Immunological Monitoring of Urinary Aflatoxins and Estimation of Liver Cancer Incidence in Koreans

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(Received September 21, 1995)

Abstract: Polyclonal antiserum R101 against aflatoxin B_1 (AFB_1) was raised in New Zealand white rabbits after injection of bovine serum albumin-AFB_1 conjugate. Competitive ELISA (enzyme linked immuno-sorbent assay) demonstrated that antiserum R101 has the highest binding for AFB_1 (50% inhibition at 170 fmol) and aflatoxicol II (50% inhibition at 112 fmol). It also reacts with other aflatoxins such as AFB_2, AFG_1, AFG_2, and aflatoxin metabolites (AFM_1, AFM_2, AFP_1, and AFQ_1), but it does not cross-react with AFG_{2a}.
Using this antiserum, aflatoxins were quantitated in 100 urine samples of undergraduate students at the College of Pharmacy, Sung Kyun Kwan University, Republic of Korea. By ELISA, AFB_1 and its metabolites were detected in human urine samples (N=100, male=89, female=11, ages=20~31 yrs) with a range of 1.4~200.6 ng/kg/day (mean± SD=18.11±33.01 ng AFB_1/kg/day in males, 3.82±2.65 ng/kg/day in females). Assuming that urinary excretion is about 7.6% of AFB_1 intake (Groopman *et al.*, 1992), we estimated that Koreans were daily exposed to a total dietary AFB_1 of 240.20±438.67 ng/kg/day in males and 50.35±29.88 ng/kg/day in females, respectively. When the human monitoring data was applied to a linear regression model of Y=21.67X−10.04 {Y=liver cancer incidence per 100,000, X=Log AFB_1 intake (ng/kg/day), r=0.99} developed from previously reported epidemiological data, calculated liver cancer incidences attributed to AFB_1 exposure were 41.56/100,000 in males and 26.84/100,000 in females. The incidences were similarly correlated with liver cancer mortality rates of 43.43/100,000 in males and 11.23/100,000 in females in Korea. These results suggest that aflatoxin exposure may be an important risk factor for the high incidence of liver cancer in Korea.
Key words: aflatoxin B_1, ELISA, human monitoring, liver cancer incidence.

Aflatoxins, metabolites of *Aspergillus flavus* and *A. parasiticus*, are present in many foods and have been implicated, in addition to hepatitis B viral infection, in the development of primary hepatocellular carcinoma (PHC) in humans (Groopman *et al.*, 1988; Wogan, 1992). An IARC working group in 1987 concluded that "there is sufficient evidence that aflatoxins are carcinogenic to humans" based on animal and human data (IARC, 1987). However, there are several reports suggesting that aflatoxins are not associated with hepatocarcinogenesis in humans (Lee *et al.*, 1989; Campbell *et al.*, 1990). To investigate the etiological role of AFB1 exposure for liver cancer, many efforts have been made to study molecular dosimetry of AFB_1 in human liver tissues and body fluids (blood, urine) (Zhu *et al.*, 1987; Gan *et al.*, 1988; Wild *et al.*, 1990).

Biological monitoring of aflatoxins exposure in humans has been studied using biological markers {aflatoxin-N7-guanine (imidazole ring-opened or -closed), aflatoxin-albumin, aflatoxins, metabolites, etc.} (Groopman *et al.*, 1985; Autrup *et al.*, 1987; Sabbioni *et al.*, 1987). Previous reports suggest that AFB_1 exposure induces macromolecular alterations in human target organs and may be one of the important causative factors for human hepatocarcinogenesis (Groopman *et al.*, 1988; Hsieh *et al.*, 1988; Wogan, 1992).

Epidemiological studies have also demonstrated a strong association between exposure to AFB_1 and human liver cancer incidence in Africa and Southeast Asia where the food contamination of AFB_1 may be higher than other areas in the world (Van Rensburg *et al.*, 1985; Groopman and Kensler, 1987; Wild *et al.*, 1989; Yeh *et al.*, 1989). A more recent prospective study in the People's Republic of China suggested that there was a strong association between hepatitis B surface antigen seropositivity and aflatoxin exposure in liver cancer risk (Ross *et al.*, 1992). It has also been reported that AFB_1 induces the transversion of G to T in codon 249 of the p53 suppressor gene in human hep-

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atocytes, suggesting the role of AFB_1 with liver carcinogenesis (Aguilar *et al.*, 1993, 1994).

