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Aflatoxin and Indian Childhood Cirrhosis[1]

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AFLATOXIN–CONTAMINATED groundnut meal is known to induce marked hepatic damage in various species of animals (1–3). The subacute toxic effects of these fungal metabolites in primates have been reported from these Laboratories (4). To date there is no direct evidence to suggest that man has suffered ill effects through consumption of aflatoxic groundnuts or groundnut products.

The high prevalence of hepatic cirrhosis in certain parts of India where groundnut is grown and consumed has been suggested as being indicative of the effects of aflatoxin on humans. There have also been suggestions that aflatoxin may perhaps be implicated in the development of primary carcinoma in man (5). Recently, the problem was investigated by Robinson (6). In view of the apparent similarity in the fluorescent property of aflatoxin B_1 and a compound appearing in the urine of cirrhotic children, it has been suggested that aflatoxin may be causally related to the liver condition. Some workers have shown that aflatoxin B_1 administered orally to rats (7), sheep (8), and cows (9) appears in urine, milk, and the liver in the form of a metabolite having fluorescent property and biological activity similar to the parent toxin. In the present investigation, the urine and liver of patients suffering from Indian childhood cirrhosis and the milk samples collected from their mothers have been examined for the presence of aflatoxin.

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MATERIALS AND METHODS

Subjects. Sixteen patients whose ages ranged from 3 months to 2.5 years with histologically proved diagnosis of Indian childhood cirrhosis were investigated. They were admitted to the hospital at various stages of severity of the disease.

All the children had been treated prior to admission with various drugs including broad-spectrum antibiotics, corticosteroids, and diuretics. Many of them had also received various types of indigenous drugs. They continued to receive the steroids and antibiotics during their stay in the hospital.

Diet history. Three infants under 1 year were entirely breast fed, whereas the others received a mixed diet. Mothers were specifically questioned regarding the consumption of groundnut by the children. Except in one case, there was no history of groundnut intake. As there is a possibility of an infant receiving the toxin through the mother's milk, breast milk samples were also collected from some of the mothers for analysis.

Clinical features. The patients could be graded into three categories depending on the clinical manifestations.

Grade I. The symptoms were vague and the children were brought to the hospital for some gastrointestinal disturbances or low grade fever. The only positive finding on examination was an enlarged, firm liver.

Grade II. The clinical findings were more definite. The child failed to thrive, had pitting edema of the lower extremities, and was undernourished. The abdomen was prominent with obviously enlarged veins; the liver was firm, markedly enlarged, and had the characteristic "leafy edge"; and the spleen was palpable.

Grade III. In this advanced stage, the abdomen was grossly protuberant because of ascites. The spleen was large and firm and the liver when

palpable was firm with a sharp margin. Persistent fever with jaundice and generalized anasarca with or without hemorrhagic phenomena were present.

Histopathological findings. A liver biopsy specimen showed varying degrees of cell destruction, fibrosis, inflammatory cell infiltration, and regenerating nodules. The cells were swollen and there was a clumping of cytoplasmic contents; the nuclei were pyknotic in some and absent in others. Mallory bodies and bird's eye nuclei were present in some sections. The liver tissue was seen to be divided into multiple small round or oval pseudolobules by fibrous tissue septa of variable thickness. In some cases, there was complete loss of architectural pattern with progressive cell destruction, collapse of reticular framework, and extensive fibrosis. Portal tracts exhibited varying degrees of cell infiltration.

Twenty-four-hour urinary collections were made in all subjects. Two patients with *grade III* manifestations died of hepatic coma within a week after admission. In one of them necropsy was done and a sample of the liver examined for presence of aflatoxin.

Eleven patients with other liver disorders and six normal patients were also studied.

Extraction and Estimation Procedures

The 24-hr urine sample collected over 5 ml HCl was concentrated to 50 ml in a porcelain dish on a hot water bath. An equal volume of methanol was added and any precipitate formed was separated by filtration. The filtrate was extracted thrice, each time with 25 ml chloroform. The chloroform extract was then concentrated to 5 ml and applied on a silica column. It was eluted first with ethyl ether to remove coloring matter and fluorescent impurities and then with chloroform containing 2% methanol to elute aflatoxin and its metabolites, if present. The extracts were concentrated and aliquot portions spotted on chromatoplates for examination of aflatoxin by the method of de Iongh et al. (10) using 5% methanol in chloroform as the developing solvent. A similar procedure was adopted for the extracts of liver specimens. Solution of pure aflatoxin B_1 was used as a reference standard, which was spotted on the same plate along with the urine and liver extracts for comparison.

The dry, developed chromatoplate was examined under long-wave ultraviolet light for the presence of blue-violet fluorescent spot of R_F 0.56, corresponding to aflatoxin B_1. For further physicochemical examination, these spots were individually eluted with chloroform and subjected to ultraviolet, infrared, and fluorescent spectroscopy.

