Aflatoxin Exposure Measured by Urinary Excretion of Aflatoxin B₁-Guanine Adduct and Hepatitis B Virus Infection in Areas with Different Liver Cancer Incidence in Kenya

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ABSTRACT

Two major etiological agents, hepatitis B virus and aflatoxin B₁, are considered to be involved in the induction of liver cancer in Africa. In order to elucidate any synergistic effect of these two agents we conducted a study in various parts of Kenya with different liver cancer incidence in order to establish the rate of exposure to aflatoxin and the prevalence of hepatitis infections. Of all tested individuals 12.6% were positive for aflatoxin exposure as indicated by the urinary excretion of aflatoxin B₁-guanine. Assuming no annual and seasonal variation, a regional variation in the exposure was observed. The highest rate of aflatoxin exposure was found in the Western Highlands and Central Province. The incidence of hepatitis infection nationwide as measured by the presence of the surface antigen was 10.6%, but a wide regional variation was observed. A multiplicative and additive regression analysis to investigate if hepatitis and aflatoxin exposure had a synergistic effect in the induction of liver cancer was negative. However, a moderate degree of correlation between the exposure to aflatoxin and liver cancer was observed when the study was limited to certain ethnic groups. The study gives additional support to the hypothesis that aflatoxin is a human liver carcinogen.

INTRODUCTION

PHC² is one of the most common cancers in the world. The highest rate has been reported in Mozambique, an area with high AFB contamination of the stable diet and where HBV infections are endemic.

Presently, it is assumed that HBV is the single most important factor, as most of the liver cancer patients show a positive serology for HBV antigens (1, 2). Additional support has come from the observation, that integrated HBV DNA has been found in cellular DNA extracted from liver tissues of patients with PHC (3, 4). However, a recent study in South Africa suggests that HBV alone cannot account for the higher incidence of liver cancer observed in the rural population (5). In Guangxi region in China the mortality rate of PHC in HBsAg positive individuals was higher when the individual lived in villages with high AFB exposure (6).

AFB is a very potent liver carcinogen in experimental animals, including nonhuman primates (7, 8), and a positive correlation between the dietary intake of AFB and the incidence of liver cancer in humans has been established (9). AFB has also been shown to be present in human liver tumor tissue by both spectrophotometric and chromatographic methods (10, 11). Human exposure to AFB has been determined by excretion of AFB and its metabolites in the urine (12-14), and unmetabolized AFB has been detected in blood samples (15). AFB-Gual has been detected in urine samples collected in Murang's district, Kenya. The presence of AFB-Gual in urine is an indication that consumed AFB is metabolized to its genotoxic metabolite that subsequently reacts with cellular nucleic acids (16).

In order to study the possible interaction of HBV and AFB in the induction of human liver cancer, a cross-sectional study in Kenya has been conducted. The incidence of PHC was determined from the medical records of patients attending the liver clinic at Kenyatta National Hospital. The prevalence of HBV infection was measured by serum HBsAg and the rate of individuals exposed to AFB by measuring the urinary excretion of AFB-Gual. The study was conducted in areas with different liver cancer incidence.

MATERIALS AND METHODS

Collection of Biological Samples. Morning urine samples (minimum volume, 25 ml) were collected at the outpatient clinic at selected district hospitals using sterile, disposable containers. The patients were attending the clinics for malaise. Patients with stomach and intestinal complaints were excluded from the study, as it could be expected that they had changed their regular eating habits. The donors represented the normal sex and age distribution of the Kenyan population. The samples were immediately put on wet ice and transferred to the Nairobi laboratory for initial processing. Samples were adjusted to pH 5 and to 7% methanol, and centrifuged at 4°C (1500 x g for 10 min). The supernatant was submitted to an initial clean up on a CIS Sep-Pak cartridge (Waters Associates, Waltham, MA) using a Sep-Pak cartridge, and a flow rate of maximally 2 ml/min. After washing with 5 ml 10% methanol and 5 ml 7% acetonitrile the samples were shipped out of Kenya for further analysis. The cartridges were kept at −70°C until analysis. The urine samples were kept at −70°C until analysis by HPLC. The recovery of AFB-Gual by this procedure was approximately 80%. Blood samples (maximum, 7 ml) were collected from the urine donors in Vacutainers blood collection tubes containing EDTA, and the plasma was prepared by centrifugation. The urine samples were collected during a 4-year period (January 1981–June 1984), while blood collection started in April 1983. No individual was sampled more than once. However, only people over the age of 10 years were included in the study.