In Korea, PLC (primary liver cancer) ranks second only to stomach cancer among all cancers and causes 8,647 deaths (6,652 males, 1,995 females) per year (National Statistical Office, 1991). Over the last 10 years, PLC mortality rates have been increasing in Korea, and as of 1991, the age-adjusted mortality rates are 43.43/100,000 in males and 11.23/100,000 in females. In addition, about 10% of Koreans are hepatitis B virus (HBV) carriers and about 30~50% of liver cancer patients of both sexes are HBV seropositive. These data suggest that the 3.8-fold sex difference in liver cancer mortality may be associated with viral infection as well as other possible risk factors such as AFB_1 and alcohol consumption. Although there may be other virological (e.g. hepatitis C virus) and chemical factors contributing to the induction of human liver cancer in Koreans, it is worthwhile to assess basic AFB_1 exposure levels and to estimate the expected liver cancer incidence attributed to AFB_1 exposure.

Recently, immunoassys have been widely used for the study of environmental levels as well as biomonitoring of human exposure to chemical carcinogens. In this paper we report the development and characterization of a highly sensitive polyclonal antiserum specific to AFB_1. Using this antiserum, aflatoxin levels were quantitated in human urine to estimate AFB_1 exposure and liver cancer risk in Koreans.

Materials and Methods

Materials

AFB_1-bovine serum albumin (BSA), AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFM_2, AFP_1, AFQ_1, AFG_{2a}, Aflatoxicol I, II, and benzo(a)pyrene were purchased from Sigma Chemicals Co. (St. Louis, USA). Fetal calf serum (FCS), diethanolamine, Freund's adjuvant, goat anti-rabbit IgG-alkaline phosphatase, p-nitrophenyl phosphate, and BSA were purchased from Sigma Chemicals Co. (St. Louis, USA). Chloroform, ethyl acetate and hexane were purchased from the Duk San Co. (Seoul, Korea) and N_2 gas was purchased from Dong A Gas Products (Seoul, Korea).

Rabbit immunization schedule

AFB_1-BSA conjugate (3 mg) obtained from Sigma was dissolved in 3 ml of phosphate buffered saline (PBS) and emulsified with an equal volume of complete Freund's adjuvant. The homogenate (100 μl/injection) was injected i.m., i.d., and s.c. into the hindquarters of three New Zealand White rabbits as previously reported (Chu and Ueno, 1977; Poirier, 1981). Four weeks after initial immunization, identical booster injections were given, followed by 2 monthly booster injections of immunogen in incomplete Freund's adjuvant. Each animal received a total of 3 mg AFB_1-BSA conjugate. Sera were obtained from the ear veins at weekly intervals starting 9 weeks after the initial immunization.

ELISA

For the competitive and noncompetitive ELISAs, polystyrene 96 microwell plates (Immulon 2, Dynatech Lab., McLean USA) were coated overnight at 4℃ with 10 ng of AFB_1-BSA in 100 μl of 0.1 M Sodium carbonate buffer (pH 9.6) as described previously (Santella *et al.*, 1988). Plates were washed with PBS-Tween and incubated with 200 μl 1% FCS in PBS containing 0.05 % Tween for 1 h (37℃) to minimize nonspecific binding of protein to the well. Mixtures of antigen in 50 μl PBS and 50 μl diluted antiserum were added to the wells and incubated for 1.5 h at 37℃. After washing the plates with PBS-Tween, 100 μl anti-rabbit IgG-alkaline phosphatase (1:500 dilution) was added and the plates incubated for 1.5 h. Finally, plates were washed with 0.01 M diethanolamine and 100 μl p-nitrophenyl phosphate dissolved in 1 M diethanolamine (pH 8.6) was added. Absorbance at 405 nm was measured with a Titertek Multiscan Microplate Reader (Labsystems, Finland).

Analysis of aflatoxin B_1 contents in human urine

A total of 100 spot urine samples were collected (10 AM, April 14, 1992) from undergraduate students (89 males, 11 females) at the College of Pharmacy, Sung Kyun Kwan University, Republic of Korea. Information on age, sex, body weight, smoking status, and alcohol consumption was collected by questionaire. To choose an appropriate solvent for extraction of aflatoxins from urine, distilled water and rat urine were spiked with known AFB_1 levels (0.00, 0.05, 0.50 ng/ml) and samples (1 ml/sample) were extracted with chloroform, ethyl acetate, or hexane, by vigorous vortexing (3 min) and centrifugation (1,500×g for 10 min). Ethylacetate extraction showed the highest recovery of AFB_1 (85%) as determined by ELISA. Therefore human urine samples were extracted 3 times with ethylacetate and the extracts were evaporated to dryness under N_2 gas. The residues were dissolved in 1 m*l* of PBS for the pH adjustment to 7.0 before ELISA. Samples with less than 20% inhibition in the ELISA were considered nondetectable (ND) and given a half value of the lowest detected level.

