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Synergistic Interaction Between Aflatoxin and Hepatitis B Virus in Hepatocarcinogenesis

Michael C. Kew

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1. Introduction

Based on the number of new cases of cancer in humans each year, hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide [20], the fifth in males and the seventh in females [37]. In the most recently reported year, 748,000 new cases of the tumour were recorded, constituting 9.2% of all new cancers [20]. Furthermore, the number of new cases of the tumour continues to increase year by year. Not only is HCC common, it also carries an especially grave prognosis, ranking third in annual cancer mortality rates. In the year mentioned, the total death rate from the tumour was 695,900. Of the patients who died, 93% did so within 12 months of the onset of symptoms. This 12 month fatality ratio in HCC (0.93 - 0.96) is the highest of any human tumour.

HCC does not have a uniform geographical distribution. Rather, of all the new cases of the cancer recorded during recent years, approximately 84% occurred in resource-constrained (developing) countries [20], particularly in sub-Saharan Africa and the Asia Pacific region. In these regions the dominant cause of HCC is chronic hepatitis B virus (HBV) infection. This infection is almost invariably acquired very early in life, either as a result of perinatal transmission of the virus or of horizontal transmission in infancy or early childhood, times at which the infection very often becomes chronic [42]. The tumour resulting from the HBV infection frequently occurs at a young or relatively young age, and it carries a particularly grave prognosis.

In addition to chronic HBV infection, the other major cause of HCC in these high-risk regions is dietary exposure to aflatoxins, the toxic secondary metabolites of the fungi, *Aspergillus flavus* and *Aspergillus parasiticus*. These viral and fungal risk factors are largely responsible for the striking geographical variation in incidence of HCC. Both aflatoxin exposure and chronic HBV infection are more common in rural than in urban dwellers in re-

source-constrained regions [42, 63]. In these regions, the association between aflatoxin exposure and the development of HCC is closest in sub-Saharan Africa [51].

Aflatoxins are structurally-related difuranocoumarin derivatives, some of which are mutagenic and carcinogenic in humans and animals [89, 87]. These toxins are widely distributed in nature. Because atmospheric humidity and moisture content of plants are important factors in determining growth of, and toxin production by, these moulds, contamination of crops occurs mainly in tropical and sub-tropical climates with high humidity and temperature. These conditions exist in sub-Saharan Africa, the Asia Pacific region, and parts of South America. Contamination is particularly likely to occur in subsistence farming communities in regions with these climates and where regulations to control exposure to the fungi are either non-existent or unenforceable in practice.

In these regions, the moulds contaminate a variety of staple foods, especially maize and groundnuts [89, 34, 87]. Because most rural dwellers can afford only limited food variation, these staples make up a significant portion of their diets. Contamination of crops with aflatoxins occurs either during their growth or as a result of their storage under conditions that promote fungal growth and toxin production [31, 32, 33]. Exposure begins *in utero* as a result of trans-placental transmission of the toxins [86] and in the postnatal period as a result of breast-feeding [85], and continues throughout life. Exposure increases with increasing age - for example, in Malaysia evidence of exposure was more common in the population aged 31 to 50 years than that aged 18 to 30 years [46].

Approximately 4.5 billion of the world's population are believed to be exposed to aflatoxins [88]. Between 25,200 new cases of HCC each year (or 4.6% of all cases of the tumour worldwide) and 155,000 new cases each year (or 28.2% of all cases of the tumour world wide) may be attributed to this exposure [51]. It has been estimated that aflatoxins play a causative role in at least 4.6% and at most 28.2% of all cases of HCC worldwide [51]. These large ranges stem from the considerable uncertainty and variability in data on cancer potency factors, HBV prevalence, aflatoxin exposure, and other risk factors [51].

