BLOOD AFLATOXIN LEVELS IN PATIENTS WITH HEPATOCELLULAR CARCINOMA IN SINGAPORE

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ABSTRACT

Aflatoxin (AF), a highly potent hepatocarcinogen, is strongly implicated in the pathogenesis of human hepatocellular carcinoma (HCC). In this study, our aim was to determine if this carcinogen is associated with cases of HCC in Singapore. Blood levels of the naturally-occurring AFs – B_1, B_2, G_1 and G_2 – were assayed in 56 cases of HCC. AF was detected in only 2/56 (3.6%) cases of HCC, one each of AF-B_1 and AF-G_1. In contrast, in a similar survey done in Singapore on normal subjects, AF was positive in 64/423 (15.1%) cases. The low frequency of AF detection in our patients suggests that HCC in Singapore is not associated with significant chronic exposure to AF.

Keywords: *aflatoxin, hepatocellular carcinoma*

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INTRODUCTION

In 1960, poultry deaths were associated with contamination of a metabolite of *Aspergillus flavus* in the peanut feeds[1], which was subsequently identified to be a mycotoxin and was named aflatoxin B_1 (AF-B_1). Since then, several studies have documented AF as an extremely potent hepatocarcinogen inciting formation of malignant hepatic tumours in both animals and humans[2,3]. Epidemiological studies have conclusively documented the positive association between exposure to AF and the incidence of human hepatocellular carcinoma (HCC)[4-6]. AF has also been implicated to potentiate the development of HCC in patients with chronic hepatitis B infection[7-9].

As HCC is a common malignancy in Singapore often associated with chronic hepatitis B infection[10] and the presence of AF exposure among normal subjects in Singapore has been documented in a survey conducted in 1986[11], we carried out a study to determine if exposure to AF has a significant contribution to the pathogenesis of HCC in Singapore.

METHODS

Fifty-six consecutive patients with HCC seen in our department during the period January 1992 to October 1992 were enrolled into the study. The diagnosis of HCC was made on the basis of the World Health Organisation (WHO) criteria (histology or alphafoetoprotein >400 ng/ml with compatible imaging studies). Venous blood was obtained by venepuncture of the antecubital vein and deposited in a EDTA tube for transportation to the laboratory. The blood specimens were then stored in a freezer at -20°C for a maximum of 2 weeks before being analysed.

Two Hewlett-Packard (HP) 1090 series 11/L liquid chromatographic (LC) instruments, each equipped with a 200mm X 2.1mm ID microbore ODS-Hypersil column of 5µm particle-size, a HP 1046A fluorescence detector and a HP 3396A integrator, were used with 32% acetonitrile in water as the mobile phase. The detector conditions used were: excitation wavelength 365nm, emission wavelength 432nm, response time 6 seconds, photomultiplier gain 14, lamp current 1 and background below 3.5. The HP3396A integrator was set as follows: zero, 20, 0.086; attenuation 5; chart speed 0.5; area rejection 1000, threshold 7 and peak width 0.15 The LC instruments were set at a flow rate of 0.2ml/min and 70kPa pressure. Blood standards containing various concentrations of each of the AF were prepared from standard solutions of AF-B_1, B_2, G_1 and G_2 of 98% purity (Sigma Chemical). Extracts of these blood standards after column clean-up and derivatisation were used to calibrate the high performance liquid chromatographic (HPLC) instruments. The detection limits for AF-B_1, B_2, G_1 and G_2 were estimated to be 3pg/ml, 10pg/ml, 3pg/ml and 10pg/ml respectively.

Each blood specimen was processed as follows: a 2ml portion of blood was placed in a centrifuge tube and 15ml of chloroform and 400pg AF-G_2 or 200pg AF-B_2 as internal standard were added. The mixture was vigorously shaken on a Voss SH/SP linear mechanical shaker for 30 minutes, followed by centrifugation at 2500rpm for 15 minutes on a Denley BS400 centrifuge. The upper blood matrix layer was aspirated off and the lower organic layer filtered through a Whatman filter paper. This organic layer was then applied to a sawn-off 50ml burette column packed with cotton wool, 2g of anhydrous sodium sulphate (Hayashi Pure Chemical Industries) and 0.6g of forisil (Merck) in chloroform. The column was washed successively with 20ml of 50% hexane in chloroform, 10ml of 10% methanol in chloroform and 10ml of 1% water in acetone. The final 9ml portion of the 1% water in acetone was collected in a large-mouthed 40ml tapered tube and the solvent admixture evaporated off on a Techne DB.3A at room temperature under a stream of nitrogen. 100µL of derivatising agent (50% trifluoroacetate in water) was then added to the evaporate and the mixture was kept at 50°C for 30 minutes. Following the removal of

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the solvent admixture on the concentrator at room temperature under a stream of nitrogen, the evaporate was taken up in 200μL of 32% acetonitrile in water which served as the HPLC mobile phase solvent. HPLC analysis was then performed.

Stringent decontamination procedures were followed to avoid cross-contamination of equipment. Disposable equipment were used whenever possible. No item was allowed to be near to or in contact with AF-rich substances. Non-disposable equipment were washed successively with chloroform, acetone, methanol, water, soaked in 1% hypochlorite for at least 12 hours, followed by washing with detergent, rinsing and drying before being re-used.

RESULTS

There were 50 men and 6 women patients with a mean (±SD) age of 58.6 (±12.1) years. Fifty-four patients were Chinese while the remaining 2 were Malay. The probable aetiological associations of the HCCs are shown in Table I.