The ultraviolet absorption spectrum was determined on a Beckman DB recording spectrophotometer from 250 to 450 mμ. Infrared spectra were obtained on a Perkin-Elmer infrared spectrophotometer, model 221. Fluorescence characteristics of the urinary spot were studied by measuring the emission and absorption spectra of the solution in chloroform in the ultraviolet region using a spectrofluorometer (Farrand Optical Co., Inc., N.Y.) with an attached graphic recorder, and they were compared with the fluorescent properties of aflatoxin B_1.

Biological Testing

Day-old ducklings were used for testing the toxicity of the fluorescent compounds appearing in the urine of cirrhotic patients. The experimental birds were housed in electric brooders and fed a laboratory duckling mash. Control birds as well as birds given 10 μg aflatoxin B_1/day were examined with each batch of experimental ducklings that received orally the fluorescent material extracted from the chromatoplate. Propylene glycol served as the medium in which the compound was dissolved for administration. The control birds received an equal amount of propylene glycol alone. Feeding of the extracts was continued for 5 consecutive days after which the experimental and control birds were killed and their livers processed for histopathological examination.

RESULTS AND DISCUSSION

Presence of a compound that gave the spot. Ten of sixteen children with Indian childhood cirrhosis had a compound in their urine that gave a fluorescent spot. Of the six that did not have this spot, four children had *grade I* cirrhosis and two had *grade II* cirrhosis. Most of the *grade II* and all *grade III* cirrhosis had the spot (Table I). One out of two having posthepatitic cirrhosis in childhood and both adult hepatic cirrhosis patients had the spot. Of seven children with nonspecific hepatomegaly, three urines were positive, whereas none of the six normal children showed the spot (Table II). All four samples of milk examined were negative.

TABLE I

Data on children with Indian childhood cirrhosis

Name	Age, years	Duration of illness, months	Severity grade	Jaundice	Liver	Spleen	Ascites	Fluorescent spot in urine
A	3/12	1	I	±	++	±	−	−
PK	1	4	I	−	++	±	−	−
S	9/12	1	I	−	++	−	−	−
G	1 6/12	3	I	±	++	−	−	−
J	1 4/12	4	II	−	++++	+	−	−
CS	2	3	II	±	+++	+	−	−
M	2 6/12	2	II	−	++++	++	−	+
M	2	4	II	−	+++	+	−	+
Tn	2	1	II	±	+++	−	−	+
P	1 6/12	3	II	−	+++	+	−	+
N	1 6/12	4	II	−	+++	+	−	+
T	1	2	III	+	++++	+	+	+
M	2	6	III	+	+++	+	+	+
Sr	1 6/12	6	III	+	+++	++	+	+
Sh	1 6/12	8	III	+	++++	++	+	+
BS	1 9/12	4	III	+	++++	++	+	+

TABLE II

Types of subjects studied for the presence of aflatoxin in urine

Disease	Number examined	Number showing a fluorescent spot
Indian childhood cirrhosis	16	10
Posthepatitic cirrhosis	2	1
Adult cirrhosis	2	2
Other hepatomegalies	7	3
Normals	6	0

Ultraviolet absorption spectrum. The ultraviolet spectra of the substance in the urine exhibiting violet-blue fluorescence and of aflatoxin B_1 are given in Fig. 1. Aflatoxin B_1 showed the peaks of absorption at 223, 265, and 363 mμ, the latter being most prominent. The urinary compound showed only one peak at 270 mμ, which is quite different from that of aflatoxin B_1.

Fluorescence spectra. The pattern of fluorescence spectra of the urinary compound and aflatoxin B_1 were measured with a view to finding out the maximum fluorescence intensities of the two compounds in chloroform solution. The measurements were made in arbitrary units.

Freshly prepared solutions of the recrystallized material were used and the concentrations adjusted in the range where a linear relationship existed between concentration and fluorescence intensity. Fluorescence spectrum and the emission spectrum of pure aflatoxin and the urinary compound were obtained using the excitation wave length between 210 and 425 mμ.

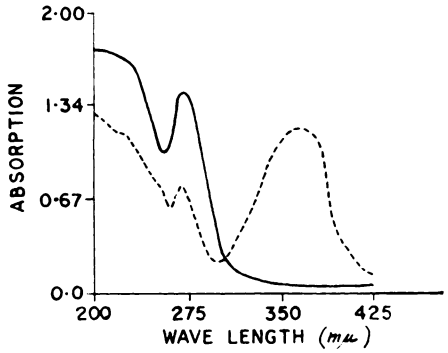

FIG. 1. Ultraviolet spectra of aflatoxin B_1 (– – – –) and the compound present in urine and in liver of cirrhotic children (——).

