Aflatoxins as a Cause of Hepatocellular Carcinoma

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ABSTRACT

Aflatoxins, metabolites of the fungi *Aspergillus flavus* and *Aspergillus parasiticus*, are frequent contaminants of a number of staple foods, particularly maize and ground nuts, in subsistence farming communities in tropical and sub-tropical climates in sub-Saharan Africa, Eastern Asia and parts of South America. Contamination of foods occurs during growth and as a result of storage in deficient or inappropriate facilities. These toxins pose serious public health hazards, including the causation of hepatocellular carcinoma by aflatoxin B1. Exposure begins in utero and is life-long. The innocuous parent molecule of the fungus is converted by members of the cytochrome p450 family into mutagenic and carcinogenic intermediates. Aflatoxin-B1 is converted into aflatoxin B1-8,9 exo-epoxide, which is in turn converted into 8,9-dihydroxy-8-(N7) guanyl-9-hydroxy aflatoxin B1 adduct. This adduct is metabolized into aflatoxin B1 formaminopyrimidine adduct. These adducts are mutagenic and carcinogenic. In addition, an arginine to serine mutation at codon 249 of the p53 tumor suppressor gene is produced, abrogating the function of the tumor suppressor gene, and contributing to hepatocarcinogenesis. Aflatoxin B1 acts synergistically with hepatitis B virus in causing hepatocellular carcinoma. A number of interactions between the two carcinogens may be responsible for this action, including integration of hepatitis B virus x gene and its consequences, as well as interference with nucleotide excision repair, activation of p21waf1/cip1, generation of DNA mutations, and altered methylation of genes. But much remains to be learnt about the precise pathogenetic mechanisms responsible for aflatoxin B1-induced hepatocellular carcinoma as well as the interaction between the toxin and hepatitis B virus in causing the tumor.

Key words: aflatoxins — hepatocellular carcinoma — sub-Saharan Africa — Eastern Asia — South America — staple foods — contaminant.

AFLATOXINS

Aflatoxins are naturally occurring secondary metabolites of the fungi, *Aspergillus flavus* and *Aspergillus parasiticus*. These difuranocoumarin derivatives are produced under certain environmental conditions and in a variety of substrates, and are common contaminants of a number of staple foods, including maize, ground nuts, rice and sorghum. The toxins are widely distributed in nature and pose serious public health hazards to humans as a result of their toxic, teratogenic, mutagenic, and highly carcinogenic properties [1, 2]. Contamination of crops and toxin production are particularly likely to occur in subsistence farming communities in tropical and sub-tropical regions with high temperatures and humidity. These environmental conditions, in addition to the moisture content of plants, are important factors in determining growth of, and toxin production by, these moulds. The mycotoxins are produced at optimum temperatures of between 25°C and 32°C, moisture contents of greater than 12% but less than 16%, and a relative humidity of 85% [2, 3]. Crops that are particularly likely to be affected are those either grown domestically or purchased at local markets.

Contamination of crops with aflatoxins is also likely to occur in communities and regions where food drying and storage facilities are suboptimal, the resources, technology, and infrastructure necessary for routine food monitoring are absent or inadequate, and regulations to control exposure to the moulds are either non-existent or unenforceable in practice [3-5]. Because most rural dwellers can afford only limited food variation, the available staple foods constitute a significant portion of their diets. In addition, storage, particularly prolonged storage, of crops in hot and humid conditions promotes growth of the aflatoxin-producing fungi and accumulation of the toxin [3, 4]. Exposure to aflatoxins is

therefore far more common and severe in rural than in urban dwellers in resource-constrained regions [5]. A strong seasonal variation in exposure to the toxin also exists. Two forms of aflatoxin poisoning are recognised. The first is acute severe intoxication which causes direct liver damage with subsequent illness and death, and the second is chronic symptomatic exposure [2].

Aflatoxins are the most common known non-infectious food-borne risk factor. It is estimated that 4.5 to 5.5 billion people worldwide are at risk of exposure to these toxins [2, 3]. The regions involved are in sub-Saharan Africa, Eastern Asia, and parts of South America, with countries located between 40° North and 40° South of the equator being at greatest risk [2-6]. Approximately 80% of all individuals exposed to aflatoxins live in these regions, with approximately one-half residing in sub-Saharan Africa. In excess of 25% of the world's crops are estimated to be contaminated by these mycotoxins [3]. Human foods are allowed between 4 and 30 parts per billion of aflatoxin, depending upon the country involved [2]. Even relatively low levels of contamination can have serious health complications when the affected food is consumed in large quantities. Exposure to aflatoxins begins in utero as a result of transplacental transmission of the toxins [7], and continues in the postnatal period by means of breast feeding [8], and thereafter throughout life, with the extent of the exposure increasing with age [9]. In addition to self-grown contaminated foodstuffs, aflatoxins are present in the meat, eggs and milk of farm animals that feed on aflatoxin-contaminated foods [3, 10, 11]. Susceptibility to aflatoxin is greatest in the young, with as many as 5 million deaths in children under the age of 5 years being attributable to aflatoxin exposure in resource-constrained countries each year [2, 8]. In addition to humans, these toxins are potent hepatocarcinogens in non-human primates, rodents, fish and birds [12].