Although the parent aflatoxin molecule is harmless, it is converted by members of the cytochrome 450 superfamily into electrophilic intermediates that are mutagenic and carcinogenic [90, 87, 40, 71]. Of the four naturally occurring aflatoxins, aflatoxin B₁ (AFB₁), B₂, G₁ and G₂, toxigenic strains of *A. flavus* typically produce only aflatoxins B₁ and B₂, whereas most strains of *A. parasiticus* produce all of the aflatoxins [18]. AFB₁ is the most potent experimental hepatocarcinogen known to man -- no animal model exposed to the toxin thus far has failed to develop HCC. AFM₁, the hydroxylation product of AFB₁, is found in milk and milk products when animals intended for dairy production consume aflatoxin-contaminated feed [70]. In rodents exposed to AFB₁ in equivalent doses to those occurring in humans, levels of aflatoxin adduct in the serum have correlated with levels of hepatic DNA damage and with development of HCC [83].

AFB₁ is the aflatoxin most often found in contaminated human foodstuffs [78], and exposure to AFB₁ is causally related to the development of HCC in humans [34]. The correlation between the degree of exposure to AFB₁ and the incidence of HCC is direct. It has been estimat-

ed that by reducing dietary AFB₁ levels to below detectable limits in Asia and sub-Saharan Africa, between 72,800 and 98,800 new cases of HCC could be prevented each year [49].

The major human cytochrome P450 (CYP) enzymes involved in aflatoxin metabolism are CYP3A4, 3A5, 3A7 and 1A2, and the predominant site of metabolism is the liver [87, 39]. AFB₁ is metabolized to an AFB₁-8,9-*exo*-epoxide and, to a lesser extent, an AFB₁-8,9-*endo*-epoxide. The *exo*-epoxide binds to DNA to form the predominant promutagenic 8,9-dihydro-8-(N⁷-guanyl)-9-hydroxy AFB₁ (AFB₁-N⁷-Gua) adduct. AFB₁-N⁷-Gua can result in two secondary forms, an apurinic site and a more stable ring-opened AFB₁-formamidopyrimidine (AFB₁-FABY) adduct, which is far more persistent *in vivo*. This adduct causes G to T transversion mutations [28, 4, 87], the most prevalent of which are targeted to the site of the original adduct. AFB₁-FABY exists as a mixture of two rotameric forms. In *Escherichia coli* AFB₁-FABY induced a six-fold higher G to T mutation frequency than AFB₁-N⁷-Gua, with mutations also occurring adjacent to the site of adduct formation [82]. AFB₁-FABY also resulted in blocked replication. Subsequent studies showed that the form of AFB₁-FABY normally present in double-stranded DNA is mutagenic, whereas the dominant species in single-stranded DNA blocks replication [5].

Chronic liver injury and regenerative hyperplasia are critical to the development of HCC [30]. AFB₁-induced DNA adducts may therefore be fixed as mutations consequent to an HBV-related increase in cell proliferation and hyperplasia. Inflammation and oxidative stress associated with chronic active hepatitis and aflatoxin exposure may also result directly in DNA damage and mutations [52].

The 'DNA damage checkpoint response' acts as an anti-tumour mechanism against genotoxic agents. By playing a central role in co-ordinating DNA repair and cell cycle progression, 'DNA damage checkpoint response' proteins play a key role in preventing mutations [66]. Genotoxic doses of AFB₁ induce an incomplete and inefficient 'DNA damage checkpoint response', which may contribute to the carcinogenic properties of the toxin [27].

AFB₁ has a geographical distribution similar to that of chronic HBV infection, colonizing a variety of foodstuffs in the same Far Eastern and sub-Saharan African countries. Accordingly, a synergistic interaction between the hepatocarcinogenic effects of HBV and AFB₁ would offer a plausible explanation for the very high incidence of HCC, and perhaps also the young age of the patients, in these regions.

