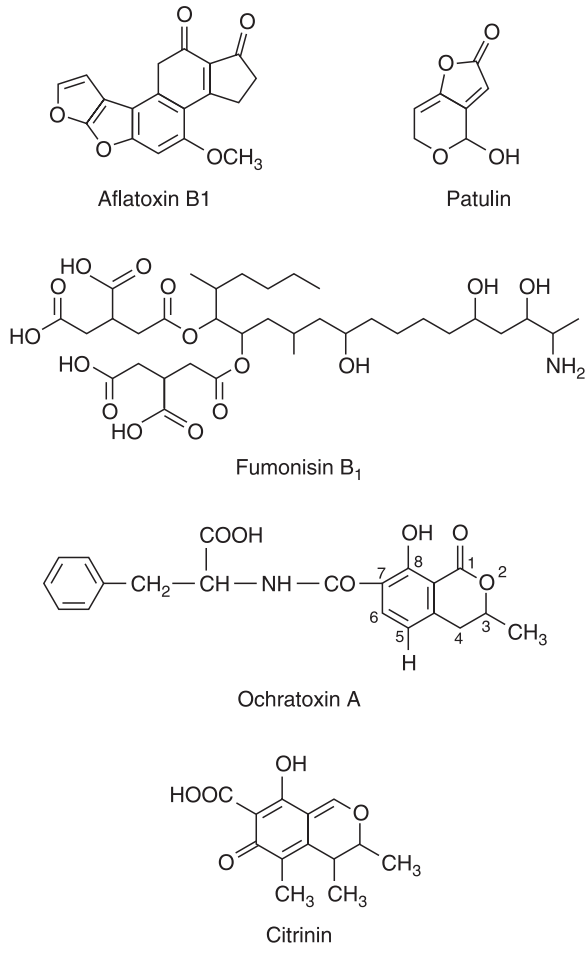

Figure 21.1 Molecular structures of the mycotoxins described in this chapter.

approximately 1.2 l/min and filtering on average 125 ml plasma/minute. The processes of reabsorption and secretion, particularly of organic acids and bases, may, however, lead to the accumulation of toxins within the tubules, making this vital organ more susceptible to toxic insults than other organs. The very nature of the kidney makes early clinical diagnosis of mycotoxin-induced nephrotoxicity difficult, if not impossible. Therefore, much research is now focusing on determining the toxic mode of action of these compounds both *in vivo* and *in vitro*,

with particular emphasis on defining the species and sex differences in toxicity that are characteristic of much mycotoxin-mediated renal toxicity.

Not least among the factors crucial in the development of renal toxicity is the presence of multiple organic anion transporters (OATs). These transporters actively eliminate drugs and toxic compounds, and their metabolites, which may become hazardous upon accumulation. Most of these transporters are confined to the proximal tubule. These highly developed transport functions, coupled with its concentrating ability, render the proximal tubule as the region of the kidney most at risk from toxic insult. Numerous organic anion and organic cation transporter systems have been found and molecularly characterized with respect to affinity, kinetics, and inhibitor specificity. Some, such as the OAT1, are classical *p*-aminohippuric acid (PAH)/dicarbonate exchangers, which mediate high-affinity PAH uptake in a sodium-dependent manner. Others function as organic anion/glutathione antiports (Oatp1) or as peptide/H^+ symports (PEPT2).

The mechanism and actual physiological function of many transporters, however, remains to be determined. A complete review of organic anion transporter molecules is beyond the intention and scope of this manuscript, however several excellent reviews and papers are available (Koepsell et al., 1999; Russel et al., 2002; Uwai et al., 1998; van Aubel et al., 2000). These transporter systems have a particular relevance for the toxicity of mycotoxins, several of which have been demonstrated to be transported by renal organic anion transporters (Loe et al., 1997; Rappa et al., 1997; Tsuda et al., 1999). Indeed, the distribution of these transporters may play a role in the preferential toxicity of certain mycotoxins for specific organ systems.

NEPHROTOXIC MYCOTOXINS

Almost all mycotoxins examined to date possess at least some nephrotoxic potential. In many cases, this is dependent on species and mycotoxin concentration, and in some cases is secondary to the effects on other organ systems. Therefore, a complete and comprehensive review of all mycotoxins that have been demonstrated to cause renal damage is beyond the intention and scope of this text. The following sections will therefore concentrate on those known to have the kidney as one of their primary sites of action.

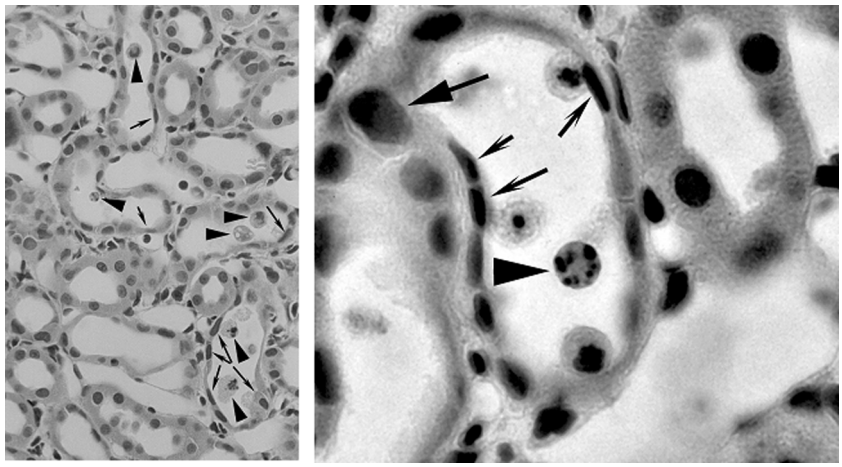

Figure 21.2 Hematoxylin-eosin stained kidney section of a male rat, treated with 1 mg/kg ochratoxin A for 7 days: (a) demonstrating a high number of necrotic (arrowheads) exfoliated or regenerative (small arrows) epithelial tubules cells in the inner part of the cortex (original magnification: ×400); (b) demonstrating exfoliated cells seemingly undergoing apoptotic necrosis (arrowheads). Regenerative epithelial cells (small arrows) as well as cells (large arrows) with giant nuclei can be observed within the same affected tubule (original magnification: ×800). (Reprinted from Rásonyi et al. (1999), with permission from Elsevier Science).

Aflatoxin B_1

After the discovery of their causal role in the deaths of thousands of turkeys, ducklings, and chicks in England in 1960, aflatoxins became the first mycotoxins to be extracted, identified, and chemically characterized (Asao et al., 1963; Nesbit et al., 1962). Up until the beginning of 2002, over 5,000 research papers on aflatoxins had been published, making them the most studied of all mycotoxins.

Aflatoxins are a family of substituted coumarins (Figure 21.1) containing a fused dihydrofuran moiety. They are produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*, but also by certain *Penicillium* and *Rhizopu* strains (Medicine, 2002; Searle, 1976). The family consists of four main members, aflatoxin B_1, G_1, G_2, and B_2. Aflatoxin B_1 (AFB_1) is the major toxin produced in culture and also the most toxic of the four – it is classified as a Group I hepatocarcinogen in

humans by the International Agency for Research on Cancer (IARC) (IARC, 1993). Consequently, it is the best studied of the group.

As is the case for many, if not all, mycotoxins, large species differences in susceptibility are evident, and the route of administration is important. Of the common laboratory species, the guinea pig is one of the most sensitive to the effects of AFB_1, with an observed LD_{50} of 2 mg/kg body weight via oral gavage and intraperitoneal administration yields an LD_{50} of 1.4 mg/kg (Netke et al., 1997). More recently, exposure to feed containing 3 mg/kg AFB_1 has been shown to be sufficient to cause severe renal damage in chicks (Valdivia et al., 2001). An LD_{50} was, however, not determined in this study. Rats are slightly less sensitive, with an LD_{50} of 7 mg/kg body weight in feeding experiments (Rati et al., 1991), although this has been determined to be age-dependent, with younger F344 rats being less sensitive (LD_{50} >150 mg/kg body weight) (Croy, 1981). Large differences within species have been reported, with intraperitoneal LD_{50} values for various allogenic mouse strains ranging from 9 to 60 mg/kg body weight (Hayes and Campbel, 1986; Mako et al., 1971). In contrast, another study reported no mortality in four different inbred mouse strains (C57B1/6, CBA/J B10A, and Balb/c) despite exposure to 60 mg/kg body weight AFB_1 (Almeida et al., 1996). Similarly, non-inbred CD-1 Swiss mice display an LD_{50} greater than 150 mg/kg body weight (Croy, 1981). These species and strain differences appear to arise due to genetic variability in the expression levels of the cytochrome P450 mixed function oxidases, of which at least five are involved in the conversion of AFB_1 to its reactive metabolite, AFB_1-8,9-epoxide (Autrup et al., 1996; Eaton and Gallagher, 1994).