It can be seen from Fig. 2 that both aflatoxin B_1 and the urinary compound showed the emission maxima at 425 mμ, whereas the excitation maxima were 365 mμ for aflatoxin B_1 and 310 mμ for the urinary compound. Identical values for emission maxima for the two compounds confirmed the similarity in their chromatic property of fluorescence. On the other hand, the difference in the excitation maxima suggested differences in their chemical nature.

Infrared absorption spectra. The infrared absorption spectrum of aflatoxin B_1 in chloroform has been studied by Hartley et al. (11). The characteristic spectrum indicated the absence of OH group unless chelated and showed two peaks in the carbonyl region at 1,755 cm^{-1} and 1,688 cm^{-1} $(\alpha)^{25°}D = -562 \pm 15$ (C, 0.115 in chloroform). It also showed characteristic peaks for difurano ring and for the coumarin structure.

The patterns of infrared absorption spectra of the urinary and liver compounds (Fig. 3) are strikingly similar, but they do not indicate any similarity to the organic moieties present in aflatoxin B_1. The infrared absorptions ($CHCl_3$) at 905, 1,125, 1,360, 1,608, 1,767, and 3,578 cm^{-1} indicate the presence of alkane group, ether linkage, CH_3 bending, C=C and C=O stretching and intramolecular hydroxy bending.

Biological testing. All the day-old ducklings receiving 10 μg aflatoxin B_1/day died on the 4th and 5th day and had developed typical biliary fibrosis and moderate fatty liver as described earlier (12). The control birds as well as the birds fed the urinary compound were apparently healthy on the 5th day, and their livers showed no abnormality.

It may be significant in this context that in all the four children with the milder stages of the disease, the compound was absent in the urine, whereas in those who were in the advanced stages, the compound was invariably present. It may also be significant that the compound was present in subjects suffering from other types of liver disorders. Whether

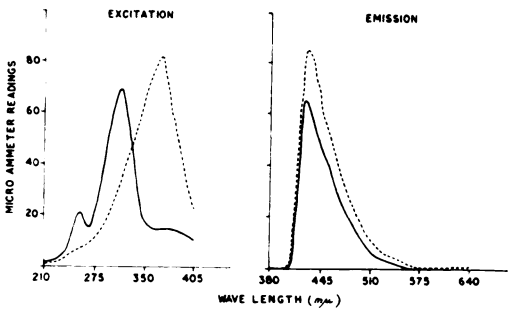

FIG. 2. Excitation and emission spectra of aflatoxin B_1 (- - - -) and the compound appearing in the urine of cirrhotic children (——).

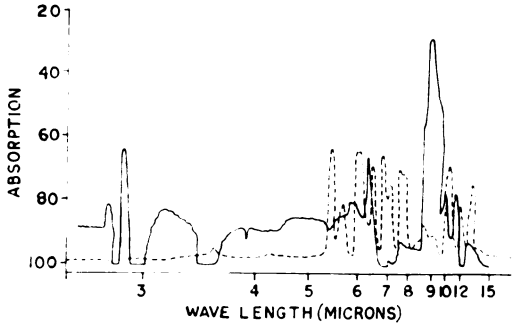

FIG. 3. Infrared spectra of aflatoxin B_1 (- - - -) and the compound present in urine of cirrhotic children (——).

the compound is an abnormal metabolite or a normal metabolite that is excreted in large amounts because of altered liver function has to be investigated. Since most subjects had had, or were receiving, steroids, the possibility of the fluorescent compound being a steroid or its breakdown product was considered. The infrared and ultraviolet spectra did not suggest that this compound was of steroid origin. More direct evidence was also obtained by giving steroids (prednisolone) to both normal children as well as to some children with *grade I* cirrhosis who had no spot in the urine. After 3 days of steroid therapy, no fluorescent spot was detected.

All these data on the physical and biological properties strongly suggest that the compound appearing in the urine, and also seen in the liver of cirrhotic patients, is chemically quite

different from aflatoxin B_1 and its metabolites. It is obviously not a steroid. Further work is now in progress to identify the compound and assess the significance of its presence in subjects with liver disease.

SUMMARY

The possible etiological role of aflatoxin in Indian childhood cirrhosis was investigated. The chloroform extracts of urine and liver of children with Indian childhood cirrhosis showed a blue-violet fluorescent spot on thin-layer chromatograms on silica gel having an R_F almost identical to that of aflatoxin B_1. The ultraviolet and infrared spectra of this compound were different from aflatoxin B_1. It was also distinguishable from aflatoxin B_1 in the fluorescent properties as evidenced by absorption spectra. Unlike aflatoxin B_1, the compound was highly soluble in ethyl ether. The biological test using day-old ducklings further confirmed the nonidentity of the fluorescent component appearing in the urine of cirrhotic patients with aflatoxin B_1.

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