Detection of Aflatoxin B$_1$-DNA Adducts in Human Placenta and Cord Blood[1]

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ABSTRACT

Human placenta and cord blood are readily available specimens that respond to maternal environmental insult and are being used to investigate metabolism, bioactivation, and transplacental transfer of procarcinogens. Enzyme-linked immunosorbent assay was used to quantitate 120 placentas and 56 cord bloods from term, uncomplicated pregnancies at Taipei Chang Gung Memorial Hospital, Taiwan, for the presence of the imidazole ring-opened form of aflatoxin B$_1$-DNA (AFB$_1$-DNA) adducts. Of the 120 samples of placentas, 69 (57.5%) contained AFB$_1$-DNA adducts in levels from 0.6 to 6.3 μmol/mol DNA. Of the 56 samples of cord bloods, 5 (8.9%) contained AFB$_1$-DNA adducts in levels from 1.4 to 2.7 μmol/mol DNA. A higher positive rate was found in samples collected in the summer than in the winter. These results indicate that a significant number of individuals in an area of high liver cancer risk have been exposed to AFB$_1$, and it is possible to transfer AFB$_1$ and its metabolites to the progeny through the transplacental unit. Thus, monitoring adduct levels in human specimens may provide information not only on carcinogen exposure but also on the relationship among infection with hepatitis B/C virus, dietary exposure to AFB$_1$, and liver cancer.

INTRODUCTION

There is a high mortality (18.5 cases/100,000/year) of PHC[3] in the Chinese population in Taiwan. PHC mortality is significantly higher among the mountainous aborigines and residents living in the Penghu islets, while hepatitis B surface antigen carrier rates are only slightly higher in these areas than for the general population (1, 2). Epidemiological studies in Taiwan have found a significant association between the development of PHC and hepatitis B virus infection, familial history of liver diseases, alcohol consumption, arsenic concentration in drinking water, and fermented bean product consumption as a source of AFB$_1$ (3). These risk factors were more significantly associated with PHC in the younger age groups than in the elderly; this suggests that they may shorten the induction period and accelerate the onset of PHC in the young. Moreover, PHC mortality for children (under age 14) is 0.4/100,000 in Taiwan, which is among the highest in the world. These studies suggest that exposure to environmental risk factors such as aflatoxin at an early age may play an important role in the development of liver cancer.

Epidemiological studies have demonstrated a strong association between exposure to AFB$_1$ in conjunction with hepatitis virus infection and an increased incidence of human hepatocellular carcinoma (4). To better understand the role of AFB$_1$ exposure with respect to human PHC incidence, immunoassays for the biological quantitation of free AFB$_1$, its metabolites, and its adduct macromolecules have been developed (5–8). There is considerable evidence that the initiating event in chemical carcinogenesis is the covalent binding of the carcinogen to cellular DNA (9), so that monitoring carcinogen-DNA adducts may be relevant to the development of cancer. The major

DNA adduct of AFB$_1$ results from binding of its 8,9-oxide to the N-7 of guanine. This adduct, which is unstable, is either lost from DNA, creating an apurinic site, or converted to the imidazole ring-opened AFB$_1$-FAPy adduct (10, 11). It is possible that AFB$_1$-FAPy, the stable form of AFB$_1$-guanine, may play an important role in the development of PHC. We have previously developed monoclonal antibodies recognizing this stable form of the adduct and used these antibodies in a competitive ELISA to quantitate DNA adducts in liver tissues of animals treated with AFB$_1$ and in human liver tissue specimens from PHC patients in Taiwan (12). A quantitative indirect immunofluorescence method using monoclonal antibody 6A10 has also been used to measure AFB$_1$-DNA adducts in liver tissues (13).

Exposure to genotoxic chemicals is widespread in human populations, and it is believed that the quantitation of carcinogen-associated DNA adduct is a valuable parameter for molecular epidemiological studies. The placenta is an important source of material for such studies because it is readily available and responsive to maternal exposures to environmental pollutants. Previous studies have shown that human and animal placentas contain enzymes which can bioactivate genotoxic carcinogens to form covalent carcinogen-DNA adducts (14–16). AFB$_1$ and its metabolites have been found in human cord sera, which suggests that transplacental transfer of AFB$_1$ may play a biological role in the initiation of PHC in progeny (17).