Heavy and prolonged exposure to aflatoxins may be complicated by the development of hepatocellular carcinoma (HCC) [5]. In many of the countries in regions at risk certain food staples, but particularly maize and, to a lesser extent, ground nuts, are highly colonized by aflatoxins, either during their growth or as a result of improper storage, or by both of these routes. These foods are the staple diet and the primary cash crop in these countries, with rural dwellers being affected to a far greater extent than urban dwellers [6]. Those countries in which the risk of exposure to aflatoxins is particularly high, have limited resources to institute prevention and control strategies. Millions of people living in these countries are estimated to be repeatedly exposed to these carcinogens and therefore at high risk of developing HCC.

By contrast, in resource-rich countries the establishment of regulatory limits on traded food, the enforcement of these limits through food monitoring, and the implementation of optimal drying and storage practices have largely eliminated harmful exposures to aflatoxins [13].

Although the aflatoxin parent molecule is harmless, it is converted by members of the cytochrome p450 superfamily into electrophilic intermediates that are mutagenic and carcinogenic [14-18]. The four major aflatoxins are aflatoxin B1 (AFB1), B2, G1 and G2. Toxigenic strains of *Aspergillus flavus* typically produce only AFB1 and aflatoxin B2, whereas most strains of *Aspergillus parasiticus* produce all of the aflatoxins. Aflatoxin B1 is the aflatoxin most often found in contaminated human foods, is the most potent of these toxins, and has the highest hepatocarcinogenic potential [19, 20].

The predominant site of aflatoxin metabolism is the liver [16, 21], and the major human cytochrome p450 enzymes involved in its metabolism are CYP 3A4, 3A5, 3A7 and 1A2 [21].

In the liver, p450 enzymes metabolize aflatoxin into an aflatoxin-8,9-exo-epoxide and, to a lesser extent, an aflatoxin-8,9-endo-epoxide. The exo-epoxide is highly reactive and can form derivatives with DNA, RNA and proteins, and can react with the p53 tumor suppressor gene [22, 23]. It binds in turn to DNA to form the predominant promutagenic 8,9 dihydro-8-(N7 guanyl)-9-hydroxy AFB1 adduct (AFB1-N7-Gua) [16]. AFP1-N7-Gua can be converted into two secondary forms, an apurinic site and a more stable AFB1-formamidopyrimidine (AFB1-FABY) adduct. This adduct causes guanine (G) to thymine (T) transversion mutations with a frequency six-times greater than AFB1-N7-Gua [24]. AFB1-FABY incorporated into double-stranded DNA is mutagenic, whereas the dominant species in single-stranded DNA blocks replication [13]. These mutations are highly reactive with DNA, causing changes that over time impose a risk of malignant transformation.

AFLATOXINS AS A CAUSE OF HEPATOCELLULAR CARCINOMA

Aflatoxin B1 is causally-related to the development of HCC in humans, and is the most potent experimental hepatocarcinogen known to man [20]. No animal model exposed to the toxin to date has failed to develop HCC. Hepatocellular carcinoma accounts for approximately 9.2 % of all new cancers worldwide, with the number increasing year by year [25]. It is the fifth most common cancer in males and the seventh in females, and occurs at a relatively young age in resource-constrained regions [26]. Approximately 84% of all new cases of HCC occur in these regions, but particularly in sub-Saharan Africa and the Asia-Pacific region, with a prevalence that is 16 to 32 times higher in these regions than in resource-rich regions [2]. The tumor carries an especially grave prognosis in high-incidence populations, ranking third in annual cancer mortality rates, and with 93% of patients dying within12 months of the onset of symptoms, the highest rate of any human tumor [25].

The interaction between the extent of exposure to AFB1 and the incidence of HCC is multiplicative or more than multiplicative. Chronic hepatitis B virus (HBV) infection and dietary exposure to the toxin are the major causes of HCC in the high-risk regions of the tumor, and are largely responsible for the striking geographical variation in the incidence of HCC. Both risk factors are more common in rural than in urban dwellers.

