

Determination of exposure to aflatoxins among Danish workers in animal-feed production through the analysis of aflatoxin B₁ adducts to serum albumin

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AUTRUP JL, SCHMIDT J, SEREMET T, AUTRUP H. Determination of exposure to aflatoxins among Danish workers in animal-feed production through the analysis of aflatoxin B₁ adducts to serum albumin. *Scand J Work Environ Health* 1992;17:436-40. Aflatoxin B₁ is suspected as an etiologic factor in the increased risk for primary liver cancer among workers in animal-feed processing plants in Denmark. Aflatoxin bound to serum albumin was therefore measured for feed-processing workers. Blood samples were collected immediately after vacation and after four weeks of work, and aflatoxin was quantified by competitive enzyme-linked immunosorbent assay. Seven of 45 individuals with an estimated exposure of 64 ng aflatoxin B₁ · d⁻¹ · kg⁻¹ body weight were positive. Three positive workers had been unloading a cargo with an aflatoxin B₁ level of 26 µg · kg⁻¹ raw material. The exposure level correlated well with the job titles. Dust samples collected at different sites showed considerable variation in the amount of aflatoxin B₁ (nondetectable to 8 µg · kg⁻¹ dust). The exposure to aflatoxin B₁ may only partially explain the increased risk of liver cancer.

Key terms: affinity chromatography, aflatoxin-albumin, biomonitoring, enzyme-linked immunosorbent assay, liver cancer.

Aflatoxin B₁, a potent liver carcinogen in experimental animals, is produced by *Aspergillus flavus* and *paraciticus* under warm and humid conditions. Aflatoxin B₁ has been classified as a group 1 carcinogen by the International Agency for Research on Cancer. A positive correlation between the dietary intake of aflatoxin B₁ and the incidence of liver cancer has been observed in African and Asian countries (1, 2). A causative association between exposure to aflatoxins and human cancer has also been established for Dutch workers in an oil mill processing peanuts and linseed; especially the incidence of lung cancer was higher than expected (3). Circumstantial evidence has been reported for an association between the inhalation of aflatoxin-contaminated grain dust and lung cancer (4). In a register-based analysis of occupational risk for primary liver cancer in Sweden, a significant excess of cases was observed for workers in grain mills, and it was postulated that potential exposure to aflatoxins may play a role (5). An increased incidence of liver cancer has been reported among Danish male workers in companies processing livestock feed. The exposure of these workers to aflatoxins through the respiratory route was estimated to be 170 µg · d⁻¹ according to conservative

estimates (6). Attempts to detect aflatoxin B₁ or its metabolites in the urine of potentially exposed workers with enzyme-linked immunosorbent assay (ELISA) were unsuccessful due to the presence of aflatoxin-like antigenic material (7, 8).

Aflatoxin B₁ is metabolized by the mixed function oxidase system to a number of hydroxylated metabolites and to the 8,9-epoxide. The latter is considered the ultimate carcinogenic form that reacts with cellular deoxyribonucleic acid (DNA) and proteins to form covalent adducts. Detection and quantification of these adducts have been suggested as alternative methods to detect human exposure to aflatoxins (9). With the use of ELISA and a monoclonal antibody against aflatoxin B₁, aflatoxin B₁ bound to albumin could be detected in blood samples collected in Gambia and Kenya, but not in samples collected in France. The sensitivity of the assay was 5 pg aflatoxin B₁ · mg⁻¹ albumin (10). In the present paper we have used a combination of affinity purification of pronase-digested albumin and ELISA to detect aflatoxin adducts to serum albumin from workers potentially exposed to aflatoxin B₁.

Subjects and methods

Subjects

A total of 45 workers at two companies processing livestock feed in Esbjerg, Denmark, 15 at company A and 30 at company B, was included in the study. The workers were either working in the factory and silo (29 workers) or in the harbor unloading ships with raw material for the production (16 workers). They were all men in the age group of 25 to 62 years. None of the

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workers had been working in any other agribusiness for three months prior to the start of this study.

Sample collection

Blood samples (5 ml) were collected from the workers when they returned to work after at least two weeks of vacation, and then again after four weeks of work.

Dust samples were collected at selected sites in the factories of company A (3 samples) and company B (9 samples). Samples of all shipments of raw material were submitted to analysis for aflatoxin B₁ at government-approved laboratories.