The relative insensitivity of mice to AFB_1-mediated hepatotoxicity toxicity has been attributed to a higher rate of transformation of AFB_1 to its demethylated derivative AFP_1 and to other water-soluble metabolites in mice than in other species, resulting in faster elimination of the mycotoxin and its metabolites (Almeida et al., 1996; Ramsdell and Eaton, 1990). These observations also correlate well with those of Wong and Hsieh (1980) who demonstrated that the mouse displays a high first-order elimination constant for AFB_1, whereas in the rat, the equilibrium transfer rate constant favors a relatively high concentration in plasma and hence a longer half-life. In contrast, Autrup and colleagues (1996), who noted a threefold higher concentration of adducts formed in murine kidney than in liver following AFB_1 exposure, have proposed that mice may in fact be more susceptible to its nephrotoxic effects than its hepatotoxic effects.

Until relatively recently, the bulk of research carried out has focused on the hepatotoxic potential of AFB_1, which greatly overshadows the renal toxicity. Early observations of renal hypertrophy, tubular congestion, and epithelial degeneration, such as those of Newberne and coworkers (1964), were made for completeness in studies on hepatotoxicity rather than as detailed investigations of renal toxicity. Hayes (1980) reported that bovine renal tissue retained the highest concentration of AFB_1 and its metabolite M_1 following feeding with radioactively-tagged AFB_1. In contrast, in pigs, most of the administered AFB_1 was to be found in the liver following oral dosing, with only minor residues in renal tissue (Lüthy et al., 1980). Based on these observations and the findings of Wong and Hsieh (1980) that species susceptibility can be correlated to tissue distribution, the first study designed specifically to investigate the renal toxicity of AFB_1 in rats was carried out by Grosman and coworkers (1983). They described decreased glomerular filtration rate, decreased tubular glucose reabsorption, and decreased tubular transport of *p*-aminohippurate (PAH) in Wistar rats following a single intraperitoneal dose of AFB_1 (100 μg/kg body weight). These authors also observed increased urinary excretion of both sodium and potassium and a twofold increase in urinary γ-glutamyl transferase activity, which persisted for more than 48 hours after injection. These authors hypothesized that the nephrotoxic effects observed in rats following AFB_1 exposure were possibly due to effects on the glomerular basement membrane. This thesis is supported by the findings of Valdiva and coworkers (2001), who noted a thickening of the glomerular basement membrane following AFB_1 exposure in chicks.

Pathology

Morrissey and coworkers (1987) described characteristic histopathological changes in the kidneys of Sprague-Dawley rats exposed to 2 mg/kg body weight/day AFB_1 for 4 days, the most obvious of which was a dark red band between the medulla and the cortex, coupled with a relatively pale renal cortex. Closer examination revealed tubular necrosis, particularly in the inner cortex, with some pycnotic nuclei. Most nuclei were swollen and many displayed a clear center with the chromatin arranged around the periphery. This degeneration was observed to progress even following discontinuation of AFB_1 exposure, with the inner cortex becoming more necrotic, an increase in the number of pycnotic nuclei, and the presence of large quantities of nuclear debris in

hematoxylin- and eosin-stained paraffin sections. These findings were supported by Rati et al. (1991) who observed nuclear enlargement in the tubular epithelium with proliferating anaplastic cells in the cortical region following subacute feeding experiments over 36 weeks with a 24 week washout phase. These authors also reported that one animal presented with massively necrotic and timorous kidneys. However, the tumor did not appear to arise as a consequence of the anaplastic cells in the cortical regions as none of the other similarly exposed animals presented with tumors up to 24 weeks following the exposure phase. Indeed, AFB_1 has not been associated with the development of renal tumors in any species tested to date. Guinea pigs respond to AFB_1 with minimal acute multifocal nephrosis of the renal tubules. This is only present following exposure to the LD_{50} dose (Netke et al., 1997).

All of the aforementioned renal effects occurred in combination with one or more severe hepatic lesions, including hepatoma and hepatocarcinoma (Rati et al., 1991), centrilobular necrosis, endothelial cell degeneration (Netke et al., 1997), and bile duct proliferation (Morrissey et al., 1987).

Mechanism of Action

It is generally accepted that both the hepatotoxicity and renal toxicity of AFB_1 are due to the generation of a reactive metabolite, namely AFB_1-8,9-epoxide (Almeida et al., 1996; Autrup et al., 1996; Busby and Wogan, 1984). This metabolite is produced rapidly by the action of at least five members of the mixed function oxidase family (Autrup et al., 1996; Eaton and Gallagher, 1994) and its concentration reaches a maximum six to twelve hours post dosing in F344 rats (Chou et al., 1993). AFB_1-8,9-epoxide reacts with DNA to yield the 8,9-dihydro-8-(N7-guanyl)-9-hydroxyaflatoxin B_1 adduct (AFB_1-N^7-Gua), which has been positively correlated with DNA strand breaks and hepatic tumor development in the rat (Bechtel, 1989). This adduct has also been shown to be positively correlated with the development of renal lesions in the mouse (Chou et al., 1997). In 1981, Croy and Wogan compared the generation of AFB_1-N^7-Gua in the livers and kidneys of both rats (F344) and mice (CD-1 Swiss). They reported a tenfold greater level of AFB_1 modification per nucleotide residue in rat liver than in rat kidney. In contrast, in the mouse, modification levels were threefold higher in the kidney than in the liver. This correlates well with both the relative species and organ sensitivities. Chou and colleagues (1993, 1997) have confirmed these

results for the rat, but in contrast found the number of adducts in mouse liver and kidney to be similar. This difference may, however, be due to the use of a different mouse strain ($B6C3F_1$) in this study.

As a result of observations that the toxicity of AFB_1 correlates with the metabolic ability of a particular species or strain (Eaton and Gallagher, 1994), several groups have investigated the effects of reduced caloric intake on AFB_1-mediated toxicity. Indeed, a reduction of food intake results in reduced metabolic activity and hence reduced carcinogenic potential for a number of genotoxic substances (Shaddock et al., 1993). In the case of AFB_1, a similar phenomenon has been demonstrated with respect to hepatotoxicity and renal toxicity in rats (Chou et al., 1993) and in mice (Almeida et al., 1996; Chou et al., 1997), respectively. A 40% reduction in dietary caloric intake significantly reduces hepatic activation of AFB_1 in B63CF1 mice and this correlates with a reduction in the total AFB_1–DNA adduct formation in the mouse kidney (Chou et al., 1997). The authors suggest this to be due to a decrease in the activity of CYP2C11-dependent AFB_1 metabolizing enzyme or an increase in AFB_1-specific glutathione *S*-transferase activity (Chen et al., 1995). This theory is supported by the observation that glutathione reduces the ability of AFB_1-8,9-epoxide to bind to DNA and proteins, both *in vivo* and *in vitro*, and its depletion is associated with increased AFB_1-N^7-Gua adduct numbers and increased tumor rates in rats (Gopolan et al., 1993) and mice (Autrup et al., 1996).

Exposure to a single dose of AFB_1 has been demonstrated to reduce renal and hepatocellular proliferation in F344. In the liver, this reduction is then compensated by extensive and massive (140 to 250% of control levels) regenerative cell proliferation following termination of exposure (Chou et al., 1993). This proliferative response, which has been suggested to form the basis for the genesis of hepatic tumors, could be prevented by restriction of caloric intake. In the rat kidney, however, the rate of DNA synthesis was not increased above control levels and caloric restriction had no effect on the level of DNA synthesis. These results support the proposed organ specificity of AFB_1 toxicity in the rat.

