Aflatoxin metabolism in humans: Detection of metabolites and nucleic acid adducts in urine by affinity chromatography

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Contributed by Gerald N. Wogan, June 11, 1985

ABSTRACT A high-affinity IgM monoclonal antibody specific for aflatoxins was covalently bound to Sepharose 4B and used as a preparative column to isolate aflatoxin derivatives from the urine of people and experimental animals who had been exposed to the carcinogen environmentally or under laboratory conditions. Aflatoxin levels were quantified by radioimmunoassay and high-performance liquid chromatography after elution from the affinity column. In studies on rats injected with [14C] aflatoxin B1, we identified the major aflatoxin-DNA adduct, 2,3-dihydro-2-(N7-guanyl)-3-hydroxy-aflatoxin B1 (AFB1-N7-Gua), and the oxidative metabolites M1 and P1, as well as the major aflatoxin species present in the urine. When this methodology was applied to human urine samples obtained from people from the Guangxi Province of China exposed to aflatoxin B1 through dietary contamination, the aflatoxin metabolites detected were also AFB1-N7-Gua and aflatoxins M1 and P1. Therefore, affinity chromatography using a monoclonal antibody represents a useful and rapid technique with which to isolate this carcinogen and its metabolites in biochemical epidemiology and for subsequent quantitative measurements, providing exposure information that can be used for risk assessment.

The aflatoxins are very high carcinogenic agents consistently found as contaminants in human food supplies in many areas of the world and epidemiologically linked to increased incidence of human liver cancer in Asia and Africa [see Busby and Wogan (ref. 1) for review]. Toxic and carcinogenic effects of aflatoxin B1 (AFB1) are thought to be related to its ability to be metabolized by constitutive cellular enzymes. Oxidative derivatives produced from the metabolism of AFB1 include hydroxylated species such as aflatoxin M1 (AFM1) and aflatoxin Q1 (AFQ1) as well as covalent macromolecular adducts with DNA and proteins (1–3). Biological consequences of oxidative metabolism of AFB1 range from cellular transformation to cell death (1–3). Furthermore, AFB1 can undergo multistage metabolism. For example, the metabolites AFM1 and AFQ1 retain the unsaturated bond at the 2,3-position of the molecule, thereby permitting further oxidation, ultimately leading to covalent adducts with cellular macromolecules (1–3).

Following elucidation of the requirement for enzymatic activation of AFB1 to mediate its adverse biological effects, many attempts have been made to develop procedures to measure its metabolites in various media. A number of investigators have attempted to monitor human exposure by quantifying AFB1 residues in serum and urine (4–7). Most of these procedures involved the use of chemical analytical techniques such as TLC, HPLC, and immunological assays for identification and quantitation. A number of earlier reports have appeared demonstrating the presence in human samples of various AFB1 derivatives. The lengthy and complex procedures used to purify the aflatoxins prior to analysis have seriously limited the application of these approaches in large-scale epidemiologic studies.

A major objective of our work has been the development of rapid, noninvasive screening procedures for assessing the exposure of humans to environmentally occurring carcinogens. Useful protocols require the ability to quantify chemical carcinogens and their metabolites, especially DNA and protein adducts, in readily accessible compartments, such as urine and serum. We have been developing these monitoring techniques through immunoassays using monoclonal antibodies (8–10). These antibodies have proven to be useful analytical tools for quantifying the aflatoxins in biological fluids and also for use with affinity chromatography matrices as preparative tools to isolate aflatoxins from biological fluids (10). We report here results of initial applications of these techniques to the analysis of human urine obtained from people environmentally exposed to AFB1 in their diet and also of urine of rats injected with AFB1. These preparative and analytical procedures permit rapid measurement of aflatoxins in complex biological fluids under conditions that can be applied to large numbers of samples collected in epidemiologic surveys seeking to evaluate aflatoxin exposure as a risk factor for liver cancer in man.

MATERIALS AND METHODS

Chemicals. Radiolabeled AFB1 was obtained from Moravek Biochemicals (Brea, CA). AFB1 was purchased from Aldrich. AFM1 was a gift from George Buchi (Massachusetts Institute of Technology) and was synthesized as described by Buchi et al. (11). AFB1-glutathione conjugate adduct standard was kindly provided by G. Neal (Medical Research Council), synthesized as described in Moss et al. (12).

