Detection of aflatoxin B_1 -DNA adducts in liver tissue from Malaysia

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Abstract

Nine liver specimens of *Macaca fascicularis* fed with various doses of aflatoxin B_1 and three controls as well as sixteen human liver biopsy specimens from Malaysians were stained for the presence of aflatoxin B_1-DNA adducts by immunohistochemistry with a monoclonal antibody against the imidazole ring-opened persistent form of the major N-7 guanine adducts of AFB_1. Livers of all monkeys fed with aflatoxin were positive while none of the controls were positive. Fourteen human liver biopsy specimens were negative for AFB_1-DNA adducts. A specimen of liver cirrhosis and one hepatocellular carcinoma specimen appeared positive. These findings show evidence of past exposure to aflatoxin B_1 in those individuals.

Key Words: aflatoxin; biomarker; hepatocellular carcinoma; immunological techniques; DNA adducts

Introduction

Aflatoxin B_1 (AFB_1) or (6aR-cis)(2,3,6a,9a)tetrahydro-4-methoxycyclo penta[c]furo- [3',2':4,5]furo[2,3-h][*l*]benzopyran-1,11-dione, is a liver carcinogen produced by *Aspergillus* spp., a fungus ubiquitous in areas of the world with hot and humid climate, including SoutheastAsia. On ingestion, AFB_1 undergoes metabolism causing the levels of the original compound to drop to low levels rapidly. However, the AFB_1-8,9-epoxide (a metabolite of AFB_1) binds to the N-7 position of guanine of DNA resulting in an unstable adduct which is either lost from the DNA or is converted to the stable imidazole ring-opened 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl-formamido)-9-hydroxy-aflatoxinB_1 adduct (iro-AFB_1-DNA). Monoclonal antibodies have been developed to detect the iro-AFB_1-DNA adduct. Monoclonal antibody 6A10[+] was found ro be highly specific for the iro-AFB_1-DNA adduct, with no cross reactivity with 8,9-dihydro-8-(7-guanyl)-9-hydroxy-AFB_1 (the N-7 AFB_1-guanine adduct), AFB_1-BSA, AFM_1-BSA, AFB_1, AFG_1 or to other carcinogen modified DNAs such as 7ß,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(*a*) pyrene-modified DNA, *N*-acetoxy-*N*-2-acetylaminofluorene-modified DNA, 1-aminopyrine-modified denatured DNA or 8-methoxypsoralen-modified DNA (Hsieh *et al.*, 1988). This antibody has been used to quantitate AFB_1-DNA adducts in fresh liver tissue of rats as well as in human hepatocellular carcinoma specimens by immunofluorescence (Zhang *et al.*, 1991a). The extent of exposure to AFB_1 in Malaysia is not known. Detection of AFB_1-DNA adducts in liver tissue sections as a marker for past exposure to AFB_1 could provide some information on the exposure to AFB_1 in the individuals. We report the use of the monoclonal antibody 6A10 against the iro-AFB_1-DNA adduct for detection of AFB_1-DNA adducts in archival liver sections of *Macaca facicularis* dosed with AFB_1, as well as in archival human liver sections of liver cancers and other liver diseases from Malaysia.

Materials and Methods

For the first part of the study, archival tissue blocks of liver from 12 monkeys were used. These were from AFB_1-fed monkeys in an experimental study carried out in 1991. (The experiment was approved by the animal use committee.) The dosages of oral AFB_1 given to these monkeys are given in Table 1. Liver sections of 5 µm thickness were deparafinized and treated with 70% cold ethanol for 10 mins. The sections were then treated with 15 mM Na_2CO_3:30 mM $NaHCO_3$ (pH 9.6) for 2 hours at room temperature to ring open the guanine adducts. The sections were rinsed with Tris- buffered saline (TBS) and treated with RNase (100 µg/ml) at 37°C for 1 hour. Following a rinse with TBS, the sections were treated with proteinase K (10 µg/ml) at room temperature for 10 mins, rinsed with TBS and 40% ethanol. The sections were then rreated with 50 mM NaOH in 40% ethanol for 30s at room temperature to denature the DNA and rinsed with 40% ethanol. The pH was adjusted with 5% aceric acid in 40% ethanol for 1 min. at room temperature followed by a rinse in distilled water and TBS. The sections were then incubated with 10% normal goat serum (Histomark, KPL, USA) in 10 mM Tris (pH 7.5) at 37°C for 45 mins. to block nonspecific binding sites. The sections were incubated with antibody 6A10 at 1/2 dilution at 37°C for 45 mins and detected using the streptavidin alkaline phosphatase system (Histomark, KPL, USA). A positive result was recorded when the pink/red staining of the nuclei was of higher intensity than the surrounding cytoplasm.

Table 1. Aflatoxin-B_1-DNA adducts in liver sections of Macaca fascicularis.