This study was designed to quantitate the levels of the imidazole ring-opened form of AFB$_1$-DNA adducts, as a marker of AFB$_1$ exposure in human placenta and cord blood from term, uncomplicated pregnancies.

MATERIALS AND METHODS

Chemicals. [³H]AFB$_1$ (25 Ci/mmol) was obtained from Moravek Biochemicals (City of Industry, CA). Dichloromethane and m-chloroperoxybenzoic acid were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). AFB$_1$, calf thymus DNA, goat anti-mouse IgG-alkaline phosphatase conjugates, and p-nitrophenyl phosphate (Sigma 104) were purchased from Sigma Chemical Co. (St. Louis, MO). RNase A and proteinase K were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). The imidazole ring-opened form of AFB$_1$-DNA was prepared as described previously (12).

Human Samples. Sixty placenta samples were collected from term, uncomplicated pregnancies at Taipei Chang Gung Memorial Hospital during August 1990 and in January 1991, respectively. Because many women do not deliver naturally, only 27 and 29 cord blood samples could be collected in parallel in August 1990 and January 1991, respectively. Placental cotyledons containing the chorionic villus (placental lobules) were dissected into small pieces to avoid nonparenchymal tissue contamination. These were thoroughly washed with 0.15 M NaCl and 0.015 M sodium citrate containing 1 mM ZnCl$_2$ at 4°C, frozen in liquid nitrogen, and stored at −70°C. Thirty-five ml of cord blood were collected per subject into a 50-ml conical tube containing 0.5 ml of 1000 units heparin sodium salt in 0.9% saline; serum, buffy coat, and RBC were separated by centrifugation and stored at −70°C.

Preparation of DNA Samples. Placental DNA nuclei were separated into the following two fractions using the technique described by Resendez-Perez *et al.* (18): nuclear fraction IV containing knotted nuclei from syncytiotrophoblasts and nuclear fraction III containing free nuclei from syncytiotrophoblasts plus nuclei from other placental cell types as well as from contaminating maternal and fetal leukocytes. The nuclear fractions were treated with proteinase K (200 μg/ml, 2 h, 37°C) in 10 mM Tris buffer (pH 8.0) containing 1 mM EDTA and 0.4 M NaCl and subjected to phenol extraction. Nucleic acids were recovered by ethanol (95%) precipitation, dissolved in Tris buffer, and treated

with RNase (25 µg/ml). DNA concentration was determined from $A_{260\ nm}$ ($\epsilon = 6500$) readings. Buffy coat DNA was prepared by a similar procedure, except that nuclei were isolated from cells treated with 0.32 M sucrose, 1 mM potassium phosphate, 1.5 mM $CaCl_2$, and 1% Triton X-100 (pH 7.5). All samples were treated with 15 mM Na_2CO_3 and 30 mM $NaHCO_3$ (pH 9.6) for 2 h at 37°C to ensure that all adducts are in the ring-opened form, neutralized, and reprecipitated with ethanol (95%) before dissolving in phosphate-buffered saline. Before analysis by ELISA, samples were sonicated and heat denatured to decrease the viscosity for the immunoassay.

Competitive ELISA. AFB₁-DNA adduct levels were measured by competitive ELISA using antibody 6A10 as described previously (12). Immulon 2 plates (Dynatech Laboratories, Chantilly, VA) were coated with 5 ng imidazole ring-opened AFB₁-DNA in phosphate-buffered saline (200 µl) by drying overnight at 37°C. Antibody 6A10 was used at $1:1.25 \times 10^6$ dilution and mixed with an equal volume of competitor (50 µl) before addition to the plate. DNA samples were assayed at 50 µg/well and quantitated relative to a imidazole ring-opened AFB₁-DNA standard, which has a modification level of 4 adducts/10^5 nucleotides. Goat anti-mouse IgG alkaline phosphatase was added at 1:1500 dilution, followed by the addition of p-nitrophenyl phosphate (1 mg/ml in 1 M diethanolamine, pH 8.6). Absorbance at 405 nm was read after 2 h of incubation at 37°C on a Bio-Tek Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT) connected to an Epson printer. The percentage of inhibition for the human samples was calculated by comparison to the nonmodified heat-denatured calf thymus DNA control. Each sample was assayed in triplicate at three different times with a variability of less than 10%. Values below 20% inhibition were considered not detectable.