2. Evidence for a synergistic hepatocarcinogenic interaction between aflatoxins and hepatitis B virus

Although a study in Guanxi, China published in the mid-1980s showed that HCC occurring in individuals infected with HBV who lived in villages with a "high" consumption of aflatoxins had a mortality rate that was 10 times higher than that in individuals living in villages with a "low" consumption [93], other early studies of the consequences of exposure to aflatoxins did not include data on the HBV status of the populations studied. All of these

studies showed a statistically significant increase in incidence of HCC in those individuals who were exposed to the fungal toxin [31, 53, 32]. But, given the high frequency of chronic HBV infection in this region at that time, the probability exists that at least some, and possibly a significant number, of the subjects included in these early studies were also chronically infected with HBV and that the virus, rather than the exposure to aflatoxins, could have caused the malignant transformation or, at least, contributed to it. In two studies, one earlier and the other later, the roles of AFB₁ and HBV in explaining the varying frequencies of HCC in different areas of Swaziland in southern Africa [61] and in Guanxi Province in China [93] were assessed. Both analyses concluded that with simultaneous exposure to the two potential carcinogenic agents, AFB₁ exposure was the more important determinant of geographical variation in the incidence of HCC than was HBV infection, at least in those regions. However, no attempt was made in either study to evaluate a possible interactive hepatocarcinogenic effect between the two risk factors.

The first published evidence consistent with synergism between AFB₁ and HBV in the genesis of HCC 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 [67]. Shortly thereafter, further experimental evidence for a positive interaction between AFB₁ and another member of the *Hepadnaviridae* family, the woodchuck hepatitis virus, in the development of HCC was presented [3]. Woodchucks infected with woodchuck hepatitis virus and exposed to AFB₁ developed, after six to 26 months of exposure, a high incidence of pre-neoplastic foci of altered hepatocytes followed by hepatocellular adenomas and HCCs. Moreover, woodchucks infected with woodchuck hepatitis virus had earlier been shown to have enhanced activation of the biologically inactive AFB₁ to AFB₁-8,9-epoxide [17]. The development of liver tumours was also reported in ducks infected with duck hepatitis virus and exposed to AFB₁ [14] and in tree shrews (*Tupaia belangeri chinensis*) infected with HBV and exposed to AFB₁ [48].

Following the introduction of methods to measure aflatoxin metabolites and aflatoxin-DNA adducts in urine and aflatoxin-albumin adducts in serum, biomarkers that were a far more accurate and reliable indicator of AFB₁ exposure than the hitherto used food sampling and dietary questionnaires, five large cohort studies were undertaken in Shanghai and Qidong county, China and in Taiwan to assess the carcinogenic effects of AFB₁ and HBV alone and in combination. In four of the studies an hepatocarcinogenic effect of AFB₁ alone was shown, with increased odds ratios ranging from 1.9 to 32.0 with a mean ratio of 13.7 [65, 64, 54, 59] (Table 1). These studies proved that exposure to AFB₁ alone could cause malignant transformation of hepatocytes in humans. The fifth study failed to show an increased odds ratio of AFB₁ exposure alone [79].

As expected, these studies (including the one that did not show an increased odds ratio for the development of HCC for AFB₁ alone [79]) confirmed an hepatocarcinogenic effect of HBV alone - odds ratios ranged from 3.3 to 17.4, with a mean ratio of 10.0 (Table 1).

	HBV alone	AFB ₁ alone	HBV and AFB ₁
	RR (95% CL)*	RR (95% CL)	RR (5% CL)
[65]	4.8 (1.2 - 19.7)	1.9 (0.5 - 7.5)	60.1 (6.4 - 561.8)
[64]	7.3 (2.2 - 24.4)	3.4 (1.1 - 10.0)	59.4 (15.6 - 212)
[80]	17.4 (3.6 - 143.4)	0.3 (0 - 3.6)	70.0 (11.5 - 425.4)
[54]	17.0 (2.8 - 103.9)	17.4 (3.4 - 90.3)	67.6 (12.2 - 373.2)
[59]	3.3 (1.3 - 8.3)	32.0 (4.0 - 255.8)	40.7 (12.7 - 130.9)

Table 1. Findings in five studies comparing the risk of HBV infection alone, dietary exposure to AFB₁ alone, and the two risk factors together in the genesis of HCC. * Relative risk (95% confidence limits).

