CHAPTER 3.

Effects of aflatoxins on aflatoxicosis and liver cancer

While there has been a very extensive focus on the role of aflatoxin exposure in hepatocellular carcinoma (HCC), over the years several cases of acute aflatoxicosis in humans have been reported in regions of some developing countries (Shank et al., 1971).

Acute aflatoxin poisoning

The clinical manifestations of aflatoxicosis include vomiting, abdominal pain, pulmonary oedema, fatty infiltration, and necrosis of the liver. In the 1970s, there was an outbreak of putative aflatoxin poisoning in western India when heavily moulded maize was consumed. There were at least 97 fatalities, all of which occurred in households where the contaminated maize was consumed. Histopathology of liver specimens revealed extensive bile duct proliferation, a lesion often noted in experimental animals after acute aflatoxin exposure (Krishnamachari et al., 1975; Bhat and Krishnamachari, 1977). An outbreak of acute aflatoxicosis in Kenya in 1981 was also associated with consumption of maize highly contaminated with aflatoxin (Ngindu et al., 1982). There were 20 hospital admissions, with 60% mortality. In a more recent report (Lye et al., 1995), the consumption of aflatoxin-contaminated noodles resulted in acute hepatic encephalopathy in children in Malaysia. Up to 3 mg of aflatoxin was suspected to be present in a single serving of contaminated noodles.

In April 2004, one of the largest documented aflatoxicosis outbreaks occurred in rural Kenya, resulting in 317 cases and 125 deaths. Aflatoxin-contaminated home-grown maize was the major source of the outbreak. In a survey of 65 markets and 243 maize vendors, 350 maize products were collected from the most affected districts. Of these maize products, 55% had aflatoxin levels greater than the Kenyan regulatory limit of 20 ppb, 35% had levels greater than 100 ppb, and 7% had levels greater than 1000 ppb. Makueni, the district with the most aflatoxicosis cases, had significantly higher aflatoxin levels in maize from markets than did Thika, the study district with the fewest cases (geometric mean aflatoxin, 52.91 ppb vs 7.52 ppb; $P = 0.0004$). Maize obtained from local farms in the affected area was significantly more likely to have aflatoxin levels greater than 20 ppb compared with maize bought from other regions of Kenya or other countries (odds ratio [OR], 2.71; 95% confidence interval [CI], 1.12–6.59). In addition to the

market survey for aflatoxin exposure, this outbreak in 2004 marked the first time that levels of aflatoxin–albumin adducts (AF–alb) independently confirmed the exposure in individuals (CDC, 2004; Azziz-Baumgartner et al., 2005; Lewis et al., 2005; Strosnider et al., 2006; Probst et al., 2007).

Hepatocellular carcinoma

For decades, it has been known that aflatoxin exposure causes liver cancer in humans and in several animal species. The International Agency for Research on Cancer (IARC) has evaluated the carcinogenicity of aflatoxins on several occasions, starting in 1972 with Volume 1 of the IARC Monographs on the evaluation of carcinogenic risks to humans. Since that time, many studies in humans and experimental animals have provided clarifying data, and naturally occurring mixtures of aflatoxins are now classified as Group 1, carcinogenic to humans (IARC, 1993). Furthermore, as described below, concomitant exposure to aflatoxin and hepatitis B virus (HBV) is common in developing countries and greatly increases HCC risk (Wu et al., 2013). Individuals who experience both exposures have a greater risk of developing HCC than those exposed to aflatoxin alone (Wogan et al., 2012).

HCC accounts for 5.6% of all reported cancer cases and is the sixth most common cancer diagnosed worldwide (Ferlay et al., 2013). The global incidence of liver cancer varies enormously, and the burden of this nearly always fatal disease is much higher in less-developed countries of Asia and sub-Saharan Africa. Overall, there are more than 780 000 new cases of liver cancer each year and more than 745 000 deaths annually (Ferlay et al., 2013). In contrast to most cancers common in developed countries, where more than 90% of cases are diagnosed in people aged 45 years and older, in high-risk regions for liver cancer, onset begins in both men and women by age 20 years, peaking at age 40–49 years in men and age 50–59 years in women (Parkin et al., 2005; Chen et al., 2006). The earlier onset of HCC may be attributable to exposures that are both substantial and persistent across the lifespan. Sex differences in liver cancer incidence have also been described; the worldwide annual age-standardized incidence rate is 15.3 per 100 000 among men and 5.4 per 100 000 among women (Ferlay et al., 2013). These epidemiological findings are also consistent with experimental animal data for aflatoxin, in which male rats have been found to have an earlier onset of cancer compared with female rats (Wogan and Newberne, 1967).