RESULTS

Sensitive immunological methods are now available for the detection of carcinogen exposure in humans. With ELISA, carcinogen-DNA adducts can be quantitated at the femtomole (10^{-15}) level. We have previously developed a monoclonal antibody 6A10 recognizing imidazole ring-opened AFB₁-FAPy adduct with 50% inhibition at 146 fmol (12). The limit of sensitivity of detection of adducts, based on 50 µg DNA/well and >20% inhibition, is 0.5 µmol/mol DNA.

To determine whether AFB₁-DNA adducts are detectable in human placenta and cord blood, placenta and cord blood samples were obtained from term, uncomplicated pregnancies at Taipei Chang Gung Memorial Hospital, Taiwan. Antibody 6A10 was used to quantitate the imidazole ring-opened form of AFB₁-DNA adducts in the placenta and cord blood. Of the 120 samples of placentas, 69 (57.5%) contained AFB₁-DNA adducts in levels from 0.6 to 6.0 µmol/mol DNA (Tables 1 and 2; Fig. 1). Of the 56 samples of cord bloods, only 5 (8.9%) contained AFB₁-DNA adducts in levels from 1.4 to 2.7 µmol/mol DNA (Tables 1 and 2; Fig. 1). A relatively higher number of positive cases was detected in both placentas and cord bloods collected in the summer (August 1990) than in the winter (January 1991). The mean levels of AFB₁-DNA adducts detected in the placentas were also slightly higher in samples collected during the summer than in the winter. Among the 5 positive cord blood samples, AFB₁-DNA adducts were detected in 3 parallel placentas.

Table 1 *Analysis of AFB₁-DNA adducts in human placenta and cord blood*

	No. of samples analyzed	Positive cases
Placenta		
Collected in August 1990	60	40(66.7%)
Collected in January 1991	60	29(48.3%)
Total	120	69(57.5%)
Cord blood		
Collected in August 1990	27	4(14.8%)
Collected in January 1991	29	1(3.4%)
Total	56	5(8.9%)

Table 2 *Levels of AFB₁-DNA adducts in positive human placentas and cord bloods measured by ELISA*

	Positive cases	AFB₁-DNA adducts (µmol/mol DNA)	
		Mean ± SD	Range
Placenta			
Collected in August 1990	40	2.55 ± 1.26	0.6–6.3
Collected in January 1991	29	2.06 ± 0.64	0.9–3.4
Cord blood			
Collected in August 1990	4	1.98 ± 0.56	1.4–2.7
Collected in January 1991	1	1.8	
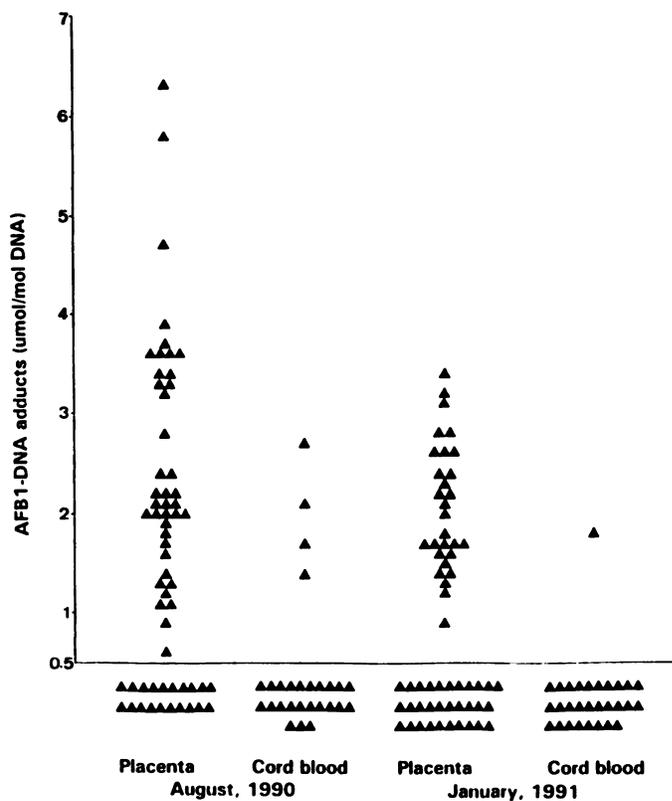
Fig. 1. AFB₁-DNA adducts detected in placenta and cord blood, measured by ELISA. Inhibition values of less than 20% were considered not detectable and are listed under the line of detection limit (0.5 µmol/mol DNA).