Analysis of the blood samples

Serum was isolated from the blood samples by centrifugation, and albumin was precipitated by the addition of saturated ammonium sulfate, final concentration 50 %. After the removal of globulins by centrifugation, albumin was precipitated from the supernatant by acidification with acetic acid (11).

Albumin was redissolved in 500 µl of 0.1 M sodium phosphate buffer, pH 7.4, and digested with pronase (Fluka, Buchs, Switzerland, 25 units per 5 mg of albumin) for 18 h at 37°C. The concentration of albumin was determined by the Biuret reagent (Sigma, St Louis, Missouri, United States, kit 540—2) assay. The digest was applied to a prewet C18 Seppak column (Waters, Milford, Massachusetts, United States). The column was washed with 5 ml of 5 % methanol to remove small peptides and amino acids, before the aflatoxin B₁-containing fraction was eluted in 80 % methanol (5 ml). This fraction was evaporated to 0.5 ml and was diluted to make 10% methanol (1 ml). The eluate was applied to an Aflatest[®] affinity column (Vicam, Sommerville, Massachusetts, United States) (10), and the aflatoxin B₁-containing fraction was eluted with 1.5 ml of methanol in phosphate buffer 0.1 M, pH 7.4 (1:1) after the column was rinsed with phosphate buffered saline (PBS) (5 ml). With the use of albumin isolated from rats treated with radiolabeled aflatoxin B₁, the recovery was estimated to be 80 %.

Enzyme-linked immunosorbent assay

Polystyrene microtiter plates (Teknunc, Roskilde, Denmark) were coated with aflatoxin B₁-bovine serum albumin (Sigma) or bovine serum albumin (BSA) as previously described (8). On each plate 100 µl of the eluates and five aflatoxin (25, 2.5, 0.25, 0.025, and 0.0 ng · ml⁻¹) standards were added immediately before 100 µl of a solution of monoclonal antibody AW-1 (12) (diluted 1:1000 with PBS) was applied to compete for binding. The plates were incubated overnight at 4°C, then washed with 0.05 % Tween 20 (polyoxyethylene sorbitan monolaurate) in PBS before incubation with the secondary antibody, goat antimouse immunoglobulin G conjugated with alkaline phosphatase

(Dakopatts, Glostrup, Denmark) (diluted 1:1000 in 1 % BSA in PBS), for 2 h at room temperature. Following washing with 0.05 % Tween 20 in PBS and 0.1 M diethylamine buffer (pH 9), 100 µl of 4-nitrophenyl phosphate substrate (Sigma (1 mg/ml) was added to each well, and the plates were incubated for 2 h at room temperature. The absorption was read at 405 nm. The intrasample variation was less than 2 %.

Analysis of the dust samples

Aflatoxin was isolated from the dust samples with a modification of the standard procedure provided by the manufacturers of Aflatest[®] affinity columns.

Dust samples (0.9 mg) were suspended in 5 ml of 80 % methanol, 0.1 mg of sodium chloride was added, and the samples were extracted by shaking for 30 min in the dark. The extracts were centrifuged for 20 min, and the supernatants were collected. This procedure was repeated, and the supernatants were pooled. Three milliliters of the extract was adjusted to 10 % methanol, and 10 ml of this solution was loaded onto the Aflatest[®] affinity column. After being washed with distilled water the aflatoxins were eluted with 100 % methanol. The quantities of aflatoxins were determined in ELISA assays as has already been described.

Results

The sensitivity of the analysis using Seppak prepurification and affinity chromatography prior to quantitative analysis with ELISA was 5 pg aflatoxin B₁ · mg⁻¹ albumin, corresponding to 30 % inhibition. It was assumed that, with the use of the AW-1 antibody, the antigenicity of aflatoxin B₁ adducts to amino acids was similar to that of aflatoxin B_{2a}, as epitope mapping indicated that modification at the 7,8 position only had minor influence on antibody recognition (8). In an attempt to increase the sensitivity of the assay, different concentrations of unmodified BSA were added. Albumin in the amount of 5 mg/incubation did not influence the quantification.