A synergistic interaction between AFB₁ exposure and chronic HBV infection in causing HCC was evident in each of the five studies - odds ratios ranged from 40.7 to 70.0 with a mean of 59.6 (Table 1). In three of these studies there was a striking multiplicative effect, and in the other two a sub-multiplicative effect between exposure to AFB₁ alone and exposure to AFB₁ in the presence of chronic HBV infection in inducing HCC, in comparison with each carcinogen alone. The study which did not show an increased odds ratio with AFB₁ alone had the highest odds ratios for both HBV infection alone and for co-existing AFB₁ exposure and HBV infection [79]. The finding in this study that exposure to AFB₁ alone did not increase the risk of HCC development [79] could conceivably be the source of the erroneous view held by some hepatologists and oncologists that AFB₁ alone does not cause HCC and is important only as a co-carcinogen with HBV.

In other investigations, also in countries with high rates of contamination of foodstuffs by AFB₁, only individuals chronically infected with HBV were studied and the influence of AFB₁ exposure in further increasing their risk of HCC development was analysed. In Qidong county, China, over a 10-year prospective follow-up period, the risk of HCC in male carriers of the virus was shown to be increased 3-fold (95% confidence limits 1.2, 8.7) in those with detectable urinary levels of AFB₁ metabolites in comparison with those without these metabolites [72]. This result was later confirmed in a longer follow-up of the same cohort of HBV carriers, when the risk of HCC was increased 3.5-fold (95% confidence limits 1.5, 8.1) [57]. A dose-response relationship between urinary AFB₁ metabolites and the risk of HCC was shown in HBV carriers in Taiwan [94]. Comparing high and low urinary levels of the aflatoxin metabolite, AFM₁, a multivariate-adjusted odds ratio of 6.0 (95% confidence limits 1.2, 29) was calculated. The risk was greater (odds ratio 10.0: 95% confidence limits 1.6 ; 60.9) when both AFM₁ and AFB₁-N⁷-gua metabolites were tested for and detected in the urine. In another study performed in chronic carriers of HBV in the same country, a statistically significant relationship was noted between detectable levels of AFB₁ adducts in serum and the risk of HCC, with an age-adjusted odds ratio of 2.0 (95% confidence limits 1.1, 3.7) [73]. A recent meta-analysis has shown that the population attributable risk of developing HCC in individuals exposed to dietary aflatoxins is 17%, with the risk being 21% in

those individuals also chronically infected with HBV [49]. Individuals infected with HBV alone have a population attributable risk of 8.8% [49]. If the one study in the meta-analysis that contributed most to heterogeneity in the analysis was excluded, the summarised odds ratio of HCC (with 95% confidence limits) was 73 (36 to 148.3) for the combined effects of AFB₁ and HBV, 11.3 (6.75 to 18.9) for HBV alone, and 6.37 (3.74 to 10.86) for AFB₁ alone [49]. The effect of a synergistic interaction between AFB₁ and HBV on the age of onset of HCC was specifically addressed in a study of Taiwanese patients. HBV-infected patients in whom tumour tissue was shown by histochemical staining to be positive for AFB₁-N⁷-gua adducts were on average 10 years younger than those with adduct-negative tumours [10].

Although they have had limitations, various animal models with natural hepatitis viral infections have been used to examine the interaction between hepadnaviruses and AFB₁ [84]. In woodchucks and tree shrews, animal species with hepadnaviral-induced liver pathology similar to that observed in HBV-infected humans, the administration of AFB₁ resulted in a higher incidence of liver tumours than in infected animals not receiving AFB₁ [92, 3]. Moreover, HBsAg transgenic mice over-expressing the large envelope protein of HBsAg in the liver developed HCC when exposed to aflatoxin, whereas their littermates not exposed to carcinogens did not [67, 47].

In those human populations in which an interaction between the fungal toxin and HBV has been described, the infection is predominantly acquired in infancy or early childhood. During the early years of HBV infection, a state of immune tolerance towards the virus exists and little if any cellular damage occurs. With loss of this tolerance, the ongoing infection results in recurring cell damage. Exposure to AFB₁ in contaminated foodstuffs also occurs in young children [81].