In high-risk regions of HBV infection, the infection is usually acquired in the early years of life, and the exposure to AFB1 begins even earlier, that is, in utero. It has been estimated that by reducing dietary AFB1 levels to below detectable limits in eastern Asia and sub-Saharan Africa, between 72,800 and 98,800 new cases of HCC could be prevented each year [4].

Of the aflatoxins, AFB1 is the most potent hepatocarcinogen [27]. The correlation between the degree of exposure to AFB1 and the incidence of HCC is direct, with an odds ratio for developing the tumor of 6.37:1.0 (range 3.74:1.0 to 10.86:1.0) [2, 3]. The overall population attributable risk is 17% (14-19%) – 21% in HBV-positive individuals and 8.5% in HBV-negative individuals [4]. It has been estimated that in between 25,200 (4.6% of all cases of the tumor world wide) and 155,000 people worldwide (28.2% of all cases world wide) HCC may be attributed to exposure to AFB1, and that approximately 40% of these people live in sub-Saharan Africa [2]. Moreover, it is believed that reducing AFB1 exposure to non-detectable levels could reduce the incidence of HCC cases in high-risk areas by approximately 23% [1].

Aflatoxin FB1 is the most potent experimental hepatocarcinogen known to man - no animal model tested has thus far failed to develop the tumor. The latency period for the development of AFB1-induced HCC is not known. Regrettably, those parts of the world in which the risk of AFB1-induced HCC is particularly high, have limited resources to implement aflatoxin control strategies [2].

Chronic liver injury and regenerative hyperplasia are central to the development of HCC. AFB1-induced adducts may therefore be fixed as mutations consequent to an HBV-related increase in hepatocyte turnover rates.

An arginine to serine (G to T) mutation at codon 249 of the p53 tumor suppressor gene (R249S; 249ser mutation) is specific for exposure to aflatoxin and is detected in as many as 64% of patients with HCC [22, 23, 28, 29]. This mutation accounts for 90% of p53 mutations in AFB1-related HCC [30], suggesting that its presence confers a selective advantage during hepatocarcinogenesis. Moreover, the mutation looses its capacity to bind to p53 response genes and to transactivate p53 target genes [30]. 249ser is present in the tumor tissue of as many as 75% of Chinese [23] and 56% of Mozambican Shangaans with HCC [22]. The mutation is present in 44% of patients with HCC who have no evidence of cirrhosis, supporting a direct, in addition to an indirect, hepatocarcinogenic effect of AFB1 [28]. The presence of both the 249ser mutation and chronic HBV infection is associated with an odds ratio for developing HCC of 399 (95% confidence interval 486 to 3270) [31]. The mutation may also contribute to hepatocarcinogenesis through interaction with HBV x protein, conferring a growth advantage in the early stages of the transformation process [30]. The 249ser mutation is rare in non-aflatoxin contaminated regions.

Aflatoxin B1 and chronic HBV infection are known to co-exist in those countries with the highest incidences of, and the youngest patients with, HCC, raising the possibility of a synergistic hepatocarcinogenic interaction between the two agents [31].

EVIDENCE FOR A SYNERGISTIC INTERACTION BETWEEN AFLATOXIN AND HEPATITIS B VIRUS IN HEPATOCARCINOGENESIS

The first published evidence for a synergistic interaction between the two hepatocarcinogenic agents was provided by experiments in which transgenic mice over-expressing the large envelope polypeptide of HBV were fed AFB1. These mice produced more rapid and extensive hepatocyte dysplasia than did their unexposed littermates, and HCCs developed [32]. Shortly thereafter, experimental evidence for a positive carcinogenic interaction between AFB1 and the woodchuck hepatitis virus was shown [33]. Woodchucks infected with the virus developed a high incidence of preneoplastic foci of altered hepatocytes, followed by hepatocellular adenomas and HCCs [32]. Liver tumors were also reported to develop in ducks infected with duck hepatitis virus and exposed to AFB1 [34], and in tree shrews infected with HBV and exposed to AFB1 [35].

The first clinical evidence of a synergistic carcinogenic interaction between HBV infection and exposure to AFB1 was provided by a study in Guanxi Province, China, which showed that HCC occurring in individuals infected with HBV who lived in villages with a 'high' aflatoxin consumption had a mortality rate that was 10-times higher than that in individuals living in villages with a 'low' consumption [36]. There is now convincing evidence for a multiplicative interaction between AFB1 and HBV as hepatocarcinogens in the populations of sub-Saharan Africa and Eastern Asia, and that this association is, in large measure, responsible for the high incidence of HCC in these regions [16, 37-41]. For example, in the report of Williams et al the odds ratio for developing HCC with exposure to aflatoxin alone was 6.37; that with HBV infection alone 11.3; and that with both risk factors 73.0 [2].