For more than 50 years, the relationship between aflatoxin exposure and human liver cancer has been examined using ecological studies, cross-sectional surveys, case–control studies, and prospective cohort investigations in exposed populations. Early studies in the Philippines demonstrated that an oxidative metabolite of aflatoxin could be detected in urine and thus had potential to serve as an internal dose marker (Campbell et al., 1970). In later studies, Autrup et al. (1983, 1987) reported the presence of aflatoxin B_1 (AFB_1)–DNA adducts in human urine samples in Kenya. Subsequent work conducted in China and The Gambia, West Africa, areas with high incidences of HCC, examined both the dietary intake of aflatoxin and the levels of urinary aflatoxin biomarkers (Groopman et al., 1992). Urinary AFB_1–DNA adduct and aflatoxin M_1 (AFM_1) levels showed a dose-dependent relationship between aflatoxin intake and excretion. Gan et al. (1988) and Wild et al. (1992) also monitored levels of AF–alb in serum and observed a highly significant association between aflatoxin intake and adduct level.

Many published case–control studies have explored the relationship between aflatoxin exposure and HCC. In an early case–control study, Bulatao-Jayme et al. (1982) compared the dietary intake of aflatoxin in cases of HCC in the Philippines with intake in age- and sex-matched controls. They found that the mean aflatoxin exposure per day in cases of HCC was 4.5 times as high as that in the controls; however, alcohol consumption may have enhanced this effect. Van Rensburg et al. (1985) and Peers et al. (1976) used a similar design for studies in Mozambique and Swaziland, respectively. Again, the mean dietary aflatoxin intakes were positively correlated with HCC rates, and the data also suggested a dose-dependent increase in liver disease associated with increased aflatoxin intake.

In the Guangxi Zhuang Autonomous Region of China, Yeh and Shen (1986) and Yeh et al. (1989) examined the interaction between HBV infection and dietary aflatoxin exposure dichotomized for heavy and light levels of contamination. Individuals whose serum was positive for the HBV surface antigen (HBsAg) and who experienced heavy aflatoxin exposure had a 10-fold higher incidence of HCC than did people living in areas with light aflatoxin contamination. People who were HBsAg-negative and who consumed diets heavily contaminated with aflatoxin had a rate of HCC comparable to that of the HBsAg-positive people consuming diets with light aflatoxin contamination (Yeh et al., 1989). In a case–control study in Taiwan, China, two

biomarkers, AF–alb and aflatoxin–DNA adducts in liver tissue samples, were measured (Lunn et al., 1997). The proportion of subjects with a detectable level of AF–alb was higher for cases of HCC than for matched controls (OR, 1.5). A statistically significant association was found between presence of detectable AF–alb and risk of HCC among men younger than 52 years (multivariate adjusted OR, 5.3).

Another study, in Qidong, China, examined 145 men with chronic HBV infection who were followed for 10 years to determine whether exposure to aflatoxin, concomitant exposure to hepatitis C virus (HCV), or family history of HCC increased the risk of developing HCC. Eight monthly urine samples collected before the initiation of follow-up were pooled to analyse for AFM_1. AFM_1 was detected in 78 (54%) of the subjects, and the risk of HCC was increased 3.3-fold (95% CI, 1.2–8.7) in those with detectable AFM_1 (> 3.6 ng/L). The attributable risk from aflatoxin exposure, defined as the presence of detectable AFM_1, was 0.553 (95% CI, 0.087–0.94). The relative risk of fatal cirrhosis for individuals whose urine contained elevated AFM_1 was 2.8 (95% CI, 0.6–14.3). Concomitant infection with HCV increased the risk of HCC 5.8-fold (95% CI, 2.0–17), adjusted for age and AFM_1 status. This study shows that aflatoxin exposure detected by the presence of AFM_1 in urine can account for a substantial portion of HCC risk in men with chronic HBV hepatitis (Sun et al., 1999).