Blood samples were collected from workers with different levels of suspected exposure to material contaminated with aflatoxin B₁ according to job descriptions (table 1). Workers exposed to grain dust for more than 50 % of their workhours were classified as highly exposed, and those exposed for less than 25 % of the workday were considered to have low exposure. Two of the 45 blood samples collected from the workers upon return from vacation indicated recent exposure to aflatoxin B₁ (50 and 54 pg aflatoxin B₁ · mg⁻¹ albumin), while all of the other samples were below the detection level of 5 pg aflatoxin B₁ · mg⁻¹ albumin. After four weeks of work 7 of 45 workers had detectable levels of aflatoxin B₁, a finding suggesting occupational exposure to aflatoxin B₁ (table 2). The mean level of the positive samples was ex-

pressed as $64 \text{ ng} \cdot \text{d}^{-1} \cdot \text{kg}^{-1}$ body weight on the assumption that the exposure was equally distributed over the four weeks of work and the average body weight was 70 kg. All of the workers with exposure to aflatoxin B₁ were employed by company B, but with different job descriptions and different types of prescribed personal protection.

In the three-month period prior to the second blood sample, the level of aflatoxin B₁ in the raw material arriving in company A was less than $5 \mu\text{g} \cdot \text{kg}^{-1}$. One shipment of peanut meal/coconut pills to company B had an aflatoxin content greater than $5 \mu\text{g} \cdot \text{kg}^{-1}$, and another had an aflatoxin B₁ level of $26 \mu\text{g} \cdot \text{kg}^{-1}$. Three out of the five workers with the highest antigenicity helped to unload the latter cargo (table 3). One of the workers with a detectable level of aflatoxin adducts to albumin but with suspected low exposure based upon his job description had a supervisory function at the grill where batches of raw material from different silos were dumped for use in production. Dust samples collected at this site had an aflatoxin B₁ content of $8 \mu\text{g} \cdot \text{kg}^{-1}$ dust. The aflatoxin B₁ content at the other sites of collection was generally low.

Table 1. Participants according to worksite and potential aflatoxin exposure. (L/M = low/medium, H = high)

	Potential exposure	Number of participants
Company A		
Factory/silo	L/M	9
Dock	H	4
Company B		
Factory/silo	L/M	8
Factory	L/M	12
Dock group 1	H	5
Dock group 2	H	5

Table 2. Individuals exposed to aflatoxin B₁ — exposure determined by amount of aflatoxin B₁ adducts bound to serum albumin. (L = less than 25 % of workhours, S = more than 50 % of workhours, ND = not detectable — less than 5 pg/mg)

Work area	Worktime in contaminated areas	Aflatoxin B ₁ adducts (pg aflatoxin B ₁ · mg ⁻¹ albumin)	
		First blood sample	Second blood sample
Company B, silo			
Worker 1	L	ND	58
Worker 2	S/L	ND	64
Company B, factory			
Worker 3	S	54	44
Worker 4	L	50	ND
Worker 5	S	ND	50
Company B, dock, team 2			
Worker 6	S	ND	88
Worker 7	S	ND	100
Worker 8	S	ND	50
Worker 9	S	ND	ND

Discussion

Human exposure to airborne or particle-borne chemical compounds can be determined either by ambient air monitoring or by the detection of the compounds or their metabolites in body fluids. However, these measurements do not give any information on the potential biological effect of the exposure. Quantification of a carcinogenic adduct to protein or DNA is an indirect measure of the amount of the active metabolite formed in the organism (13). A linear relationship between hepatic aflatoxin B₁ adducts to DNA and the risk for developing liver cancer among rodents has been reported. In the present study the binding of aflatoxin B₁ to serum albumin has been used as the monitor. One of the advantages of using serum albumin rather than, for example, lymphocyte DNA is that the measured level represents the accumulated dose during the half-time of the serum albumin, as no repair pathways are known, whereas the half-time of aflatoxin adducts to DNA in experimental animals is less than 24 h. A good correlation between the binding of aflatoxin B₁ to serum albumin and liver DNA has been reported for experimental animals (14). This finding suggests that binding to serum albumin is a good monitor for the binding to target cell DNA even at low doses of aflatoxin B₁. It has been estimated that approximately 5 % of the administered dose binds to albumin (10). The major aflatoxin B₁ adduct to albumin has been identified as being formed between the active form of aflatoxin B₁ and lysine (15). With the use of affinity purification of albumin hydrolysate of aflatoxin B₁ in combination with radioimmunoassay, a correlation was observed between the intake of aflatoxin, as measured by food analysis, and aflatoxin adduct to lysine in a Chinese population (16). However, this assay gave an overestimate in comparison with affinity chromatography combined with high-pressure liquid chromatography with fluorescence detection (17). Aflatoxin B₁

adducts to lysine have not been detected in people living in Western Europe.