In a study carried out by Grosman and colleagues (1983), AFB_1 was shown to impair the function of the organic acid transport system as measured by reduced PAH accumulation in renal slices. This was coupled with increased intracellular sodium and decreased intracellular potassium, suggesting a loss of normal control of membrane permeability; which could result in the observed renal effects. This is supported by a further study, which showed AFB_1 to dose-dependently reduce

Na^+/P_i cotransport in opossum kidney (OK) cells, which were used as a model for proximal renal epithelial cells. This effect does not appear to be due to a generalized inhibition of sodium cotransport as sodium-dependent uptake of L-alanine was not affected, but rather a specific inhibitory effect on renal reabsorption of inorganic phosphate (Glahn et al., 1994). The authors suggest that this effect is also due to the conversion of AFB_1 to its active metabolite AFB_1-8,9-epoxide, which then binds to DNA and RNA. This alters cellular functions, such as hormone synthesis, the responsiveness of renal tissue to hormones such as insulin and parathyroid hormone, and the activity of protein kinases, adenylate cyclases, and cyclic nucleotide phosphodiesterases, leading to the observed renal toxicity.

In conclusion, the nephrotoxicity and hepatotoxicity of AFB_1 appear both to be mediated by the generation of the major active metabolite AFB_1-8,9-epoxide. However, whereas the downstream events leading to the generation of hepatic tumors have been relatively well characterized, much work remains to be done to deduce the order of events involved in its nephrotoxicity.

Citrinin

The organic anion citrinin is a benzopyran metabolite produced by toxic strains of *Penicillium* and *Aspergillus* species. As such, it can be coproduced with ochratoxin A and an isocoumarin ring is common to the structure of both (Figure 21.1). Both of these mycotoxins have been associated with the development of Balkan endemic nephropathy and urothelial tumors in humans. This is discussed in the section dealing with ochratoxin A and mycotoxin interactions (see below). Originally, citrinin was suggested for use as an antibiotic due to its marked antibacterial activity (Hetherington and Raistrick, 1931). However, animal tests demonstrated it to be severely nephrotoxic, with the detrimental effects far outweighing any potential benefits (Ambrose and DeEds, 1946; Blanpin, 1959). Citrinin has been demonstrated to be acutely toxic in several species, including rabbit, rat, mouse, and hamster, with LD_{50} values for intraperitoneal administration of 50, 64, 80, and 75 mg/kg body weight, respectively (Hanika and Carlton, 1983; Jordan and Carlton, 1977, 1978; Jordan et al., 1978b). Toxicity varies considerably with route of administration, with 134 mg/kg representing the oral LD_{50} in rabbits.

Some hepatotoxic effects have been reported for citrinin, however the lethal effects are largely due to a severe nephrotoxicity that is very

similar in manifestation and progression in all species tested to date. It is characterized clinically by an increased excretion of dilute urine, which is thought to result from an impaired capacity for urine concentration. Other features include reduced glomerular filtration rate and renal blood flow resulting in increased blood urea nitrogen concentration, urinary lactic acid dehydrogenase, aspartate amino transferase, and isocitric dehydrogenase activities, as well as proteinuria, glucosuria, lowered urinary specific gravity, and the presence of necrotic cells in the urinary sediment (Jordan et al., 1978a; Kogika et al., 1996; Lockard et al., 1980).

These functional changes are associated with acute tubular necrosis. The location of the most severe tubular damage varies from species to species. In the mouse (Jordan and Carlton, 1977) and in hamsters (Jordan et al., 1978b) the distal portion of the nephron is mostly affected, whereas proximal segments are most at risk in the rat (Lockard et al., 1980), rabbit (Ambrose and DeEds, 1946), guinea pig (Thacker et al., 1977), and pig (Krogh et al., 1973). Despite this, the specific pathologies resulting from acute citrinin exposure are remarkably similar in all species. Initial pathological observations report the kidneys of acutely exposed mice to be swollen and pale, with stippling of the capsule cortex and outer medulla (Jordan and Carlton, 1977; Hanika and Carlton, 1983). Similar findings have been reported for other tested species (Hanika and Carlton, 1983; Jordan and Carlton, 1978; Jordan et al., 1978b).

Closer histopathological examinations of the kidneys of exposed animals of several species reveals a similar picture of citrinin-related damage, including necrosis and desquamation of renal epithelial cells of the proximal tubules, basement membrane thickening, tubule dilation, and proliferation of cells in the interstitium. In rats, the primary site of action appears to be the renal cortex and the outer part of the renal medulla, particularly the straight segments and the distal convoluted tubule (Jordan and Carlton, 1978), with the tubules in these areas displaying marked necrosis and deposition of protein casts. Almost identical observations have been made in hamsters (Jordan et al., 1978b).

Necrosis of individual cells or small groups of renal tubular cells has also been described in the straight segments and the distal convoluted tubule of the mouse (Jordan and Carlton, 1977) and in anesthetized dogs. In dogs, this is confined to the S_2 cells and is characterized further by a loss of brush border and apical vacuoles and a displacement of the organelles away from the luminal margin (Krejci et al., 1996). The protein

casts observed in mice following citrinin exposure are, in contrast to the observations made in the rat, more commonly located to the tubules of the inner medullary area (Jordan and Carlton, 1977). This variation in the primary site of citrinin-mediated damage may be due to species differences in the function of various segments. Experiments carried out by Hanika and colleagues (Hanika and Carlton, 1983) demonstrated the renal injury caused by exposure to citrinin to be nonprogressive, and indeed reversible, at least in the rabbit. Renal tubule regeneration, starting in the convoluted segments and proceeding into the straight portions, could be observed as early as 3 days after exposure and recovery was almost complete by the seventh day. A similar regenerative response has also been made in one study using rats (Lockard et al., 1980). Hanika and colleagues also described the occurrence of a slight heterophilic inflammatory response in the kidneys of exposed rabbits. This has not been described for other species and the authors suggest that this may be responsible for the mild interstitial fibrosis evident in some animals following recovery.

A curious observation made by Jordan and Carlton (1977) is that although the number and severity of citrinin-induced renal lesions and pathology in mice can be positively correlated with the dose, multiple injections of a similarly toxic dose increases neither the number nor the severity of observed lesions nor does it increase mortality. These authors suggest two possible explanations. A citrinin-sensitive population of cells may be destroyed by the initial dose and the remaining cells are insensitive to subsequent doses of citrinin. Alternatively, the initial citrinin exposure may induce increased production of enzymes responsible for citrinin detoxification leading to a more rapid metabolism and excretion of subsequent doses.

Mechanism of Action

The mechanism of action of citrinin remains unclear. Direct effects on the kidney have been observed and the extrarenal vascular and parasympathomimetic (e.g., reduction in blood pressure) and local irritant actions of citrinin noted in *in vivo* experiments have also been suggested to indirectly affect renal function and ultrastructure (Ambrose and DeEds, 1946; Hanika and Carlton, 1983; Krejci et al., 1996). Indeed, some of the electrolyte disturbances that have been associated with renal effects may actually be due to dehydration as a result of emesis and diarrhea

immediately following citrinin administration (Kitchen et al., 1977; Krejci et al., 1996).

Studies carried out by Berndt and Hayes (1982; Berndt, 1983) indicated the toxicity of citrinin to be related to its active tubular transport and accumulation in the kidney. Pretreatment of rats with the organic anion transporter blocker probenecid significantly reduces renal but not hepatic accumulation. In this study, probenecid was demonstrated not only to reduce citrinin accumulation in renal tissue, but also to ameliorate the effects of the toxin on rat mortality. These results correlated well with experiments into renal function, as measured by renal slice transport, carried out *in vitro* by the same authors, which demonstrated that accumulation of PAH into renal slices could be suppressed by citrinin and that this effect could be significantly reduced by preincubation with probenecid. These authors also described a reduced toxicity of citrinin in newborn rats and hypothesized this to be a result of either the lower level of renal transport (Tune, 1974) of the parent compound or to reduced renal or hepatic metabolism of citrinin to reactive metabolites in newborns.