DISCUSSION

Previous studies have shown the ability of monoclonal antibody 6A10 to detect iro AFB₁-DNA [8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl-formamido)-9-hydroxy-AFB₁-DNA] adducts formed in animals and humans (12, 13). This study indicates that competitive ELISA is valuable in the assessment of exposure to AFB₁. Rather than measuring the aflatoxin levels in foods or its metabolites in body fluids, which can be extremely variable, it is now possible to measure levels of the covalent AFB₁-DNA adducts. Measuring carcinogen-DNA adducts may be more relevant to the development of cancer, since the initial event is thought to involve DNA damage, as evidenced by the ability of the majority of chemical carcinogens to give rise to covalent chemical addition products in the DNA of experimental animals (9).

Monoclonal antibody 6A10 was used to quantitate 120 placentas and 56 cord bloods from term, uncomplicated Taiwanese pregnancies. A higher positive rate of adducts was found in samples collected in the summer than in the winter. It may be linked to the variation in the amount of AFB₁ in food, which seems to be higher during the rainy

season in the summer than in the winter months, which are usually dry and chilly in Taiwan. Similar observations have been made in Kenya and Thailand (19, 20).

It has been reported that the presence of DNA adducts in the placenta of animals exposed to environmental carcinogens predicts the occurrence of adducts in both fetal and maternal tissues (16). Previous studies demonstrated that AFB_1 and its metabolites as well as AFB_1-albumin adducts have been found in human umbilical cord sera (17, 21, 22). Some cord bloods (8.9%) were positive for AFB_1-DNA adduct in this study, while 70% were positive for AFB_1-albumin adduct (22) and 12%–48% were positive for AFB_1 and its metabolites (17, 21). It is difficult to compare levels of DNA adducts from this study to levels of albumin adducts or free AFB_1 and its metabolites quantitated in other studies. However, the level of AFB_1-DNA adducts found in this study is comparable to those reported for liver samples from PHC patients (12, 23). The present study also indicates that a significant number of individuals in an area of high liver cancer risk have been exposed to AFB_1 and that it is possible to transfer AFB_1 and its metabolites to progeny through the transplacental unit. Previous studies (17, 21, 22) and this work provide evidence of *in utero* exposure, indicating the capacity of fetal liver to metabolize aflatoxins. Thus, quantitating adduct formation in placenta is appropriate to studying carcinogen exposure if fresh tissue is available from healthy subjects, which facilitates multiple assays.

Evidence supporting a role for aflatoxin in human liver cancer incidence has come from recent studies of PHC that demonstrate a specific point mutation at the third base of codon 249 in the *p53* tumor suppressor gene (24). It was also shown that the frequency of codon 249 *p53* mutations in PHC samples from Taiwan is lower than that found in the high AFB_1 exposure area but higher than that found in the low AFB_1 exposure area (25). Taken together, AFB_1 exposure levels may play a role in the development of PHC in Taiwan.

ACKNOWLEDGMENTS

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