Data analysis

Correlations of risk factors (alcohol consumption,

smoking status) with urinary aflatoxins in humans were examined by multivariate linear regression analysis and correlation coefficient. The Mann-Whitney Rank Sum Test was also performed to analyze sex differences.

Results

Development of polyclonal antisera against AFB_1

New Zealand White rabbits were immunized with commercially available AFB_1-BSA and three antisera (R101 R102, R103) were obtained. When tested by noncompetitive and competitive ELISA, antisera R101 showed the highest sensitivity for AFB_1 at an optimal dilution of $1:10^7$. The sensitivity and specificity of antiserum R101 was determined by competitive ELISAs and indicated the highest reactivity with AFB_1 (50% inhibition at 170 fmol) and aflatoxicol II (50% inhibition at 112 fmol) (Fig. 1). R101 also reacted with other aflatoxins such as AFB_2, AFG_1, AFG_2 (50% inhibitions; at 1,010 fmol, 1,190 fmol, 865 fmol) and aflatoxin metabolites (AFM_1, AFM_2, AFP_1, and AFQ_1: 50% inhibitions at 3,810 fmol, 3,490 fmol, 11,470 fmol, 426 fmol). However, it did not cross-react with $AFG_{2\alpha}$ or benzo(a)pyrene at the highest concentrations tested (Table 1).

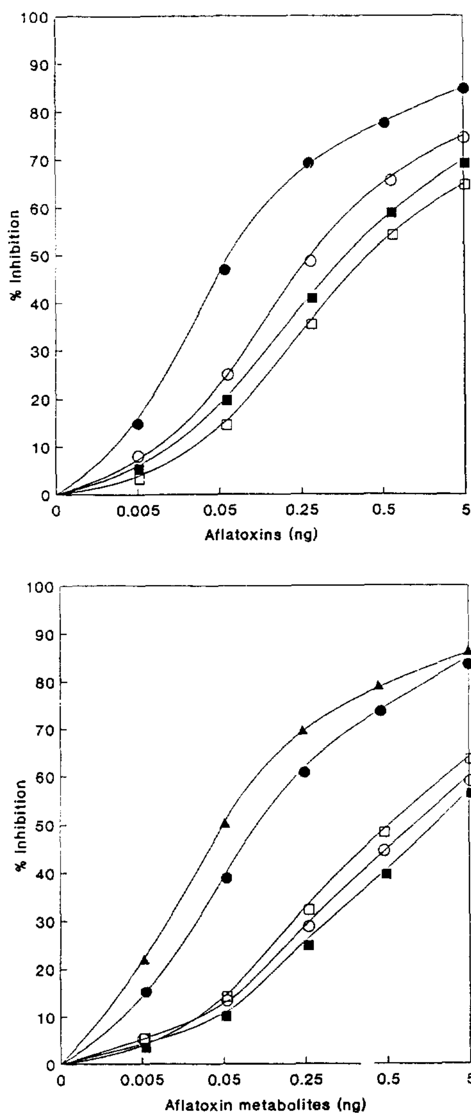

Fig. 1. Competitive inhibition of polyclonal antiserum R101 binding to aflatoxin B_1-BSA. (A): The competitors were aflatoxins; aflatoxin B_1 (●), aflatoxin B_2 (■), aflatoxin G_1 (□), aflatoxin G_2 (○). (B): The competitors were aflatoxin metabolites; aflatoxin Q_1 (●), aflatoxin M_1 (○), aflatoxin M_2 (□), aflatoxin P_1 (■), aflatoxicol II (▲). The antiserum was diluted $1: 10^7$.