In more recent studies, Chagas et al. (1992a, 1992b, 1995) have demonstrated citrinin to disrupt normal management of the mitochondrial membrane and hence normal membrane potential function in both liver and kidney mitochondria. The effects were primarily directed toward monovalent cation permeability, resulting in a disruption of the fluidity of the inner mitochondrial membrane. In these investigations, the kidney was clearly far more sensitive to these effects and this correlates well with the known organ specificity of citrinin toxicity.

Citrinin-mediated cytotoxicity has been demonstrated in an *in vitro* test system using Madin-Darby bovine kidney (MDBK) cell line and primary fetal bovine kidney (PFBK) cells (Yoneyama et al., 1986). Although these experiments were carried out using extremely high concentrations ($EC_{50} = 3.8 \times 10^{-4}$ M), and their relevance to the *in vivo* situation is hence questionable, the authors reported several findings that reflected those seen *in vivo*, including cellular swelling and loss of cell–cell contact. Moreover, PFBK cells were noted to require tenfold higher concentrations of citrinin to display similar levels of cytotoxicity to that observed in the continuous cell line, although primary cells would generally be expected to be more sensitive to toxic insult than continuous cell lines. The authors did not attempt to explain this difference, however there are two possible explanations. One possibility is that immature animals have an inherently lower expression level of

transporter molecules, thus allowing reduced access of citrinin to the mitochondria. Alternatively, the preparation and culture procedure itself may reduce the number or activity of the membrane-bound transporters. Either way, the net result is a reduction in intracellular citrinin accumulation and an apparently reduced sensitivity. This supports the importance of the role of organic anion transporters in the renal toxicity mediated by citrinin and other mycotoxins.

The relative roles of the parent compound and reactive metabolites in citrinin-mediated toxicity remain to be definitively elucidated. Berndt and colleagues (Berndt and Hayes, 1982; Berndt, 1983) support the theory that citrinin itself is responsible as no renal metabolism of citrinin could be detected in their investigations. In contrast, the presence of the urinary metabolite dihydrocitrione has been demonstrated (Dunn et al., 1983), however, only 10 to 20% of citrinin is excreted as metabolites hence the possible role in renal toxicity is as yet, unknown.

In summary, the nephrotoxic actions of citrinin appear to result from active uptake into the kidney, probably of the parent compound, which then impairs the normal regulation of mitochondrial metabolism.

Ochratoxins

Like many other mycotoxins, ochratoxins belong to those that are produced by *Penicillium* and *Aspergillus* species (Scott et al., 1972). They may be produced in the field or, more commonly, as a result of improper or suboptimal storage conditions of grain, coffee, dried fruits, etc., and are known to be common contaminants of human foodstuffs such as bread, cereals, beer, wine, etc. (Speijers and van Egmond, 1993; Studer-Rohr, 1995; Wolff et al., 2000). Three different ochratoxins, (A, B, and C) with differing toxicities have been isolated and characterized. Of these, ochratoxin A (OTA) is both the most commonly detected and the most toxic, followed by ochratoxin B (OTB) and C (OTC) (Li et al., 1997; van der Merwe et al., 1965). OTA is a weak organic acid and consists of a dihydroisocoumarin moiety linked to L-phenylalanine (Figure 21.1) and is classified under Group 2B (possibly carcinogenic to humans) by the IARC (IARC, 1993). A variety of other toxic effects, including teratogenesis (Fukui et al., 1992; Hood et al., 1976; Shreeve et al., 1977) and immunotoxicity (Harvey et al., 1992; Müller et al., 1999a, 1999b; Stoev et al., 2000), in several species have been attributed to OTA. It is, however, the renal toxicity, and in particular renal carcinogenic effects, that have commanded the most attention. OTB, differing from OTA only

by the lack of a chloride at the 5-position on the isocoumarin-ring moiety (Figure 21.1), is significantly (10- to 20-fold) less toxic *in vitro* and *in vivo*.

OTA generally displays a relatively low acute toxicity, although large species differences are apparent in sensitivity (oral LD_{50} values range from approximately 20 and 46 to 58 mg/kg body weight in rats and mice, respectively, to 0.2 to 1 mg/kg body weight in pigs, cats, rabbits, and dogs). In contrast, it is the subchronic and chronic effects of OTA that are of greatest concern. OTA is considered causal for the nephropathies observed in several species of agricultural animals, particularly in pigs (Krogh et al., 1976; Stoev et al., 1998a), resulting in huge financial losses in agriculture and consequently in the food industry.

Functional deficits resulting from OTA exposure include increased urinary concentrations of glucose, proteins, leucine aminopeptidase, and γ-glutamyl transferase, coupled with a decrease in serum cholesterol and protein concentrations. Creatinine clearance rates are reduced and urinary specific gravity is markedly reduced. This decreased ability to produce concentrated urine is a direct consequence of impaired tubular function (Stoev et al., 1998b).

The renal pathological lesions caused by chronic OTA exposure, characterized by progressive tubular atrophy coupled with proliferation of fibroblastic connective tissue (Krogh et al., 1976) and progressing to activation and proliferation of vascular endothelial and adventitial cells (Stoev et al., 1998a), were first observed in pigs, and are described in the classical Danish model of mycotoxic nephropathy. This nephropathy results in reduced food intake and hence reduced weight gain by the animals. Furthermore, detection of OTA in meat and meat products leads, in certain countries (e.g., in Scandinavia), to the condemnation of this produce as unfit for consumption.

Chronic exposure to OTA is also thought to be involved in the etiology of two human kidney disease states, namely Balkan endemic nephropathy (BEN) and urothelial tumors. BEN is a chronic progressive kidney disease, first described for populations in the lowland regions of the river Danube. It is characterized by progressive tubulointerstitial nephropathy, leading to tubular atrophy, periglomerular fibrosis, and cortical cysts, inevitably leading to end-stage renal failure (Tatu et al., 1998). Urothelial tumors, i.e., the malignant tumors of the upper urinary tract, which often accompany BEN, are extremely aggressive in nature (Sostaric and Vukelic, 1991; Vukelic et al., 1991) and some studies have suggested a slightly higher disease incidence in females.

Although no direct link has been established, epidemiological data correlates a moderate increase in serum OTA levels with a higher incidence of nephropathy and urothelial tumors in humans. Studies carried out in several countries, where climatic conditions or suboptimal storage of grain and grain products promote OTA production by fungal species, have also indicated a link between dietary intake of OTA and the development of renal and urothelial tumors (Kuiper-Goodman, 1999; Maaroufi et al., 1995a, 1995b; Radic et al., 1997; Tatu et al., 1998).

The wealth of research that has been carried out into the effects and mode of action of OTA to date reflects its economic and social importance. Despite this, however, many questions remain unanswered.

Pathology

The pathology associated with OTA exposure varies between species. Initial examinations following feeding experiments in pigs revealed enlarged kidneys, which, when decapsulated, had a greyish appearance, indicative of fibrosis (Krogh et al., 1976; Elling, 1983). Closer examination revealed that the initial lesions occur in the proximal tubules and are characterized by desquamation and focal degeneration of the epithelial cells, coupled with focal peritubular fibrosis and thickening of the basement membrane. The severity of these lesions is dose- and time-dependent. Elling and colleagues also demonstrated the effects of OTA in young pigs to be more severe than in adult animals and that the renal damage caused by OTA exposure appears to be permanent as a return to contamination-free fodder did not reduce the incidence and severity of pathological changes (Elling, 1979, 1983). Renal tumors have so far not been observed in pigs. This may, however, be due to the relatively young age at which commercially raised pigs are slaughtered, thus not allowing for the relatively long latency and developmental periods typical for renal tumors.

Apart from a reduction in kidney size (in contrast to the increase seen in pigs), the pathomorphological effects noted in humans suffering from BEN are similar in nature to those noted in pigs. Kidneys also have a greyish appearance and are difficult to cut – giving the first indication of the underlying diffuse cortical fibrosis, which extends into the corticomedullary junction. In more advanced stages of the disease, the epithelium becomes severely degenerative and necrotic and hyperplastic arteriopathy is evident (Vukelic et al., 1991).

