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MONITORING OF AFLATOXIN EXPOSURE BY BIOMARKERS

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ABSTRACT — Epidemiological studies have demonstrated a strong association between exposure to AFB1 and an increased incidence of human hepatocellular carcinoma (HCC). This association has led to a need for accurate techniques relating AF exposure to an individual's risk of developing disease. With the understanding of the progressive processes of carcinogenesis, opportunities for the identification of molecular biomarkers reflecting events from exposure through clinical disease are provided. However, the development of biomarker methods to monitor human exposure to AFs requires techniques which are sensitive, specific, and amenable to large numbers of samples.

To better understand the role of AF exposure with respect to HCC incidence, immunoassays for the biological quantitation of free AFB1, its metabolites, and its adduct macromolecules have been developed. ELISA appears to offer a suitable method for use in epidemiological studies for monitoring short-term exposure to AFs, as it has the appropriate sensitivity and specificity. However, the presence of substances that are presumably not AFs and which are inhibitory in the ELISA system has necessitated the development of purification techniques, usually based on adsorption onto Sep-Pak C18 cartridges and immunoaffinity chromatography. Many protocols have been developed for the assay of soluble AF metabolites in urine, milk and blood. However, these assays only indicate recent exposure, whereas the presence of albumin-AFB1 adducts in peripheral blood could present a useful material for assessing longer-term exposure. Among the various possible biomarkers of AF exposure, the measurements of AF-DNA and -protein adducts are of major interest because they are direct products of damage to a critical cellular macromolecular target.

In Thailand, AF contamination of foods was reported to be high. More recent data using biomarkers as measures of AF exposure will be discussed. The data from epidemiological studies, AF exposure assessment using AF-albumin adduct and urinary AF level as exposure markers as well as the prevalence of p53 mutation at codon 249 are all suggestive of a limited importance of AF in the etiology of HCC in this country compared to other areas, including parts of Africa and China. These results also indicate that research on other potential hepatocarcinogens should not be neglected.

KEY WORDS: aflatoxin monitoring, biomarkers, Thai population

INTRODUCTION

There is considerable evidence indicating an association between AF ingestion and liver cancer in humans. Most of the evidence linking these two factors is based on correlation between cancer incidences and the levels of exposure to AF contamination in food. But the presence of AF-contaminated food in general food surveys does not give information con-

cerning specific individual exposures which can only be obtained from individual monitoring¹. Although epidemiological studies may provide evidence for an association, they cannot give direct proof of a cause and effect relationship. In addition, this is usually retrospective. In such circumstances, they can detect only relatively large increases in relative risk and huge numbers of subjects will be required to evaluate a substance having a small risk.

In Thailand, liver cancer is the most common fatal neoplasm reported in the National Cancer Registry. It occurs in males three times more frequently than in females². According to histological diagnoses, HCC was found more frequently than cholangiocarcinoma in both sexes³. The geographical variations in the incidence of HCC in Thailand are rather small.

Aflatoxin monitoring in biological fluids

In the past, monitoring of human exposure to AFs was achieved indirectly by analysis of raw agricultural commodities and food products ready for consumption. However, the drawbacks of this approach are that it relies on dietary recall for the amounts and types of food consumed and involves the laborious sampling and analysis of individual foods prepared for consumption. More importantly, it provides little or no information about interindividual differences in intake or metabolism of AF⁴.

The survey of AF-contaminated foodstuff in various parts of the world revealed that the proportion of contaminated food in Thailand seemed to be lower than in other countries⁵. During 1967-1969, average levels of AF in groundnut were more than 1,000 (g/kg with more than 50% of samples being positive, whereas in rice only 2% of samples were positive with an average level of 10 (g/kg in the positive samples⁶. More recent data similarly showed a high contamination of groundnuts and corn but not of rice⁷. Since rice is the dietary staple and consumption of corn or groundnuts is infrequent, this is reflected in the relatively low daily individual exposures of between 5 and 45 ng AF/kg body weight/day⁷.

The development of methods which permit the monitoring of exposure to a suspected or known carcinogen at an individual level may overcome some of the limitations of cancer epidemiology. Exposure monitoring provides a bridge between laboratory animals and human studies. The incorporation of biological monitoring methods into conventional epidemiological studies can increase their power to detect carcinogenic risk earlier, and at lower exposure, as well as to estimate more accurately the magnitude of human risk⁸. However, biological monitoring may not be straight forward and should be used in parallel with environmental monitoring, especially in the case of carcinogens, for which the latency between exposure and effects may last for decades.