Human monitoring of AFB_1 in urine

Urinary levels of aflatoxin metabolites were quantitated by ELISA using polyclonal antiserum R101. Ninety three percent of the samples showed detectable antigenicity in a range of 1.4~200.6 ng AFB_1 equivalents/kg /day. Aflatoxin levels were 5-fold (18.11± 33.01 ng/kg /day) higher in male than in female students (3.82± 2.65 ng/kg/day) (Table 2). Assuming that urinary AFB_1 excretion is 7.6% of total intake (Groopman *et al.*, 1992), expected daily AFB_1 intake was 240.20± 438. 67 ng/kg/day in males and 50.35± 29.88 ng/kg/day in females. Alcohol consumption, smoking status, body weight, and age were not statistically associated with AFB_1 excretion.

Table 1. Competitive inhibition of polyclonal antiserum R101 binding to aflatoxins and metabolites

Antigen[a]	fmol causing 50% inhibition[b]
AFB_1	170± 26[c]
AFB_2	1010± 342
AFG_1	1180± 276
AFG_2	865± 192
AFM_1	3810± 122
AFP_1	11470± 801
AFQ_1	426± 80
AFM_2	3480± 1685
AFG_2	$>1.7\times10^6$
Aflatoxicol II	112± 54
Benzo(a)pyrene(B(a)P)	$>1\times10$
3-OH-B(a)P	$>1\times10^8$
B(a)P-7,8,9,10-tetrahydrotetraol	$>1\times10^8$
7,12-dimethylbenz(a)anthracene-3,4-dihydrodiol	$>1\times10^8$
Chrysene-1,2-dihydrodiol-3,4-epoxide	$>1\times10^8$

[a] The abbreviations used are: AFB_1, aflatoxin B1; AFB_2, aflatoxin B_2, AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2; AFM_1, aflatoxin M_1; AFP_1, aflatoxin P_1; AFQ_1, aflatoxin Q_1; AFM_2, aflatoxin M_2; AFG_{2a}, aflatoxin G_{2a}.

[b] Antiserum was diluted $1:10^7$ and 50 ng of aflatoxin B_1-BSA was coated on the plate for ELISA.

[c] Values are means± SD of three experiments.

Table 2. Daily aflatoxin B_1 exposure and calculated liver cancer incidence in Koreans

	Urinary AFB_1 equivalents (ng/ml)[a]	Urinary AFB_1 equivalents (ng/kg/day)[b]	AFB_1 intake (ng/kg/day)[c]	Calculated[d] incidence
Male (N=88)	0.86±1.39	18.11±33.01[e]	240.20±438.67	41.56
Female (N=11)	0.16±0.09	3.82± 2.65	50.35± 29.88	26.84
Total (N=100)	0.78±1.39	17.80±40.09	219.07±420.08	40.68

[a]Data are expressed as equivalents of AFB_1 quantitated by ELISA in ml urine. AFB_1 equivalents are used because antisera R101 majorly reacts with AFB_1, but the data may be possibly affected by its cross reactivity with aflatoxicol II.
[b]Daily AFB_1 excretion in urine was estimated assuming that a total volume of urinary excretion was 1,200 ml/day/person (body weight. kg).
[c]Daily AFB_1 intake was estimated from urinary AFB_1 assuming that 7.6% of AFB_1 intake was excreted in urines.
[d]Liver cancer incidence per 100,000 was calculated by the application of AFB_1 intake levels to the linear regression model of $Y=21.67X-10.04$ with a correlation coefficient of 0.99 ($p<0.001$) obtained from epidemiological data (Zhu *et al.*, 1987: Sabbioni *et al.*, 1987; Groopman *et al.*, 1992).
[e]Statistically different from female by Mann-Whitney Rank Sum Test ($p<0.001$).

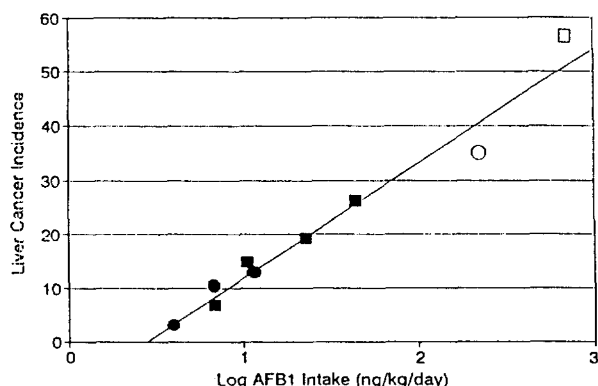

Fig. 2. A linear regression model of $Y=21.67X-10.04$ developed from epidemiological data of Kenya (●), Swaziland (■), Mozambique (○), and China (□). The correlation coefficient was 0.99 ($p<0.001$) {Y represents liver cancer incidence per 100,000 and X for AFB_1 intake (ng/kg/day)} (Sabbioni *et al.*, 1987; Zhu *et al.*, 1987; Groopman *et al.*, 1992).