The similarities between the pathology observed for BEN and urothelial tumors in humans and that observed in pigs following chronic dietary intake of OTA have led to a number of laboratory studies that aimed to establish a definitive link between OTA exposure and renal disease. Chronic (two-year) studies in rats have demonstrated a clear causal relationship between OTA exposure and renal cortical tumor development, with 60% of male rats developing renal cell carcinoma (RCC), coupled with a distinct pathology of the pars recta (P_3) of the proximal tubule (Boorman, 1989; Boorman et al., 1992; Rásonyi et al., 1993). While no urothelial tumors or preneoplastic lesions of the transitional epithelium or the renal pelvis were reported, several prominent nonneoplastic lesions were observed in the renal cortex. These include degeneration of the renal tubular epithelium in the inner cortex and the outer stripe of the outer medulla, protein casts, karyomegalic nuclei, and renal cortical cysts, which were morphologically distinct from those present in aging rats. Hyperplastic lesions, which became apparent following nine months of exposure, were restricted to a single tubule. Longer exposure resulted in malignant renal cell adenomas and carcinomas which were often bilateral and/or multiple. In the same study, female rats were found to be much less susceptible to OTA-mediated toxicity, displaying a milder P_3 pathology and only a 6% tumor incidence. Even more pronounced sex differences have been noted in the carcinogenic response in mice where, despite being exposed to a 20-fold higher dose (4,800 mg/kg body weight/day for 2 years) than that employed in Boorman's study, only 28% of male mice presented with renal tumors, while female mice were totally refractive (Bendele et al., 1985).

Despite intensive research, the mechanism of OTA-induced carcinogenicity in rodents remains to be elucidated. In view of the decisive species differences and the lack of knowledge of the underlying mechanism, any human risk assessment is, at best, unreliable. The currently accepted virtually safe dose for human renal cancer risk of 0.2 ng/kg/day has been extrapolated from the rodent studies cited above. However, two important factors were not considered in this extrapolation. First, in rodents, OTA is primarily excreted via the biliary route, whereas in humans, the primary route of excretion is urinary (Appelgren and Arora, 1983; Fuchs and Hult, 1992; Fuchs et al., 1988), resulting in the delivery of higher concentrations of OTA to the human kidney. Second, and perhaps more critical, the half-life of OTA in humans (35.3 days) is approximately 14 times longer than that of the rat

(DFG, 1990; Li et al., 1997; Studer-Rohr et al., 2000). Hagelberg and Hult (Hagelberg et al., 1989) have proposed that this enormous species variation in half-life may be caused by differences in renal clearance rates due to different plasma protein binding characteristics. These authors also demonstrated that OTB possesses a far lower affinity for plasma proteins and is more rapidly eliminated than OTA in the species tested (fish, quail, mouse, rat, and monkey), observations that correspond well with the comparatively lower toxicity of OTB.

Mechanism of Action

It is currently unknown how OTA mediates its toxicity. The mechanistic background to the stark species and sex differences remains enigmatic. One possibility is that these differences are governed by specific renal handling of OTA; for example, through variations in the transporter and binding protein complements of renal cells from different species. Indeed, early experiments indicated OTA to be a substrate for the organic anion transport system (Sokol et al., 1988; Stein et al., 1985). Accumulation of OTA in rabbit renal basolateral membrane vesicles (BLMV) (Sokol et al., 1988) and OK cells (Gekle et al., 1994) was reported to occur solely via the PAH transport pathway. In contrast, Groves and colleagues, working with suspensions of isolated rabbit renal proximal tubules (Groves et al., 1998), concluded OTA accumulation to be a combination of passive diffusion and nonspecific binding and carrier-mediated processes. A more recent study has indicated OTA to be accumulated into mouse P_2 and P_3 renal cells stably transfected with the human organic anion transporters hOAT1 and hOAT3 (Jung et al., 2001). The authors describe saturable, dose- and time-dependent uptake of ^{3}H-OTA, which they assume to be localized to the basolateral membrane of the proximal tubule. The K_m values determined for the hOAT1 and hOAT3 in this study were two- to threefold higher than that determined by Groves et al. in their study with rabbit proximal tubule cells.

Heussner and colleagues (2002) describe the presence of at least one homogeneous binding component with low affinity but high capacity for OTA in renal cortical homogenates from pig, mouse, rat, and human, of both sexes. The binding of ^{3}H-OTA to these proteins could be competed by a range of substances known to have affinity for steroid receptors or for various organic anion transporters previously reported to be responsible for the transport of OTA (Tsuda et al., 1999). Heussner and

colleagues reported a capacity ranking for specific OTA-binding of human > rat > pig ≥ mouse, which correlates with the toxicity ranking for experimental animals *in vivo* and, furthermore, suggests an even higher sensitivity for humans. Sex differences could, however, only be detected for the rat, with males having a higher binding capacity. Based on the pattern of protein binding competition, the authors suggested that the binding component does not belong to organic anion transporters previously described.

Similarly, a further study has demonstrated that primary renal epithelial cells originating from pigs or rats accumulate more OTA than their continuous cell line counterparts (O'Brien et al., 2001). Moreover, in this study, primary human renal epithelial cells were also shown to accumulate 10 to 15 times more OTA than the other cell types tested (LLC-PK_1, NRK-52E, NRK-49F, primary porcine kidney cells). Indeed, primary renal epithelial cells obtained from female donors were the most sensitive to the cytotoxic or antiproliferative effects of OTA in this study. A significant decrease in cell numbers could be detected following just 48 hours exposure to 1 nM OTA, a concentration that reflects normal serum levels of OTA in BEN areas. In agreement with the known toxicities of OTA and OTB, an approximately tenfold higher concentration of OTB was required to induce a comparable reduction in cell numbers. In this study, a maximal reduction in cell number was achieved following 48 hour exposure to 10 μM OTA. Approximately 15% of cells from all species tested survive and can reenter the cell cycle and proliferate following removal of the toxin, even following exposure to higher concentrations ($\leq 100\ \mu$M) and longer exposure times (≤ 96 hours). The authors suggest the remaining cells to represent a subpopulation that are resistant to OTA-mediated toxicity and possibly apoptotic-defective (Dreger et al., 2000; O'Brien et al., 2001). Interestingly, although NRK-49F cells (a rat renal fibroblast cell line) accumulated comparable amounts of OTA to their epithelial (NRK-52E) counterparts, these cells were relatively insensitive to OTA-mediated cytotoxicity (O'Brien et al., 2001). Similar observations have been made for primary human renal fibroblasts (O'Brien, personal communication). The authors thus propose that BEN could be caused by a cytostatic or cytotoxic effect of OTA in epithelial cells, while fibroblasts are more refractive. This would lead to increased or maintained proliferation of fibroblasts coupled with reduced epithelial proliferation or epithelial cell death, resulting in the gradual and progressive replacement of healthy tissue with the fibrotic tissue characteristic for BEN.

The molecular mechanism of action remains controversial. OTA has been reported as both nonmutagenic (IARC, 1993; Kuiper-Goodman and Scott, 1989) and mutagenic (Obrecht-Pflumio et al., 1999) in a variety of microbial genotoxicity tests. The formation of DNA adducts by a reactive metabolite (Castegarno et al., 1998; Creppy et al., 1985; Pfohl-Leszkowicz et al., 1991, 1993), sister chromatid exchange (Föllmann et al., 1995), unscheduled DNA synthesis (Doerrenhaus et al., 2000), and the generation of reactive oxygen species have been proposed as candidate mechanisms. However, neither the oxidative changes nor the DNA adducts reported could be corroborated using HPLC-MS or LC-MS/MS (Gautier et al., 2001a; Zepnik et al., 2001). Furthermore, although the levels of DNA-adducts formed in male rats (determined by the ^{32}P postlabelling method) appeared to be higher than that in females, thus suggesting a correlation between DNA-adducts and the known sex differences in OTA-mediated renal carcinogenicity, no clear correlation could be determined between the level of DNA-adducts and the incidences of adenocarcinoma or karyomegaly reported (Castegarno et al., 1998). Indeed, while approximately 42% of female Dark Agouti (DA) rats were reported to present with DNA-adducts, some of which were at the same levels as the exposed male rats, none of the female DA rats presented with either karyomegaly or renal epithelial tumors, thus questioning the relevance of the adducts in the ^{32}P postlabeling method.