Analysis of Urine. The urine samples were analyzed as previously described (17, 18). The C₁₈ Sep-Pak cartridges were eluted with 80% methanol (10 ml), and the eluate was concentrated to 0.5 ml using a Speedivac Evaporator. Further cleanup was performed by HPLC using a C₁₈-Bondapak column and 18% ethanol-10 mM ammonium formate (pH 5.1) as eluent with a flow rate of 1 ml/min. AFB-Gual eluted at 23 min, and fractions from 22–24 min were collected. Several urinary compounds absorbing at 365 nm comigrated with AFB-Gual on the C₁₈-Bondapak column. This peak was resolved into several components on an Ultrasil-Si column using 4.5% acetonitrile (flow rate, 1 ml/min). The eluate was monitored at 365 nm. AFB-Gual eluted at 5.5 min on this column.

To verify that the chemical identity of the product had the same retention time as AFB-Gual in the two different HPLC systems the eluate at 5.5 min in the second HPLC system was scanned by fluorescence spectrophotometry using synchronous luminescence and photon counting (19). The spectra were obtained by using a fluorescence spectrophotometer.
spectrophotometer (Perkin-Elmer MPF 44B) and a photon counter (EG and G Ortec). The fluorescence was measured in a quartz cell (10 mm light path). Both the excitation and emission monochromators were set at 5 nm band width. The wavelength difference was 34 nm with the emission wavelength being 34 nm higher than that of excitation. The emission and excitation were scanned simultaneously (synchronous scanning). Under these conditions, both AFB-Gual isolated by HPLC from chemically modified DNA and the positive urine samples gave a characteristic fluorescence with a peak emission of 415 nm when excited at 381 nm.

Determination of HBV Serological Markers. Ausria II-125 and Corab kits (Abbott Laboratories, North Chicago, IL) were used for the assays of HBsAg and anti-HBc, respectively. The general procedure described by the manufacturer was followed. The samples were counted on an Aloka Auto Well Gamma System ARG-500.

Liver Cancer Incidence. Patient records from more than 2000 patients attending the liver clinic at Kenyatta National Hospital in Nairobi during the period 1978–1982 were analyzed. The diagnosis of primary hepatocellular carcinoma was confirmed by two criteria: (a) presence of α-fetoprotein and (b) histological examination of needle or surgical biopsies. The 1981 census (Kenyan Government report) was used for the calculation of the incidence rates of PHC in the various districts.

Statistical Evaluation. The χ² test was used to compare serological markers for HBV in AFB-Gual positive and negative individuals. Multiplicative and additive regression analyses were conducted in order to study if HBV and AFB had a synergetic effect in the induction of PHC. The individual role of AFB and HBV in the Bantu people was analyzed by Spearman's nonparametric rank correlation analysis because the distribution of the observations are unknown.

RESULTS

Urine samples were collected in different districts in Kenya during a 4-year period. A total of 983 samples have been analyzed for the presence of AFB-Gual. A sample was considered positive for AFB-Gual if it cochromatographed with authentic AFB-Gual in two different HPLC systems and had the characteristic synchronous fluorescent spectrum (16). About 85% of the samples positive in both HPLC systems were also positive by the third criterion giving 15% false positive if the chemical verification was excluded. The lower level of detectability for AFB-Gual by this assay is 0.3 pmol/25 ml urine. A total of 122 samples (12.4%) were positive for AFB-Gual by these criteria, with the highest prevalence of exposure in Kitale, Kericho, and South Nyanza districts (Table 1). Males had a significantly higher rate of exposure to AFB than females. The male:female ratio ranged from 0.9–4.5, with only the Kitale region below 1, and Rerichio at 4.5. The seasonal variation in the prevalence of AFB exposure is shown in Table 2.

The seroepidemiology of HBV in the same regions is shown in Table 3. Males in general had a higher rate of HBV infection than women. The distribution of the serological markers for HBV infection was the same in both AFB-Gual positive and negative cases (Table 4). Of the tested individuals 8.2% were positive for both AFB exposure and one of the HBV serological markers.