Animals. Male Fischer 344 rats were injected i.p. with 1 mg of [14C]AFB1 per kg of body weight (specific activity, 2.8 mCi/mmol; 1 Ci = 37 GBq) in 50 μl of dimethyl sulfoxide (Me2SO). These animals were then placed in metabolic cages and the urine was collected for 20 hr in flasks suspended in ethanol/dry ice.

Isolation and Purification of Monoclonal Antibodies. Production and characterization of an aflatoxin-specific IgM monoclonal antibody (designated 2B11) was the subject of a previous report (10). Briefly, the antibody-secreting hybridoma was grown as ascites tumor cells in BALB/c mice, previously injected with 0.5 ml of pristane (Aldrich). Ascites

Abbreviations: AFB1, aflatoxin B1; AFM1, aflatoxin M1; AFB1, aflatoxin P1; AFQ1, aflatoxin Q1; Me2SO, dimethyl sulfoxide; AFB1-N7-Gua, 2,3-dihydro-2-(N7-guanyl)-3-hydroxy-AFB1.

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fluid collected from these mice was pooled and either used directly in immunoassays or fractionated by saturated ammonium sulfate precipitation followed by dialysis against phosphate-buffered saline at pH 7.2 (P_/NaCl). The IgM (2B11) monoclonal antibody was further purified by HPLC with a TSK 4000SW column and isocratic elution using P_/NaCl. Proteins were monitored at 280 nm, and IgM and IgG standards purchased from Cappel Laboratories (Cochranville, PA) were used as molecular weight standards. Using this procedure, we were able to isolate up to 1 mg of chromatographically pure 2B11 per HPLC run.

Preparation of Monoclonal Antibody Affinity Column. The 2B11 monoclonal antibody affinity column was also prepared as described (10). The antibody was dissolved in coupling buffer (0.1 M NaHCO3, pH 8.3/0.5 M NaCl) at 1 mg/ml. This solution was coupled to 2.5 g of cyanogen bromide-activated Sepharose 4B (Sigma). The resin had been incubated in 8 ml of 1 M ethanolamine (pH 8.5). The studies described in this paper employed a column with a 2-ml bed volume, eluted with a flow rate of 0.5 ml/min at ambient temperature.

Affinity Chromatography Purification Procedure. Samples were applied to the monoclonal antibody affinity chromatography column in 2 ml of P_/NaCl. The column was then washed with 10 ml of P_/NaCl to remove any nonspecifically bound materials. Aflatoxin derivatives were eluted off the column with 2 column vol of 50% Me2SO/P_/NaCl; generally, the aflatoxins were quantitatively eluted in the first 2 ml of the eluant. The eluate was analyzed by competitive RIA and/or preparative HPLC.

Competitive RIA. A [3H]AFLB1 tracer was used to quantify the aflatoxins in rat and human urine samples. We routinely used an assay with a 400-μl total volume, 100 μl of which consisted of the [3H]AFLB1 tracer (specific activity 24 Ci/mmol). The tracer was diluted in 1% normal mouse serum/0.1% bovine serum albumin in P_/NaCl to a level of about 10,000 cpm/100 μl. The monoclonal antibody was diluted to a concentration that precipitated 40–60% of the AFLB1 tracer. The antibody was added to the reaction mixture in 100 μl of 10% horse serum in P_/NaCl. The test samples, either unlabeled AFLB1 for standard curves or antibody column eluates, were added in 200 μl of a P_/NaCl mixture, and the reaction mixtures were incubated for 2 hr at ambient temperature. An equal volume of ice-cold saturated ammonium sulfate was added to the reaction vessel, and the antibody was allowed to precipitate for 15 min. The precipitate was then centrifuged for 15 min at 12,000 × g, and the radioactivity in 400 μl of the supernatant was counted by using a LKB Model 1211 β-counter to quantify the amount of antibody inhibition.

HPLC. All HPLC procedures used a Beckman model 322MP gradient liquid chromatograph equipped with a Beckman model 160 UV detector. Chromatograms were recorded with both a strip chart recorder and a Hewlett Packard model 3390A integrator. Preparative HPLC was performed by using a 10 mm × 25 cm ODS 5-μm Ultrasphere reversed-phase column operated at 3 ml/min. Analytical HPLC was performed by using a 2.5 mm × 25 cm ODS 5-μm Ultrasphere reversed-phase column operated at 1 ml/min. For analysis of aflatoxin metabolites, an 18% ethanol isocratic elution for 20 min followed by an 18–25% ethanol gradient generated over 25 min was used. The mobile phase was buffered to pH 3.0 with 20 mM triethylammonium formate (Aldrich). To quantify radioactivity, 1-min fractions were collected and radioactivity was quantified as described.