Obrecht-Pflumio and Dirheimer (2001) have reported the generation of DNA and deoxyguanosine-3′-monophosphate adducts using mouse microsomes. The incidence and number of adducts reported, using the ^{32}P postlabeling method, was much higher than that previously reported for the Lewis, Sprague-Dawley, or DA rat, which is in complete conflict with *in vivo* observations of renal tumor incidence. Furthermore, other researchers have reported poor metabolism of OTA via P450 peroxidases and little glutathione conjugate formation with liver or kidney microsomes or postmitochondrial supernatants from rat, mouse, or human tissue (Gautier et al., 2001a; Zepnik et al., 2001), making it unlikely that an OTA metabolite is responsible for the supposed DNA-adduct generation. Gautier and colleagues (Gautier et al., 2001a, 2001b) also reported an absence of OTA-induced DNA-adducts and have suggested that many of the ^{32}P-postlabeled adducts observed in previous studies with OTA may indeed be the result of cytotoxic effects of OTA or the ability of OTA to generate an oxidative stress response in rats or mice. This was corroborated by the absence of such adducts

when animals are pretreated with superoxide dismutase, catalase, or other antioxidants.

Research into possible epigenetic mechanisms has so far concentrated on the demonstration of OTA-mediated inhibition of tRNA synthase (Creppy et al., 1979, 1983), on lipid peroxidation (Omar et al., 1990; Rahimtula et al., 1988) and on cytoskeletal changes (Heussner et al., 1998). Many of these studies however, used unrealistically high concentrations of OTA, which are irrelevant to the *in vivo* situation and are, in fact, close to the acute lethal doses in rats and mice. In contrast, several researchers have recently reported *in vitro* effects of OTA at nM (dietary-relevant) concentrations. OTA induced apoptosis in dedifferentiated MDCK-C7 cells (Gekle et al., 2000), immortalized human (IHKE) cells (Schwerdt et al., 1999) and human kidney epithelial (SB3) cells (Horvath et al., 2002). These observations, and some by other authors, were made in the absence of serum, in cells that had been previously transformed, or in cells that had been pretreated with hydroxyurea (Bondy et al., 1995; Doerrenhaus and Föllmann, 1997; Doerrenhaus et al., 2000; Dopp et al., 1999; Gekle et al., 1998; Maaroufi et al., 1999; Ueno et al., 1995), and as such may not be ideal model systems for the situation *in vivo*. In contrast to these reports, Seegers and colleagues (1994) reported that only a maximum of 5% of hamster kidney cells can be induced to undergo apoptosis *in vitro* following OTA exposure. These findings support the thesis proposed by Rásonyi and colleagues (1999), who described apoptotic cells in the lumen of affected tubules in rats following OTA exposure (Figure 21.2) and speculated that apoptosis may be a secondary or tertiary event in OTA toxicity, resulting from disruptions in cell–cell interactions or cell–basal lamina adhesion. This view is shared by other authors, who have found no evidence of the induction of apoptosis by OTA *in vitro* at dietary-relevant concentrations (Dreger et al., 2000; Heussner et al., 2000; O'Brien et al., 2001; Wolf et al., 2002). Cytotoxicity, however, could be demonstrated in primary cells exposed to nanomolar concentrations of OTA in serum-replete medium (O'Brien et al., 2001; Wolf et al., 2002). An interesting side-note is that following OTA exposure under these conditions, many floating cells can be seen in the culture flasks. If these are collected and reseeded, they will proliferate (Wolf and O'Brien, personal communication). It is possible that these cells could be resistant to OTA-mediated cytotoxicity. The occurrence of such cells *in vivo* could form the rudiments of the observed tumors via transformation or invasion into the transitional epithelium.

Several other possible mechanisms have been suggested, including an increase intracellular pH via a disruption in membrane anion conductance (Gekle et al., 1993), inhibition of mitochondrial transport (Meisner and Chan, 1974; Moore and Truelove, 1970), inhibition of mitochondrial respiration (Wei et al., 1985), and disruption of gap junction intercellular communication (Horvath et al., 2002). These theories could correlate to the previously described role of organic anion transporters. It remains to be seen if any or indeed all of these play a role in the toxicity of OTA.

Fumonisins

It can almost be said that the discovery of the fumonisins occurred as a byproduct of research into other mycotoxins produced by *Fusarium* species, namely the trichothecenes, which were responsible for several outbreaks of alimentary toxic aleukia in humans in Russia during World War II (Joffe, 1986). Alimentary toxic aleukia is caused by T-2 toxin and other, related trichothecenes. Of the various toxins produced by *Fusarium* species, the fumonisins are arguably the most significant having a variety of toxic effects in several species.

Fumonisins are a group of water-soluble bifuranocumarin mycotoxins (Figure 21.1) which, under suitable environmental or storage conditions, may be produced by several *Fusarium* species, in particular *Fusarium moniliforme* and *F. proliferatum* (Marasas et al., 1988). Several fumonisins have been identified to date, however of these fumonisin B_1 and B_2 (FB_1, FB_2) are the most abundant, making up 70% of the total concentration of fumonisins detected (Prozzi et al., 2000), and also the most toxic and hence the most investigated of the group (Sydenham et al., 1991). Commonly found as a contaminant of corn and in particular overwintered grain, it is thought that FB_1 is produced as a result of an endophytic relationship with grain (Bacon and Hinton, 1996), imparting an increased resistance to diseases or insects.

FB_1 has been determined to be neurotoxic, hepatotoxic, and toxic to the lung, and has also been associated with the development of esophageal cancer, particularly in areas where corn forms part of the staple diet (Gelderblom et al., 1988; Haschek et al., 1992; Norred, 1993). As in the case with many other mycotoxins, the concentrations required to cause these toxic syndromes vary both in effect induced and with the species tested.

One of the earliest diseases demonstrated to result from the feeding of animals with fumonisin-contaminated fodder is equine leucoencephalomalacia (Kellerman et al., 1990; Wilson et al., 1992). A similar syndrome has more recently been determined in rabbits (Bucci et al., 1996). Another disease of particular economic importance is porcine pulmonary edema, a fatal disease characterized by pulmonary edema and hydrothorax (Harrison et al., 1990). The economic relevance of fumonisins, coupled with their consistent presence in human foodstuffs (Sydenham et al., 1991), has resulted in fumonisins being one of the best-studied classes of mycotoxins. Although the kidney and the liver are primary organs for FB_1-mediated toxicity in several species, including mice (Howard et al., 2000b), sheep (Edrington et al., 1995), and rabbits (Gumprecht et al., 1995), display FB_1-mediated renal toxicity (Riley et al., 1994; Voss et al., 1993). The nephrotoxic aspects have only relatively recently been intensively investigated.

The feeding of FB_1-contaminated corn to rats has been reported to result in reduced body and absolute kidney weight, coupled with increased enzyme activities and elevated serum bilirubin levels (Norred et al., 1996). Gumprecht and colleagues (1995) demonstrated a number of specific renal effects in rabbits following intravenous administration of FB_1 (once-daily for 5 days). Serum creatinine and urea nitrogen concentrations were elevated and urinary glucose and protein concentrations were markedly increased following either single or multiple doses. Urine output was reduced. In contrast, Bondy and colleagues (1995) reported an increased production of dilute urine in Sprague-Dawley rats exposed to FB_1 under a similar dosage regimen (once-daily for 4 days) albeit at higher concentrations (7.5 to 10 mg/kg body weight) with intraperitoneal administration. These authors also reported an increased excretion of dilute urine coupled with elevated blood urea nitrogen concentrations and increased serum levels of several enzymes including alanine aminotransferase and serum alkaline phosphatases, well as chloride and potassium imbalances. These observations, and similar ones made by other researchers (Suzuki et al., 1995; Voss et al., 1998), imply a reduced concentrating ability, which is indicative of tubular damage.