No	Aflatoxin dose (mg/kg/day)	Total aflatoxin dose (mg/kg)	AFB_1-DNA Adducts
M439	0	0	-
M511	0	0	-
M559	0	0	-
M509	05	4	+
M541	05	35	+
M68	10	4	+
M519	10	4	+
M523	10	4	+
M561	10	7	+
M382	30	12	+
M504	30	12	+
M518	30	12	+

For part two of the study, sixteen archival human liver sections kept at the Institute for Medical Research were used. These were from patients from the state of Sabah, Malaysia, who were suffering from various liver diseases. Of these, 12 were hepatocellular carcinoma, while the others were diagnosed as hepatitis, fatty liver or cirrhosis. Sections from livers of *Macaca fascicularis* were used as positive and negative controls and included in every batch of slides stained. The specimens were stained following the same protocol.

Results and Discussion

All specimens from monkeys dosed with AFB_1 were positive for the AFB_1-DNA adduct. The negative control specimens remained negative (Table 1). Of the specimens of human liver, fourteen specimens were negative for AFB_1-DNA adducts. A specimen of liver cirrhosis and one hepatocellular carcinoma specimen was positive (Table 2).

Aflatoxin contaminates food leading to a chronic and possibly intermittent exposure. Assessment of AFB_1 exposure of individuals by measuring AFB_1 levels in food is tedious and inaccurate. The use of AFB_1-DNA adducts as a biomarker of aflatoxin exposure is a useful alternative approach. In our series, two individuals were positive for AFB_1-DNA adducts. As the half-life of adducts is not known, it could not be ascertained whether AFB_1 exposure caused the particular liver disease. If, however, intraindividual variation of the AFB_1-DNA adducts is minimal, such finding would suggest that the two individuals could have had significant exposures to AFB_1 leading to liver cirrhosis and hepatocellular carcinoma. While liver cancer itself could alter the metabolism of AFB_1 and thus affect adduct formation (Kirby *et al.*, 1993), the detection rate of AFB_1-DNA adducts appeared similar in liver cancers and their adjacent non-tumourous liver tissue (Zhang *et al.*, 1991b). The detection of AFB_1-DNA adducts in hepatocellular carcinoma has been reported previously. In a series from the Kaohsiung Medical College Hospital in Taiwan, 35 out of 50 cases of hepatocellular carcinoma had detectable levels of AFB_1-DNA adducts. The authors suggested that AFB_1 could be involved in the pathogenesis of hepatocellular carcinoma in those cases

Table 2. Aflatoxin B_1-DNA adducts in human liver sections.

No	Year	Age	Ethnic	Diagnosis	AFB_1-DNA adducts
1	1984	65	Malay	Hepatocellular carcinoma	-
2	1984	56	Kadazan	Hepatocellular carcinoma	-
3	1984	29	Bugis	Hepatocellular carcinoma	-
4	1984	89	Unknown	Hepatocellular carcinoma	+
5	1984	8	Dusun	Hepatocellular carcinoma	-
6	1984	47	Kadazan	Hepatocellular carcinoma	-
7	1985	26	Bajau	Fatty liver	-
8	1985	63	Chinese	Fatty liver	-
9	1985	59	Kadazan	Liver cirrhosis	+
10	1985	34	Jawa	Hepatocellular carcinoma	-
11	1985	41	Unknown	Hepatocellular carcinoma	-
12	1985	32	Chinese	Chronic persistent hepatitis B	-
13	1985	61	Bolongan	Hepatocellular carcinoma	-
14	1986	67	Kadazan	Hepatocellular carcinoma	-
15	1985	47	Kadazan	Hepatocellular carcinoma	-
16	1986	56	Malay	Hepatocellular carcinoma	-

(Chen *et al.*, 1992). In another series, from the National Taiwan University Hospital, Taiwan, 8 out of 27 hepatocellular carcinoma specimens had detectable levels of AFB_1-DNA adducts (Zhang *et al.*, 1991b). Meanwhile, Santella *et al.* (1993) noted that none of the 8 liver specimens (of which one was hepatocellular carcinoma) from the United States had detectable levels of AFB_1-DNA adducts. In our series, only 1 out of 12 cases of hepatocellular carcinoma had detectable levels of the adducts. All the reported studies and ours were case series, thus, the difference in the detection rate could be purely incidental. Nevertheless, it could still be possible that the difference is due to a difference in the extent of AFB_1 exposure, the length of storage in the specimens or the difference in the detection systems. While not being able to quantitate the AFB_1-DNA adducts, our method could be carried out in laboratories without fluorescence microscopy. The slides could also be kept for further review. Our findings provide evidence of past exposure to AFB_1 in two individuals in Malaysia. The exposure to AFB_1 was significant enough in the two cases to lead to the development of AFB_1-DNA adducts at detectable levels. In other words, AFB_1 associated DNA insult occurred in the individuals. Further studies would be needed to estimate the extent of AFB_1 exposure in relation to liver diseases in Malaysia. Our study demonstrates the use of an immunological method for detection of an important DNA adduct in local specimens. The development of monoclonal antibodies to detect other important DNA adducts in human specimens could be useful area for biotechnologists in the region.

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