In this paper, we report the presence of aflatoxin B₁ adducts to lysine in the serum albumin of some workers who have been unloading aflatoxin B₁-contaminated raw material for animal-feed production. Aflatoxins are frequently found in respirable grain dusts that are common in grain silos and are produced by the handling of the raw material for animal feed pellets (3, 18). Two of the workers had a detectable level of aflatoxin B₁ adducts to serum albumin upon return from vacation, but the adducts disappeared or their concentration dropped slightly in the subsequent four weeks. The source of this aflatoxin B₁ exposure is unknown, as the workers were neither involved in any other type of agribusiness during vacation nor had their own farms. It should be noted that one of these workers had only had two weeks of vacation and that the detectable level of aflatoxin B₁-albumin could have been caused by exposure prior to vacation. The half-time of albumin is more than three weeks. Only three out of five workers unloading the heavy contaminated cargos had a detectable level of exposure, although the same type of personal protection was in use. However, these workers implemented different job functions. Another explanation for the different adduct levels with the same exposure could be an inter-individual difference in the metabolism of aflatoxin B₁ to biologically active metabolites or detoxified products (19). One of the workers in the silo at factory B had a fairly high level of aflatoxin B₁ adducts to albumin although his estimated exposure to dust particles was low, less than 25 % of the workhours. He had a supervisory function controlling the ramp for unloading the raw material and grain for transport to the factory. Dust samples collected at this site showed a fairly high level of aflatoxin B₁ (8 µg · kg⁻¹) compared with the 1 µg · kg⁻¹ or nondetectable level at the other collection sites in the silo or factory. As such, there appears to be a good association between the level of aflatoxin B₁ adducts to albumin and exposure, as measured by the analysis of dust or raw material. An excess number of liver cancer cases has been reported in this group of workers (6), and attempts were made to explain the number of cases on the basis of the level of exposure to aflatoxin B₁. The mean level of exposure, only including the individuals with detectable levels, was 64 ng aflatoxin B₁ · kg⁻¹ · d⁻¹ on the assumption that 5 % of the dose binds to albumin and that the exposure was equally distributed over the 30 workdays between the two blood samples. This estimated level was slightly higher than the level of exposure in an area with an intermediate liver cancer rate; in this area the exposure had been estimated to be 16.5 ng · kg⁻¹ (20).

On the assumption that the measured exposure is representative for the aflatoxin B₁ exposure of the workers at the animal-feed manufacturing companies in Denmark over an extended period of many years,

Table 3. Control measurements of aflatoxin B₁ in raw material unloaded during the four weeks prior to the second blood sample.

Company	Amount of raw material (t)	Amount of aflatoxin B ₁ ^a (µg · kg ⁻¹)	Number of workers (positive/exposed)
Company A			
Team 1	4.612	<5	—/4
Company B			
Team 1	8.850	<5	—/5
	2.100	8—9	—/5
Team 2	1.900	<5	3/5
	2.200	26	

^a This analysis is required by the Department of Agriculture, and the results have been provided by the companies.

85 liver cancer cases per million exposed individuals per year would be expected according to the linear regression data from correlation studies on aflatoxin B₁ exposure and liver cancer incidences (21). Among the 2000 members of the trade unions representing the workers, only 1.7 new cases of liver cancer should be expected from the monitoring data in comparison with the observed value of 3 cases (6). Exposure to carcinogens normally takes place 10—30 years prior to the diagnosis of cancer. The quality of the raw material, the number of cargoes contaminated with aflatoxins, and the work environment have changed considerably in the last 10 years so that the measurements made today are not representative of the period in which the liver cancer patients were exposed. As only a few of the trade union members are currently exposed, a lower number than 1.7 cases/10 years is to be expected.

In addition to its carcinogenic effect, aflatoxin B₁ has been suggested to influence the immune system (22). The efforts made by the companies to reduce the level of aflatoxin B₁ in the raw material, as well as encapsulating the work process, will remove some of the health hazards associated with work in the animal-feed manufacturing companies.