Two major cohort studies incorporating aflatoxin biomarkers have clearly demonstrated the etiological role of this carcinogen in HCC. The first study, comprising more than 18 000 men in Shanghai, China, examined the interaction of HBV and aflatoxin biomarkers as independent and interactive risk factors for HCC. The nested case–control data revealed a statistically significant increase in the relative risk of 3.4 for those HCC cases in whom a urinary aflatoxin biomarker (AFB_1–N7-guanine) was detected. For men whose serum was HBsAg-positive but whose urine did not indicate aflatoxin exposure, the relative risk was 7.3, but in individuals exhibiting both the urinary aflatoxin biomarker and positive HBsAg status, the relative risk was 59.4 (Ross et al., 1992; Qian et al., 1994). These results strongly support a causal relationship between the presence of carcinogen- and viral-specific biomarkers and the risk of HCC. Subsequent cohort studies in Taiwan, China, have substantially confirmed the results from the Shanghai investigation. Wang et al. (1996) examined HCC cases and controls nested within a cohort and found that in HBV-infected people there was an adjusted odds ratio of 2.8 for detectable compared with non-detectable AF–alb, and an adjusted odds ratio of 5.5 for high compared with low levels of aflatoxin metabolites in urine. In a follow-up study, there was a dose–response relationship between urinary AFM_1 levels and risk of HCC in chronic HBV carriers (Yu et al., 1997). As in the Shanghai cohort, HCC risk associated with AFB_1 exposure was most striking among HBV carriers with detectable AFB_1–N7-guanine in urine.

Furthermore, the relationship between aflatoxin exposure and development of HCC has been highlighted by molecular biological studies on the *p53* tumour suppressor gene, the gene most commonly mutated in many human cancers (Greenblatt et al., 1994). Many studies of *p53* mutations in HCC occurring in populations exposed to high levels of dietary aflatoxin have found high frequencies of G:C → T:A transversions, with clustering at codon 249 (Bressac et al., 1991; Hsu et al., 1991). In contrast, no mutations in codon 249 were found in *p53* in HCC from Japan and other areas where there was little exposure to aflatoxin (Ozturk, 1991; Aguilar et al., 1994).

Thus, studies of the prevalence of codon 249 mutations in HCC cases from populations in areas of high or low exposure to aflatoxin suggest that a G → T transversion at the third base of codon 249 is associated with aflatoxin exposure, and in vitro data would seem to support this hypothesis. Application of these specific mutations as biomarkers for early detection also offers great promise for HCC prevention (Sidransky and Hollstein, 1996). In a seminal study, Kirk et al. (2000) reported for the first time detection of *p53* codon 249 mutations in plasma of liver tumour patients residing in The Gambia; however, the mutational status of their tumours was not determined. The authors also reported the presence of this mutation in the plasma of a small number of cirrhosis patients. Given the strong relationship between cirrhosis and future development of HCC, the possibility of this mutation serving as an early detection marker needs to be explored. Jackson et al. (2001) examined 25 HCC tumours for specific *p53* mutations. Analysis of 20 additional plasma–tumour pairs showed that 11 tumours and 6 plasma samples contained the specific mutation. This group (Jackson et al., 2003) further explored the temporality of detection of this mutation in plasma before and after clinical diagnosis of HCC in the same patients. This study was facilitated by the availability of longitudinally collected plasma samples from a cohort of 1638 high-risk individuals in Qidong, China, who have been followed since 1992. The results showed that in samples

collected before liver cancer diagnosis, 21.7% (95% CI, 9.7–41.9%) of the plasma samples had detectable levels of the codon 249 mutation in *p53*, whereas this mutation was detected in 44.6% (95% CI, 21.6–70.2%) of the plasma samples collected after the diagnosis of liver cancer. This percentage of positive samples after liver cancer diagnosis compares with about 50% of all liver tumours in Qidong, suggesting a nearly 90% concordance between plasma and tumour *p53* codon 249 mutation outcome.

Finally, recent work has taken advantage of a population-based cancer registry to track primary liver cancer mortality in Qidong, China, a region of 1.1 million residents. This database indicates that a greater than 50% reduction in HCC mortality rates occurred across birth cohorts from the 1960s to the 1980s for Qidongese younger than 35 years. The prevalence of HBV infection was unchanged, since all were born before universal vaccination of newborns. Randomly selected serum samples from archived cohort collections from the 1980s to the present were analysed for aflatoxin biomarkers. Median levels of the aflatoxin biomarker AF–alb decreased from 19.3 pg/mg in 1989 to non-detectable (< 0.5 pg/mg) by 2009. A population-attributable benefit of 65% for reduced primary liver cancer mortality was estimated from a government-imposed switch of the dietary staple from maize to rice. These data reinforce the role that aflatoxin plays in high-exposure regions with populations at high risk for HCC (Chen et al., 2013).