The information on liver cancer incidence was established on the basis of information from the medical records of patients attending the liver clinic at Kenyatta National Hospital over a 5-year period (1978–1982). This hospital serves as a general hospital for the central part of Kenya, but patients with special diseases (such as liver disorders) are referred from district and provincial hospitals from all over Kenya. However, the incidence of PHC in Busia and South Nyanza may be underestimated due to the long distance to Nairobi and good major hospitals in the areas. Patients with liver diseases may prefer to be treated locally, and their medical record will not enter the data at Kenyatta Hospital. These areas have been excluded from the correlation analysis.

In 399 patients of the 2000 attending the liver clinic at Kenyatta Hospital, the diagnosis of PHC was confirmed. PHC was predominant among males (299 cases) with a male:female ratio of 3:1. A high male:female ratio has also been shown on a distinct basis. The PHC incidence in different districts and the sex ratios are shown in Table 5.

Taking female and male data as separate entries, a Spearman rank correlation analysis of the rate of AFB exposure and PHC incidence in Bantu peoples living in different districts gave a
moderate association ($r = 0.75$). The same type of analysis did not indicate any association between HBV and PHC ($r = 0.190$).

A multiplicative and additive regression analysis of the incidence of PHC with the prevalence of HBV infection and rate of AFB exposure in different districts for both sexes did not indicate that combined exposure to AFB and HBV increased the risk for developing PHC.

**DISCUSSION**

Epidemiological data support a multifactorial origin of liver cancer (20), but the existing data on the role of HBV and AFB are only suggestive and need further substantiation.

AFB has been classified as a potential human carcinogen because a close association between the intake of AFB and the incidence of PHC has been observed in several parts of the world, including Kenya (21, 22). AFB does require metabolic activation to become oncogenic, and the suspected mutagenic action of AFB in rats (24). An identical adduct profile has been observed when cultured human tissues including fetal liver from rats treated with AFB and from humans living in an area unstable and AFB-Gual has been detected in the urine collected on May 29, 2017. © 1987 American Association for Cancer Research. cancerres.aacrjournals.org Downloaded from

The present study in Kenya includes districts in Central, Eastern, and Western provinces. The areas have different incidences of PHC and represent different ethnic, cultural, and social groups. The highest rate of AFB exposure was observed in the Western and Central parts of Kenya. The stable diet is assumed to be important for induction of PHC. However, regression analysis of the present data did not reveal any synergistic or additive action of HBV and AFB in the induction of PHC.

The other etiological agent in the induction of PHC is proposed to be HBV. A strong seroepidemiological association between HBV and PHC exists (30), and it is estimated that persistent HBV infection can account for 80% of all PHC in another high risk area (28). However, Bagshawe et al. (31) did not find any association between PHC and HBV infection in Kenya using HBsAg as serological marker. These and other observations suggest that HBV alone could not be responsible for induction of liver cancer. The incidence of HBV infection in this study was determined by the serology of HBsAg and anti-HBc carriers does exist in the experimental group. Sixty-six percent of the HBsAg positive patients were also positive for anti-HBc. Our carrier rate for HBsAg positive cases (9%) is slightly lower than the carrier rate reported by Zumla and Voller (32) in noncancerous groups in neighboring Zambia and lower than in a non-African high risk area (33). HBV was responsible for 70% of all cases of acute hepatitis in Kenya (34). Forty-six percent of the patients excreting AFB-Gual had a positive serology for HBV. The rate of AFB exposure was identical in the HBV positive and negative individuals. A prospective study following these individuals would have been of great interest in elucidating the interplay between HBV and AFB in the induction of PHC.

The results indicate that people living in high risk areas for PHC are exposed to both major etiological agents considered to be important for induction of PHC. However, regression analysis of the present data did not reveal any synergistic or additive action of HBV and AFB in the induction of PHC in Kenya, but rank correlation indicates that exposure to AFB may play an important role. The validity of this test can be questioned as we compare biological data collected in 1981–1984 with incidence data from 1978–1982. However, AFB
contamination of the stable diet has not changed during the
last generation. No information on the prevalence of HBV
infection 10–20 years ago is available. A case-control study or
a prospective study measuring AFB exposure over several sea-
sons and years may be required to establish the role of these
two agents in the induction of PHC in humans. However, on
the basis of this study, it is apparent that improvement in food
hygiene may be just as important as vaccination against HBV
in preventing liver cancer in Third World countries.

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