In order to monitor human exposure to any toxic substance there are two important considerations to

bear in mind. Firstly, the methods used must be sensitive and specific enough to allow detection of human exposure to any substance of interest, which will often be at an extremely low level. Secondly, possible modifying and/or confounding factors, such as dietary considerations, drinking and smoking habits must be taken into account when the results of the biological monitoring are being analysed⁹.

Epidemiological studies have demonstrated a strong association between AFB1 exposure and an increased incidence of human HCC. It is now possible to assess individual exposure to AFB1 with comparative simple assay that can be applied in field studies to a relatively large number of samples. This should allow clarification of the role of AFB1 in the etiology of liver cancer.

At present, the biological samples of interest for human monitoring of AF are urine, blood, milk and tissues samples. The possibilities for the measurement of AF exposure in biological samples include the measurement of parent AF and/or their metabolites in these samples or the measurement of AF adducts with DNA or protein. Immunoassays for the biological quantitation of free AFB1, its metabolites, and its adduct macromolecules have been developed. ELISA appears to offer a suitable method for use in epidemiological studies for monitoring short-term exposure to AF, as it has the appropriate sensitivity and specificity.

Urinary AF levels in Thai population

Different Thai population had been monitored for urinary AF levels. All urine samples were cleaned-up by passing through Sep-Pak C18 cartridges before analyzing for AFB1 equivalent with competitive ELISA using polyclonal antibody raised against AFB1. The anti-serum showed cross-reactivity with various AF metabolites but with differing degree of sensitivity¹⁰. For comparison purpose, creatinine concentrations were determined and used as a reference point to correct for individual variations in volume voided. The lower limit of significant detection was 8 ng AFB1 equivalent /mg creatinine.

When this system was applied to urine samples collected from 100 neonates within 24 h after birth, the levels detected were in the range of 0.69-6.90 ng /mg creatinine. These levels were lower than the ones (0.48-19 ng AFB1/mg creatinine) detected in samples from healthy infants who were fed on AF-free powder milk and lived in the same areas as the neonate. The differences in urinary AF levels of these two groups

may reflect false positive finding in ELISA due to the presence of interfering substances which were recognized by the polyclonal antibody used in this study.

Since AFs have been reported to contaminate mainly vegetarian products such as peanut, rice and maize and vegetarians consume more of these food-stuff than the meat-eaters, it is likely that the former have a greater risk of exposure to AFs than the latter. Therefore, the level of AF excreted in vegetarians living in one community in Bangkok were monitored. The estimated levels of AF in food samples analyzed by HPLC were in the range of 0.25-2.15 ppb. The total AF intakes were in the range of 0-1.8 ug/day with the average of 0.44 ug/day. Urinary AF levels in these vegetarians were in the range of 0.28-15.71 ng AFB1/mg creatinine.

With the detection limit of 8 ng/mg creatinine, only 19% of urine samples from the infant group and 5% from the vegetarian group were found to be positive (Table 1). When some of these urine samples were sent to be analysed at the MRC Toxicology Unit in UK. for the contamination of AFB1 by HPLC technique using fluorescence and UV detectors, no AF was detected. These findings indicated that the detectable values shown by ELISA in this study were due to the presence of the interfering substances.

In order to solve the problem regarding the urinary AF-like substances, immunoaffinity chromatography had been developed and being included in the clean-up procedure. When uncontaminated human urine samples were passed through Sep-Pak C18 cartridges and immunoaffinity columns before analyzing for AFB1 by ELISA, the apparent AFB1 levels were reduced by 97%. The lower limit of significant detection for the presence of AF assayed by this system was 0.2 ng/mg.

With this new system, another group of vegetarians from the same community was reinvestigated for

their urinary AF levels. The results showed that the highest level detected was 3.22 ng/mg creatinine. When the results were compared with the one obtained from the non-vegetarian group, it was found that the levels of AF excreted in urine of these two groups were not significantly different. With the detection limit of 0.2 ng/mg creatinine, 30% of the samples from the vegetarian and 26% from the non-vegetarian groups were found to be positive (Table 2).