References

1. Munoz N, Bosch FX. Epidemiology of hepatocellular carcinoma. In: Okuda K, Ishak KG, ed. Neoplasma of the liver. Tokyo: Springer, 1987:3—19.
2. Autrup H, Seremet T, Wakhisi J, Wasunna A. 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. *Cancer Res* 1987;47:3430—6.
3. Hayes RB, Van Nieuwenhuize JP, Raatgever JW, Kate FJW. Aflatoxin exposures in the industrial setting: an epidemiological study of mortality. *Food Chem Toxicol* 1984;22:39—44.
4. Dvorackova I, Stora C, Ayraud N. Evidence for aflatoxin B₁ in two cases of lung cancer in man. *J Cancer Res Clin Oncol* 1981;100:221—4.
5. McLaughlin JK, Malker HSR, Malker BK. Registry-based analysis of occupational risk for primary liver cancer in Sweden. *Cancer Res* 1987;47:287—93.
6. Olsen JH, Dragsted L, Autrup H. Cancer risk and oc-

- cupational exposure to aflatoxins in Denmark. *Br J Cancer* 1988;58:392—6.
7. Dragsted LO, Larsen AI, Bull I, Autrup H. Aflatoxin-udskillelse hos erhverksponerede havnearbejdere. *Ugeskr Laeger* 1985;147:4148—50.
 8. Dragsted LO, Bull I, Autrup H. Substances with affinity to a monoclonal aflatoxin B1 antibody in Danish urine samples. *Food Chem Toxicol* 1988;26:233—42.
 9. Autrup JL, Autrup H. Human exposure to aflatoxins — biological monitoring. In: Bhatnager, ed. *Handbook of applied mycology; vol 5 (Mycotoxins in ecological systems)*. New York, NY: Marcel Dekker, in press
 10. Wild CP, Jiang Y-Z, Sabbioni G, Chapot B, Montesano R. Evaluation of methods for quantification of aflatoxin-albumin adducts and their application to human exposure assessment. *Cancer Res* 1990;50:245—51.
 11. Shearson D, Sabro P, Sigsgaard T, Johansen F, Autrup H. Biological monitoring of foundry workers exposed to polycyclic aromatic hydrocarbons. *Br J Ind Med* 1990; 47:448—453.
 12. Sun T, Wu Y, Wu S. Monoclonal antibody against aflatoxin B1, and its potential applications. *Chin J Oncol* 1983;5:401—5.
 13. Autrup H. Human exposure to genotoxic carcinogens: methods and their limitations. *J Cancer Res Clin Oncol* 1991;117:6—12.
 14. Bechtel DH. Molecular dosimetry of hepatic aflatoxin B1-DNA adducts: linear correlation with hepatic cancer risk. *Regul Toxicol Pharmacol* 1989;10:74—81.
 15. Sabbioni G. Chemical and physical properties of the major serum albumin adducts of aflatoxin B1 and their applications for the quantitation in biological samples. *Chem Biol Interact* 1990;75:1—15.
 16. Gan LS, Skipper PL, Peng X, et al. Serum albumin adducts in the molecular epidemiology of aflatoxin carcinogenesis: correlation of aflatoxin B1 intake and urinary excretion of aflatoxin M1. *Carcinogenesis* 1988; 9:1323—5.
 17. Sabbioni G, Ambs S, Wogan GN, Groopman JD. The aflatoxin-lysine adduct quantified by high-performance liquid chromatography from human serum albumin samples. *Carcinogenesis* 1990;11:2063—6.
 18. Sorensen WG, Jones W, Simpson J, Davidson JJ. Aflatoxin in respirable airborne peanut dust. *J Toxicol Environ Health* 1984;14:525—33.
 19. Forrester LM, Neal GE, Judah DJ, Glancey MJ, Wolf CR. Evidence for the involvement of multiple forms of cytochrome P-450 in aflatoxin B1 metabolism in human liver. *Proc Natl Acad Sci USA* 1990;87:8306—10.
 20. Van-Rensburg SJ, van-Schalkwyk GC, van-Schalkwyk DJ. Primary liver cancer and aflatoxin intake in Transkei. *J Environ Pathol Toxicol Oncol* 1990;10:11—6.
 21. Report from a working group: health evaluation of aflatoxins in foodstuffs. Copenhagen: Danish Institute of Toxicology, National Food Agency, 1989:1—50.
 22. Pestka JJ, Bondy GS. Alteration of immune function following dietary mycotoxin exposure. *Can J Physiol Pharmacol* 1990;68:1009—16.

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