Some authors have gone so far as to suggest that a hepatocarcinogenic role for the AFB1 may be confined to regions where chronic HBV infection is endemic [16, 42].

Five large studies have been performed in Qidong County, China and in Taiwan. All showed a markedly increased incidence of HCC in those individuals positive for both HBV and AFB1, compared with those positive for HBV alone or AFB1 alone [43-47]. Three of the studies showed a striking multiplicative effect in patients positive for both HBV and AFB1, with odds ratios of tumor development ranging from 59.4 to 70 and with a mean ratio of 63.2 for patients positive for HBV/AFB1, compared with 9.5 for HBV alone, and 1.9 for aflatoxin alone [43-45].

In the other two studies there was a sub-multiplicative carcinogenic effect between exposure to HBV and AFB1 (67.6 and 40.7; mean 54.2) compared with 10.2 with exposure to HBV alone and 24.7 for AFB1 alone [46, 47]. These results were later confirmed in other studies [24, 48]. In a study in Taiwanese patients, those with HBV infection and exposure to aflatoxin were on average 10 years younger than those without exposure to aflatoxin [49].

POSSIBLE MECHANISMS FOR THE INTERACTION BETWEEN AFLATOXIN B1 AND HEPATITIS B VIRUS

Hepatitis B virus infection may directly or indirectly sensitize hepatocytes to the carcinogenic effects of AFB1, although the biology underlining the synergistic interaction between the two carcinogens is not fully understood [38]. The observation that Gambian and Taiwanese children and

adolescents chronically infected with HBV have higher concentrations of AFB1 adducts than uninfected individuals is consistent with this phenomenon [50, 51], although some clinical studies and a study in woodchucks infected with woodchuck hepatitis virus failed to confirm this observation [52]. AFB1-8,9 epoxide has been shown to bind to DNA causing changes that increase the risk of integration of viral DNA and hence malignant transformation [37].

The 249ser mutation is believed to be a primary and early genetic event in hepatocarcinogenesis. This mutation is present in 36.3 to 66% of patients with heavy exposure to AFB1 [22, 23, 31, 52-54]. It abrogates the normal functions of p53, including those in cell cycle control, DNA repair, and apoptosis, and thus contributes to the multistep process of hepatocarcinogenesis. Evidence has supported an interaction between this mutation and AFB1 in hepatocarcinogenesis [4, 30, 31, 53, 54]. For example, in a study in the Gambia, patients with HBsAg alone had an increased relative risk of HCC development of 10, those with 249 ser mutation of 13, and those with both of 399 [53]. Other studies, however, failed to confirm this finding [55].

The HBV x gene is frequently included in sequences of the virus that are integrated into cellular DNA [56]. Nuclear excision repair, which is normally responsible for removing AFB1-DNA adducts, is inhibited by HBV x protein, favouring the persistence of existing mutations or damaged DNA [56, 57]. It may also contribute to uncontrolled cell proliferation [56]. The transcription of p21waf1/cip1, which induces cell cycle arrest, is activated by HBV x protein in a dose-dependent manner in the presence of functional p53, although the transcription is repressed by HBV x protein when p53 is absent or present at a low level [58]. The expression of HBVx protein also correlates with an increase in the overall frequency of DNA mutations in transgenic mice and a two-fold increase in the incidence of the 249ser mutation in transgenic mice exposed to AFB1 [59].

Another possible mechanism for an interaction between AFB1 and HBV is that increased hepatocyte necrosis and proliferation resulting from HBV infection increase the likelihood of both AFB1 mutations, including 249ser, and the subsequent clonal expansion of cells containing these mutations [60]. Chronic inflammatory hepatic disease, including that resulting from HBV infection, results in the generation of oxygen and nitrogen reactive species [61]. Both of the latter are mutagenic but, in addition, increased oxidative stress has been shown to induce 249ser mutations [62]. Altered methylation of genes may play a role in hepatocarcinogenesis. A statistically significant association exists between ras association domain gene 1A (RASSF1A) methylation status and the level of AFB1-DNA adducts in HCC tissues [63].

CONCLUSION

An understanding of the mechanisms responsible for the heightened risk of malignant transformation in patients chronically infected with HBV and persistently exposed to AFB1 is far from complete, and there is clearly a need for further research to be undertaken into the pathogenesis of the interaction of the two hepatocarcinogens.

Conflicts of interest. None to declare.

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