Pathology

The clinical pathology effects associated with FB_1-exposure in rabbits have been shown to be coupled with a distinct pathology, which is

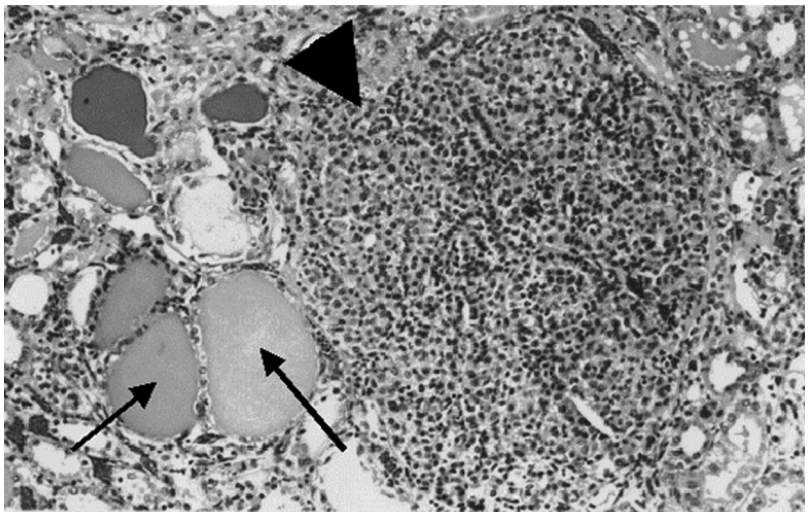

Figure 21.3 Hyaline casts (arrow) and a solid renal adenoma (arrowhead) in a male F344 rat treated with 150 mg FB_1/kg body weight for 2 years.

dependent on the number of doses applied as well as on the actual dose (Gumprecht et al., 1995). The primary lesion observed was necrosis of the proximal tubule, which was multifocal in the cortex and more extensive in the outer regions of the medulla. These authors also described the occurrence of mitotic figures and individual cell necrosis in the proximal tubular epithelium and vacuolization of tubular epithelial cells. The severity of the lesions was observed to increase, progressing into severe necrosis of the distal proximal tubule and denudation of the basement membrane, coupled with extensive hyaline casts (Figure 21.3), with repeated dosing. These authors also reported tubular regeneration.

Several authors have made similar observations in calves (Mathur et al., 2001), as well as in rats (Voss et al., 1998) and mice (Howard et al., 2000b), with mice being less sensitive to the effects of FB_1. Voss and colleagues (1998) described lesions in the outer medulla of the kidney of Sprague-Dawley rats, consisting of basophilic epithelial cells and the presence of condensed or pycnotic nuclei, indicating apoptosis. These apoptotic cells were sloughed off into the tubular lamina, and tubular cells displayed an altered morphology, appearing lower and cuboidal in shape. Mitotic figures were occasionally present and the

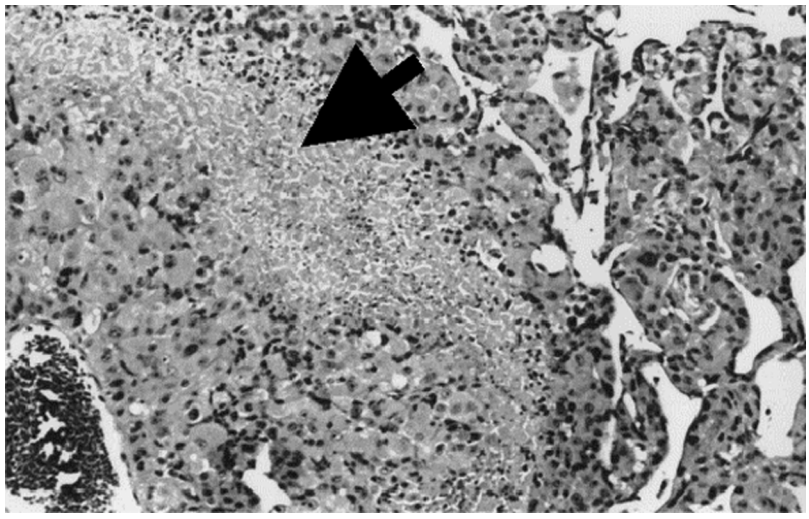

Figure 21.4 Invasive renal cell carcinoma presenting with areas of focal necrosis (arrow) in a male F344 rat treated with 150 mg FB_1/kg body weight for 2 years.

cells of the zona fasiculata showed cytoplasmic vacuolization. In contrast, apoptosis was not observed following exposure to the less toxic analogue FB_2.

Vacuolar degeneration of the tubular epithelium, detachment of epithelial cells and the presence of pycnotic nuclei have also been described by Prozzi and colleagues (2000) and by Hard and colleagues (2001) following chronic exposure to FB_1. Both of these groups also reported that the renal lesions were slightly more prominent in male than in female rats. In addition to the pathological changes previously described by other authors, Hard et al. (2001) observed the generation of a neoplastic response in the form of solitary foci of atypical tubule hyperplasia. These were generally located to the deep or midcortex and the corticomedullary in the kidneys of rats exposed to FB_1 in a two-year carcinogenicity study. These lesions progressed to yield renal tubule carcinomas (Figure 21.4), of a rare and highly malignant form, with marked cellular pleomorphism, locally invasive growth and a high rate of mitosis, as well as conventional solid (Figure 21.3) or papillary adenomas with higher doses of FB_1. Further publications from the same group report apoptotic cell death to predominate in short term exposure and conclude that these tumors observed in long term studies

are a result of compensatory regenerative hyperplasia (Howard et al., 2000a, 2000b).

In all of the investigations discussed here, primary renal effects were accompanied by hepatic lesions. Based on these rodent studies, a provisional human tolerable daily intake for FB_1, FB_2, or FB_3, alone or in combination, of 2 μg/kg body weight has been established (Creppy, 2002).

Mechanism of Action

Considering the extremely short period since the discovery of fumonisins, the mechanism of FB_1-mediated toxicity is surprisingly well elucidated. As with many carcinogenic substances, the initial investigations were carried out into a possible genotoxic mechanism of action. The nonmutagenic nature of the fumonisins has been determined using a range of testing methods. The *Salmonella* mutagenicity test (Gelderblom and Snyman, 1991), *in vitro* DNA repair assays in primary rat hepatocytes (Gelderblom et al., 1992; Norred et al., 1992), the DNA repair assay in *Escherichia coli* (in the presence or absence of rat liver S9 fractions), and the micronuclei assay in primary rat hepatocytes have all proved negative.

The *in vivo* observations of apoptosis, necrosis, and regenerative processes gave rise to a number of *in vitro* investigations into the underlying mechanisms of FB_1 toxicity. FB_1 has been demonstrated to be antiproliferative (10 to 35 μM), cytotoxic (>35 μM), and to disrupt cell–cell contact in the LLC-PK_1 cell line (Yoo et al., 1992). These authors also demonstrated actively proliferating cells to be more susceptible to FB_1-mediated toxicity, which suggests a disruption of normal cell cycle control. FB_1 has been reported to cause rat hepatocytes to arrest in the G1 phase of the cell cycle and then to undergo apoptosis (Gelderblom et al., 1995). In contrast, other authors have demonstrated the induction of apoptosis without G1 arrest in cultured human keratinocytes (Tolleson et al., 1996). These differences may, however, be due to the use of differing cell types and dosage regimens, as both apoptosis and necrosis have been observed *in vivo*.

Further investigations have shown that, like so many other mycotoxins, the mechanism of fumonisin toxicity appears to be related to its structure. The fact that the backbone of fumonisin closely resembles that of sphinganine, sphingosine, and sphingoid bases, and that FB_1 inhibits the activity of ceramide synthase, which is a key enzyme in the

generation of sphingomyelin and complex sphingolipids (Wang et al., 1991), gave the first clue to the nature of fumonisin toxicity. Interestingly, the *N*-acetylated forms of FB_1 (FA_1 and FA_2) have little or no cytotoxicity, whereas the aminophenol derivatives (AP_1 and AP_2), which share the backbone structure of FB_1, display a level of cytotoxicity similar to that of the parent compound (Yoo et al., 1992).