Nevertheless, it is likely, certainly in China and Taiwan, where perinatal transmission of HBV is the predominant mode of infection, and also probably in Africa, where slightly later horizontal infection is the major route of infection, that the HBV carrier state is established, not before exposure to, but before heavy exposure to the toxin.

3. Possible mechanisms of interaction between AFB₁ and HBV in hepatocarcinogenesis

A number of possible mechanisms for the interaction between HBV and AFB₁ in causing HCC have been suggested. One is that HBV infection directly or indirectly sensitizes hepatocytes to the carcinogenic effects of AFB₁. One way in which this may be accomplished is that the specific cytochrome P450s that metabolize AFB₁ to AFB₁-8,9-epoxide may be induced either by chronic hepatitis caused by HBV infection or by the presence of the virus itself. Induction of these phase I enzymes has been described in HBV transgenic mice [21, 6], where this effect appeared to result from hepatocyte injury induced by the virus rather than the presence of the virus *per se* [6]. The observation that Gambian and Taiwanese children and adolescents chronically infected with HBV have higher concentrations of AFB₁ adducts than uninfected individuals [2, 76, 11] is consistent with this mechanism. But studies in adults in China, Taiwan

and The Gambia have either failed to show a significant difference in serum AFB₁ adduct levels between HBsAg-positive and -negative subjects [23, 79, 12] or showed only a marginally significant difference [74]. Moreover, a study in woodchucks with chronic woodchuck hepatitis virus infection did not show enhanced activation of AFB₁ [75, 44].

The generated aflatoxin-8,9-epoxide has been shown to bind to proteins, causing acute toxicity, or to DNA inducing changes that over time increase the risk of malignant transformation [26]. DNA damage can also increase the chance of integration of the viral DNA into the host genome [16]. This effect could be exerted directly by AFB₁ or indirectly by oxidative stress induced by chronic viral hepatitis.

A guanine to thymine transversion at the third base of codon 249 of the p53 tumour suppressor gene (arginine to serine substitution; 249^{ser}, R249S) is present in between 40 and 66% of HCC patients in regions with heavy dietary exposure to AFB₁ [28, 4, 45, 30]. The mutation is also detectable in circulating cell-free DNA from the plasma of HCC patients and healthy subjects from these regions [77]. The exact timing of the development of the 249^{ser} mutation remains uncertain, although it has been shown to be an early event. The mutation abrogates the normal functions of p53, including those in cell cycle control, DNA repair, and apoptosis, thereby contributing to the multistep process of hepatocarcinogenesis. This mutation is extremely uncommon in tumors other than HCC [58].

A specific and close association between this inactivating mutation, the presence of AFB₁ biomarkers, and the development of HCC was recognised in epidemiological studies in regions with high or low AFB₁ exposure rates [4, 60, 15, 19, 45, 30, 62, 22], and evidence that the mutation induced chromosomal instability was found [62]. Arising from the observation of the co-existence of the p53 mutation and AFB₁ exposure, the presence of the 249^{ser} mutation was believed to be a primary genetic event in hepatocarcinogenesis. It occurs early in the series of events leading to AFB₁-associated HCC, and may thus provide an early biomarker of exposure to the fungal toxin and AFB₁-induced hepatocarcinogenesis [36].

But the findings have been inconsistent with support for an aetiological association being provided by some but not all studies. In an investigation of Taiwanese patients with HCC the mutation was present in 36.3% of HBV-infected patients with HCC, compared with 11.7% of those without HBV markers [79]. In a second analysis in Taiwan, all of the 249^{ser} mutations occurred in patients positive for HBsAg, giving an odds ratio of 10.0 (95% confidence limits 1.6; 17.5) [54]. In a study in The Gambia patients positive for HBsAg alone had an increased relative risk of 10, those with 249^{ser} mutation alone of 13, and those with both an estimated risk of 399 [45]. Other studies, however, showed a similar, but not a statistically significant trend [68, 19], and in yet other analyses from a variety of countries no association could be found [listed in reference: 69]. Furthermore, in a meta-analysis of 49 published studies using a method that takes into account both within-study and study-to-study variability, little evidence for HBV-AFB₁ interaction in modulating the 249^{ser} mutation was found [69]. In addition, the absence of the 249^{ser} mutation from the serum of patients from countries with a low incidence of HBV-induced HCC to date suggests that chronic HBV infection alone is insufficient to result in the development of the 249^{ser} mutation [82].