The levels of AF excreted in urine specimens of patients with or without liver disease were also compared in 47 samples from the former and 68 samples from the latter who were admitted to the hospitals during the same periods of time with matching age and sex. Morning urine specimens were collected on the day after their admissions and were analysed for AF levels using immunoaffinity column and ELISA. The results showed that the levels of urinary AF excretion were in the range of 0.02-0.84 and 0.02-1.69 ng AFB1 equivalent /mg creatinine in the liver disease and non-liver disease groups, respectively. The levels were not significantly different between both groups. However, the results showed a trend toward a wider distribution in the levels of AFB1 equivalent in samples obtained from the non-liver disease patients. Only 17% of samples from the liver disease group and 28% from the other group were positive (Table 2). This may be due to the fact that the patients with liver disease came to the hospitals when they were too sick to have their normal diet and the levels of AF excreted in the urine might not represent the normal level of exposure. These results suggested that patients with liver disease may not be the group that should be monitored for urinary AF levels.

With all these results, it can be concluded that the urinary AF levels in Thai population are quite low when compared to the results obtained from other parts of the world¹¹.

Table 1. Urinary AF levels in different population in Thailand.

Population	No. of subject	No. of subject with different urinary AF levels (%) (ng AFB1 equivalent/mg creatinine) ^a			
		<8 ^b	8-12	12-16	16-20
Neonate	100	100(100)	-	-	-
Infant	101	82(81)	13 (13)	4 (4)	2 (2)
Vegetarian	186	177(95)	6 (3)	3 (2)	-

^a Samples were cleaned-up with Sep-Pak C18 cartridges before analysing with competitive ELISA .

^b Lower limit of detection = 8 ng AFB1 equivalent/ mg creatinine

AF-albumin adduct and p53 gene mutation

Among the various possible biomarkers of AF exposure, the measurement of AF-DNA and -protein adducts is of major interest because they are direct products of damage to a critical cellular macromolecular target¹³. In Thailand, analyses of sera from adults throughout the country revealed a much lower prevalence of positive samples and the levels of adducts were lower than those in Africa. Only 13% of the samples analyzed were positive with the mean adduct level of 2 pg/ mg albumin. The prevalence of HBsAg was 9% which is much lower than the one reported in China¹².

In patients with HCC from regions where high exposure to AFs is expected to occur as determined by levels of contamination in food; almost all mutations of p53 gene were G to T transversions in the 3rd nucleotide of codon 249. The presence of this specific G to T transversion is consistent with the occurrence of DNA damage induced by AFB1 since the mutagenic metabolite induces this type of base change¹⁴. In Thailand, only one G to T transversion at codon 249 was detected among the 15 samples (7%) of HCC. This may reflect a lower exposure to AFs in Thailand than in China or the Gambia, rather than differences in HBsAg carrier rates¹⁵.

Recently a case-control study² and a correlation study³ of HCC in Thailand were reported using biomarkers to assess AF exposure. Both studies failed to identify an AF-associated risk of HCC. Therefore, the data from epidemiological studies, AF exposure assessment and p53 gene mutations are all suggestive of a limited importance of AF in the etiology of HCC in Thailand compared to southern China and west Africa and also indicate that research on the other

potential hepatocarcinogens should not be neglected.

Conclusion

The availability of data to elucidate the relative contribution of AF exposure and HBV infection and their mechanisms of interaction in liver carcinogenesis will influence decisions regarding the most appropriate public health measures for prevention of HCC in any given country. Prevention methods that lower AF-DNA adduct formation in HBV-positive people may be important goal to ameliorate synergy between these two potent carcinogens in causing liver cancer. And the data on AFB1 exposure in populations that have undergone vaccination against hepatitis B will be important in the assessment of the final effectiveness of HBV vaccination in the prevention of liver cancer.

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Table 2. Urinary AF levels in different population in Thailand.

Population	No. of subject	No. of subject with different urinary AF levels (%) (ng AFB1 equivalent/mg creatinine) ^a			
		< 0.2 ^b	0.2-0.5	0.5-1.0	> 1.0
Non-liver disease	68	49 (72)	13 (19)	3 (4)	3 (4)
Vegetarian	45	32 (71)	11 (24)	1 (2)	4 (4)
Non-vegetarian	45	138 (74)	39 (21)	6 (3)	3 (2)

^a Samples were cleaned-up by Sep-Pak C18 cartridges and immunoaffinity columns before analysing with competitive ELISA.

^b Lower limit of detection = 8 ng AFB1 equivalent/ mg creatinine.

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