This disruption of sphingolipid metabolism leads to a depletion of complex sphingolipids and an accumulation of sphingoid bases. Indeed elevations of the ratio of free sphinganine to free sphingosine in serum, urine, kidneys, and liver can even be used as an indicator of exposure to FB_1 (Riley et al., 1993, 1994). Accumulation of sphingoid bases results in a cascade of events including inhibition of protein kinase C and Na^+/K^+-ATPase, release of intracellular calcium, promotion of retinoblastoma protein dephosphorylation and culminating in the induction of apoptosis (Merrill Jr. et al., 1995; Riley et al., 1996). All of these events have been observed *in vivo* or *in vitro* following FB_1 exposure (Riley et al., 1993, 1994; Mathur et al., 2001; Norred et al., 1998). Indeed, in the study carried out by Yoo and colleagues (1992), the cytotoxicity in LLC-PK_1 cell line exactly paralleled the inhibition of ceramide synthase. The regenerative response resulting from cell loss is a known risk factor for tumorigenesis, suggesting a tumor-promoting role for FB_1 (Howard et al., 2000a).

Sphingolipids play a crucial role in the regulation of cell–cell and cell–substrate contact, as well as in cellular growth and differentiation. Furthermore, many sphingolipids are uniquely expressed in the kidney (Shayman, 2000). Hard and colleagues (2001) have therefore proposed that apoptosis may not be the primary event leading to the observed pathology. These authors suggest that, instead, apoptosis occurs as a secondary event to anokisis – the loss of cell–substrate contact.

Two further theories have been proposed to explain the mechanism of action of FB_1: disruption of fatty acid metabolism and the induction of oxidative stress, and modulation of gene expression (Abado-Becongnee et al., 1998; Mobio et al., 2000). In contrast to the sphingolipid imbalance theory, which can account for all of the known effects of FB_1 either *in vivo* or *in vitro*, these alternative theories cannot.

Patulin

The cyclic γ-lactone mycotoxin, patulin (Figure 21.1) is also produced by members of the *Penicillium* and *Aspergillus* families,

and is frequently found as a contaminant of a variety of foods and, in particular, processed apple products (e.g., apple juice and cider) (Le Bourhis, 1984; Thurm et al., 1979). The name comes from *Penicillium patulum*, where the toxin was first identified, and the substance is highly toxic to Gram-negative bacteria, certain fungi, and protozoans, and also to certain plants. Like citrinin, however, it has also been demonstrated to have toxic effects in several animal species.

Subacute and acute exposure leads to a massive increase in the incidence of fundic ulcers, associated with duodenal dilation and activation of the mesenteric and pancreatic duodenal lymph nodes in rats (Speijers et al., 1988). This is thought to be a result of a direct irritant action of patulin on the gastric mucosa. The presence of fibrosis in the submucosa and underlying muscle layer was also described in the same study. Due to the primarily gastric effects of patulin, relatively little research has been carried out into the renal effects. In contrast to many other mycotoxins, patulin has been determined to be noncarcinogenic in rats (Becci et al., 1981).

Initial investigations carried out into patulin-mediated toxicity revealed functional deficits of the kidney, including a dose-dependent occurrence of oligouria, decreased serum sodium levels (Becci et al., 1981; McKinley et al., 1982), and a decrease in creatinine clearance (Speijers et al., 1984). Becci and colleagues (1981) reported these effects to be of a transient nature, with urinary parameters returning to normal levels within two years. The effects of patulin have been reported to be independent of the route of administration although pulse dosing appears to increase the rate of mortality resulting from the gastric effects in gavage experiments when compared with administration via drinking water in subacute studies in rats (Speijers et al., 1984). Subsequent studies have aimed to identifying the pathological changes responsible for the functional effects observed in the kidney.

Speijers and colleagues (1988) observed an increase in relative and absolute kidney weights in rats following chronic exposure to patulin via drinking water. This was coupled with a slight increase in creatinine clearance in animals of the high-dose (approximately 27 mg/kg body weight/day) group. A slight reduction in urinary output and an increase in urinary protein and bilirubin concentration were also noted. Males appeared to be slightly more sensitive with respect to these effects.

Pathology

Curiously, despite the use of several different histological and staining techniques, no renal histopathological changes have been demonstrated. As a result of the lack of demonstrable pathology, research into the mechanism of action of patulin has been neglected. It seems unlikely that functional defects are not associated with at least some pathological indications. Future studies using more precise histopathological techniques could reveal these changes.

Mechanism of Action

Despite the lack of evidence of pathological changes resulting from patulin exposure, some research has been carried out into the mechanism of action of patulin using relevant *in vitro* model systems. Phillips and Hayes (1979) proposed that patulin interferes with transepithelial sodium transport in mouse brain and also directly inhibits Na^+/K^+-ATPase. These authors suggest that such an effect could explain the alterations in sodium and potassium levels observed *in vivo* (Becci et al., 1981; McKinley et al., 1982) and *in vitro* (Kreisberg and Wilson, 1988). This theory is supported by the work of Riley and colleagues (1990), who demonstrated that patulin altered the membrane function of the LLC-PK_1 cell line as manifested by a reduction in transepithelial transport of sodium. These and other authors (Hinton et al., 1989) have also demonstrated that patulin caused an increase in potassium efflux, relative to sodium, resulting in a transient hyperpolarization of the affected cells.

More detailed investigations by Riley and Showker (1991) described lipid peroxidation, abrupt calcium influx, extensive blebbling, depletion of nonprotein sulfhydryls, and a total release of cellular LDH in LLC-PK_1 and L6 cells. These observations, which occurred sequentially, were made following exposure to the relatively high concentration of 50 μM. Lower concentrations (5 to 10 μM) have been demonstrated to induce the same effects, albeit over a longer time span (Riley et al., 1990). The authors suggest that a progressive loss of integrity of the plasma membrane occurs rather than a specific interaction with Na^+/K^+-ATPase. The lipid peroxidation observed in this study could be prevented by coincubation with antioxidants, however this could not prevent the cells eventually dying as a result of patulin exposure. This is probably due to the continued ability of patulin to deplete nonprotein sulfhydryls,

resulting in the observed cation leaks, and inhibition of ion pumps, changes in cellular volume and the occurrence of blebbing. Of particular relevance with respect to pathological potential is the ability of patulin to alter normal cellular calcium regulation, as described by Riley and Showker (1991), as alterations in calcium homeostasis have previously been demonstrated to be sufficient to cause DNA strand breakage (Cantoni et al., 1989).

The effects outlined above have been noted following exposure to very high concentrations of patulin, which are unlikely to be of relevance to the *in vivo* situation. It is disturbing that little or no research has been directed toward determining the pathological or toxic potential of patulin following chronic, low-level exposure. Indeed the long-term carcinogenic and the pathological potential of patulin have not been adequately investigated. Although Becci and colleagues (1981) found no evidence of tumorigenesis in rats, other species remain to be examined. The *in vitro* observations of Riley and colleagues (1990) that exposure to lower concentrations over a longer time period gives rise to similar effects to those observed following acute exposure to high concentrations also indicate an urgent need for further investigation.

Thus, patulin does appear to have at least the potential to cause pathological effects in the kidney, and this aspect of its toxicity should perhaps be more closely studied with state-of-the-art techniques in order to quantify and qualify possible latent risks that have long been neglected.

MYCOTOXIN INTERACTIONS

From the sections above, it is clear that many mycotoxins may be produced by a single mould. In particular, many of the *Aspergillus* and *Penicillium* species can produce several toxins simultaneously, depending on the environmental and substrate conditions. This begs the question of how do these mycotoxins interact with each other? Moreover, what effect do these interactions have on the nephrotoxic potential of these toxins? As most research has concentrated on the actions of pure toxins on the kidney, relatively little is known about potential additive or synergistic effects, which are arguably more relevant to the real-life situation. Some mycotoxins have, however, been shown to interact (Morrissey et al., 1987; Prozzi et al., 2000; Raju and Devegowda, 2000; Rati et al., 1991; Shinohara et al., 1976; Stoev et al., 2001) and exacerbate the toxicity resulting from individual administration. In view

of the fact that under natural conditions single pure mycotoxins occur only very rarely, future investigations should aim to further elucidate whether additive or synergistic effects can occur so that relevant and accurate risk assessments may be carried out – as Oscar Wilde once said: "*The truth is rarely pure, and never simple*" (Wilde, 1895).

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