Table I – Probable aetiological associations of the HCCs (n=56)

Probable aetiology	Number of cases
HBsAg positive	38
Anti-HBcIgG positive alone	8
Anti-HCV IgG positive alone	2
Anti-HCV and anti-HBcIgG positive	3
Alcohol (≥60g/day for 5 years)	3
No apparent disposition to HCC	2

Two patients with HCC, who were also HBsAg positive, had measurable levels of AF in their blood. One had a AF-B_1 level of 7.4pg/ml while the other had a AF-G_1 level of 17pg/ml (Table II).

Table II – Mean blood AF levels and positive rates of detection of blood AF

Country	Blood AF level Mean±SD (pg/ml)	Range (pg/ml)	% of samples found positive
Present study			
HCC patients	AF-B_1:7.4 AF-G_1:17	– –	3.6% (2/56)
Singapore[19]			
Normal subjects	AF-B_1:5.4±3.2	3.0-17	15.1% (64/423)
Japan[20]			
Normal subjects	AF-B_1:119.5±37.8	82-180	8.0% (4/50)
HCC patients	0	–	0% (0/12)
Indonesia[20]			
Normal subjects	AF-B_1:220.6±185.3	78-616	20.0% (13/65)
HCC patients	AF-B_1:231.3±121.1	68-358	6.3% (3/48)
Philippines[20]			
Normal subjects	AF-B_1:277.1±258.0	50-864	25.0% (14/56)
HCC patients	AF-B_1:85.0	76- 94	5.7% (2/35)

DISCUSSION

AFs are metabolites of the ubiquitous fungal strains *Aspergillus flavus* and *Aspergillus parasiticus* and are also potent hepatocarcinogens[12]. AF occurs naturally in four major forms (B_1, B_2, G_1 and G_2). AF-B_1 is the most potent carcinogen[13] and also the most commonly occurring AF[12], being present whenever there is food contamination by AF[14]. AF has long been classified by the WHO International Agency for Research in Cancer (IARC) as a definite human carcinogen causing HCC[2].

Several epidemiological studies have documented a positive relationship between AF ingestion and HCC[4-6]. However, these studies were confounded by the presence of chronic hepatitis B infection, which in itself is a risk factor for HCC. Later studies specifically addressed this issue and showed an increased risk from AF exposure itself over and above chronic hepatitis B infection[15,16]. Recently, a codon 249 mutation of the p53 gene has been shown in hepatitis B-related HCCs which were also strongly associated with dietary AF intake, thus suggesting a possible potentiating mechanism[7]. Other recent studies have also implicated the potentiation by AF of the development of HCC in patients with chronic hepatitis B infection[8,9].

HCC is a common malignancy in Singapore, being the fourth most common cancer in males and ninth in females[17]. Most of our HCCs are associated with chronic hepatitis B infection[10] (personal unpublished data). Singapore has to import almost all her food supplies and despite stringent checks on the quality of food imported, a number of aflatoxin-contaminated food items have slipped through our surveillance[18]. Furthermore a 1986 survey showed the presence of aflatoxin exposure in Singapore[11].

With these background in mind, we did a study to document for the first time whether our patients with HCC are associated with chronic exposure to AF. A previous survey conducted in Singapore on healthy blood donors revealed a positive AF exposure rate of 15.1%[19].

In contrast to the results obtained from the healthy subjects, only 2 of the 56 (3.6%) HCC patients had measurable serum AF. One of the patients had AF-B_1 measured at 7.4pg/ml whilst the other patient had AF-G_1 measured at 17pg/ml. Both these patients had chronic hepatitis B infection as well. As some of the blood specimens were obtained from the HCC patients after they had been on a few days of a controlled hospital-prepared diet, the positivity rate may be under-estimated. However, a similar study performed by another group of workers on hospital patients with HCC showed that they can still have detectable AF levels (Table II), implying that our cases of HCC have a true likelihood of non-association with AF exposure.

In this study, our HCC patients have a lower frequency of positive blood AF levels compared to healthy subjects. This was also the case in Japan, Indonesia and Philippines (Table II). Although this may be taken to argue against a significant contribution of AF to the pathogenesis of HCC in these countries, it is also possible that the AF levels were falsely low in the patients with HCC because of the controlled hospital-prepared diet that they were taking. In contrast, the normal populations studied were hospital staff, medical students, families of patients[20] and in our case, blood donors[19] – all of whom are well and consume an uncontrolled diet. Another factor that could account for a falsely low AF level in HCC patients is poor intake of food due to constitutional symptoms associated with the malignancy, hence minimising the ingestion of AF-contaminated items.

Similar studies done in Indonesia and the Philippines that also used HPLC for detection of AF-B_1 in blood have revealed higher rates of detection of AF-B_1 both in their normal subjects and patients with HCC compared to Singapore (Table II), probably reflective of their dietary customs and socio-economic situations. This conclusion is supported by the fact that Japan, which has a higher standard of living than Singapore, has lower rates of detection of AF (Table II).

Due to their short periods of persistence in the circulation,

blood levels of the parent AF compounds may not be the best method of detecting low levels of AF exposure in individuals[20,21]. The major urinary AF DNA adduct, AF-B_1 N^7-guanine (AFB_1-N^7-Gua), which persists longer, may be a more suitable marker for low levels of AF exposure[22] and indeed has already been shown to be a useful biomarker for risk of HCC[23]. Alternatively, assaying serum albumin adducts to AF which have a similar half-life to albumin may better assess dietary AF intake in the longer term[24]. We are presently looking into improving detection of AF exposure with the measurement of these AF adducts.

In conclusion, we have shown that our patients with HCC do not have an association with chronic exposure to AF. As an extension to the present study, the use of better markers of AF exposure may be explored. Further studies are also being considered to measure AF levels in HCC tissues, which would give a more direct indication of the association of this carcinogen with our cases of HCC.

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