Another suggested possibility is that the activity of phase II detoxification enzymes (glutathione S transferase (GST) and epoxide hydrolase (EPHX)) may play a role in the genesis of HCC induced jointly by AFB₁ and HBV [94; 73; 56]. A multiplicative interaction in the genesis of HCC in West African and Chinese patients was demonstrated between HBV infection and mutations of EPHX [56]: patients with chronic HBV infection but with normal EPHX alleles were at a 15-fold increase in risk, and those with both HBV infection and at least one EPHX mutant were at a 77-fold increased risk. In further studies in these patients a positive interaction between HBV and AFB₁ seemed to depend on the presence of a polymorphism of the GST M1, GST T1, and EPHX genes that are normally responsible for converting the carcinogenic AFB₁-8,9-epoxide to non-reactive metabolites [56, 8, 94, 73]. But again no consistent pattern has emerged. In one analysis in Taiwan the risk of HCC formation was greater in HBV carriers who had the GST M1 null genotype compared with the non-null genotype [94], in a second study the risk appeared to depend on the presence of a GST T1 null genotype [73], and in a third the risk was considerably greater in those with null genotypes of both GST M1 and GST T1 [8].

Another possible mechanism for a carcinogenic interaction between AFB₁ and HBV is that increased hepatocyte necrosis and proliferation cause by chronic HBV infection increases the likelihood of both AFB₁ mutations, including 249^{ser}, and the subsequent clonal expansion of cells containing these mutations [13]. Chronic necroinflammatory hepatic disease, including that resulting from HBV infection, results in the generation of oxygen and nitrogen reactive species [50, 35]. Both of the latter are mutagenic, but, in addition, increased oxidative stress has been shown to induce 249^{ser} mutations [29].

The HBV x gene is frequently included in sequences of the virus that are integrated into cellular DNA [43]. AFB₁-DNA adducts are normally repaired by the nucleotide excision repair pathway. The HBV X protein interferes with the nuclear excision repair pathway [38; 43] and might, by this means, favour persistence of existing mutations or impaired DNA. DNA repair is also compromised by the rapid cell turnover rate in chronic hepatitis. In the presence of dietary exposure to AFB₁, the HB X protein may contribute to the uncontrolled cell proliferation in other ways. The transcription of p21^{waf1/cip1}, which induces cell cycle arrest at the G₁-S checkpoint, is activated by HB X protein in a dose-dependent manner in the presence of functional p53. This transcription is, however, repressed by HB X protein when p53 is not functional or is functional at a low level [1]. The expression of HB X protein also correlates with an increase in the overall frequency of DNA mutations in transgenic mice and a 2-fold increase the incidence of the 249^{ser} mutation in transgenic mice exposed to AFB₁ [55].

Altered methylation of genes may play a role in hepatocarcinogenesis [43]. For example, the methylation status of the human *ras* association domain gene (RASSF1A) and the P16 gene has been incriminated in the pathogenesis of HCC [95]. No association was found between methylation status and P53 status [95]. A statistically significant association was, however, found between RASSF1A methylation status and the level of AFB₁-DNA adducts in HCC tissues [95].

An understanding of the mechanisms responsible for the heightened risk of malignant transformation in patients chronically infected with HBV and exposed to AFB₁ is far from

complete, and there is clearly a need for further research to be undertaken into the pathogenic mechanisms involved in this interaction between the two common hepatocarcinogens in resource-constrained geographical regions.

Author details

Michael C. Kew^{1*}

Address all correspondence to: michael.kew@uct.ac.za

1 Department of Medicine, Faculty of Health Sciences, University of Cape Town, Department of Medicine, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

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