Estimation of liver cancer incidence in Koreans exposed to AFB_1

Liver cancer incidence was estimated in Koreans exposed to AFB_1. Here, we assumed that AFB_1 might be associated with liver cancer incidence in Koreans even if the hepatitis virus (B,C) and other chemical carcinogens were involved. To estimate liver cancer incidence, we collected epidemiological data of liver cancer incidence and AFB_1 intake levels previously reported (Sabbioni *et al.*, 1987; Zhu *et al.*, 1987; Groopman *et al.*, 1988, 1992). Using epidemiological data from Kenya, Swaziland, Mozambique, and China, a linear regression equation of $Y=21.67X-10.04$, where Y is liver cancer incidence per 100,000 and X is Log AFB_1 intake (ng/kg/day), was obtained with a correlation coefficient of 0.99 (Fig. 2). When the human monitoring data (240.20 ng/kg/day in males and 50.35 ng /kg/day in females) was applied to the equation, attributable liver cancer incidences due to AFB_1 exposure were 41.56/100,000 in males and 26.84/100,000 in females.

Discussion

Research on molecular epidemiology has played an important role in the area of cancer research (Calleman *et al.*, 1978; Perera *et al.*, 1982). This area has greatly progressed by the introduction of sophisticated techniques such as ^{32}P-postlabelling methods, ELISA, Synchronous fluorescence technique, etc. (Reddy and Randerath, 1986; Santella *et al.*, 1988; Weston *et al.*, 1989). Using these techniques, many carcinogen-DNA or -protein adducts have been quantitated in target tissues and body fluids of cancer patients as well as industrial workers (Perera *et al.*, 1982; Harris *et al.*, 1985; Hsieh *et al.*, 1988; Perera *et al.*, 1988; Wild *et al.*, 1990; Lee *et al.*, 1991). Human body fluids (urine, blood) are readily available and thus measurements of carcinogens and metabolites in body fluids can be more appropriate for monitoring and risk assessment.

For these reasons, in this study a highly sensitive polyclonal antiserum R101 was developed and used for human monitoring of AFB_1 in urine. The intake levels of AFB_1 equivalents (240.20 ng/kg/day in males, 50.35 ng/kg/day in females) were estimated for risk assessment of AFB_1 exposure assuming that 7.6% of ingested AFB_1 was excreted in urine. These levels are close to a daily intake of 222 ng in Mozambique but lower than those of 691.4 ng AFB_1 in China (Groopman and Kensler, 1987; Groopman *et al.*, 1992). For the precise estimation of AFB_1, it is apparent that antibody-crossreactivities and interindividual differences in AFB_1 excretion rates are factors to be considered, but they are difficult to assess comprehensively. In addition, the 7.6% of ingested AFB_1 might vary depending on intraspecies and interspecies.

Risk assessment models for liver cancer were previously evaluated from experimental data and a virtual safety dose was estimated (Carlborg, 1979). Also, the annual incidence of liver cancer in USA was predicted from extrapolation models (Dichter, 1984). In this paper, we immunologically estimated exposure levels of AFB_1 in small groups of humans, formulated a liver cancer incidence model due to AFB_1 exposure based on epidemiological data, and calculated liver cancer incidences in Koreans from an incidence model.

For the risk assessment, it is possible that primary etiological agents for human liver cancer may vary from country to country and thus direct application of AFB_1 intake levels to the linear regression model prepared in this report could be highly biased. In a given situation, however, calculated liver cancer incidence (41.56/100,000) is very reasonable when compared with liver cancer mortality rates in Korea (43.43/100,000). Although the risk may be overestimated, these data at least indicate that Koreans are exposed to high levels of AFB_1 which may be implicated as a possible risk factor for hepatocarcinogenesis in Korea. This preliminary report only provides limited evidence of AFB_1 exposure and requires further research.

In the future, further investigation of other risk factors such as HBV, HCV, heavy metals, and other hepatocarcinogens should be comprehensively carried out in liver cancer patients for the etiology of human hepatocarcinogenesis and correct risk assessment.

Acknowledgement

The authors are grateful to Dr. Regina M. Santella, Columbia University for critical reading of the manuscript and excellent advice.

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