Human Urine Sample Collection Procedure. People known to be exposed to AFLB1-contaminated foods were identified in the Guangxi Province in China. AFLB1 content had been determined previously in corn and peanut oil being consumed by the 20 individuals selected for this pilot study. Levels of AFLB1 contamination from 10 to 250 parts per billion (109) were recorded. From daily food intake data, we estimated that between 13 and 87 μg of AFLB1 had been ingested on the day before collection. The following morning the first urine was collected and an aliquot of 25 ml was passed through a C3 Sep-Pak column prepared previously by washing with 5 ml each of water, then ethanol, and finally water. After the urine was applied to the columns, they were washed with 5 ml of water, then washed with 15 ml of 10% acetonitrile, and sealed with Parafilm. They were stored at −20°C, until processing, which began when columns were rinsed with 5% acetonitrile and the aflatoxins were eluted with 80% methanol. This eluate was then dried in vacuo with a rotary evaporator. The aflatoxin-containing residue was redissolved in 2 ml of P_/NaCl prior to applying the sample to the monoclonal antibody affinity column.

RESULTS

Affinity Chromatography and HPLC Analysis of in Vitro Spiked AFLB1 Samples. Our earlier studies [Groupman et al. (10)] provided data showing that affinity chromatography on the monoclonal antibody column effectively isolated aflatoxins from complex mixtures such as urine and serum. This approach has been extended to the quantitative analysis of aflatoxins in rat and human urine. We first quantitatively recovered the derivatives of interest by monoclonal antibody affinity chromatography and then quantified their levels individually by immunoassay and highly sensitive HPLC methodologies. Use of both approaches provides complementary information on both identity and level of each substance present.

Elution of the aflatoxins from the preparative affinity column produced a 50% Me2SO/P_/NaCl solution, which was then subjected to both preparative and analytical reversed-phase HPLC chromatography. A typical profile of authentic standards of the aflatoxin–DNA adducts and other metabolites resolved by this analytical HPLC procedure is shown in Fig. 1. Using these chromatographic conditions the AFLB1–glutathione adduct chromatographed with a retention time of 8.6 (peak not shown). These procedures have an operational limit of detectability of about 1 ng of aflatoxin per ml of 50% Me2SO/P_/NaCl. We validated this method of quantitative analysis by using in vitro samples spiked with 0, 100, and 1000 ng of AFLB1. More than 90% of AFLB1 applied to the antibody affinity column could be recovered and quantified by the HPLC analysis. These recovery data were further extended by application of the same methods to in vivo samples obtained from rats dosed with [14C]AFLB1.

Antibody Affinity Chromatography and Preparative HPLC Analysis of in Vivo Aflatoxin Rat Urine. Two adult male Fischer 344 rats were each injected with 1 mg of [14C]AFLB1 per kg of body weight and their urine was collected for 20 hr, at which time 10–12% of the 14C from the initial dose had been excreted into the urine. These urine samples were initially fractionated on the affinity column and then were analyzed by competitive RIA; the results from this assay were compared to the amount of aflatoxin determined from the radioactivity data. Data from both rats indicated that the majority of the aflatoxin derivatives in rat urine would be recognized by the monoclonal antibody. Specifically, rats 1 and 2 contained 14.9 and 16.8 pmol of aflatoxin equivalents in 100 μl of the 50% Me2SO fraction quantified by radioactivity and 17.0 and 16.0 pmol of aflatoxin equivalent content measured by RIA, respectively. These data also indicated that the competitive RIA had the requisite sensitivity to determine aflatoxin content in biological samples.
Aliquots (100 μl) of each urine, containing 290 and 310 ng of [14C]aflatoxin equivalents, respectively, were diluted with 1.9 ml of Pi/NaCl and applied to the antibody affinity column, and 65% of the applied 14C became bound to the affinity matrix. Recycling of unretained 14C through a second passage over the column failed to increase the binding of aflatoxin to the column, indicating that the unretained aflatoxins were not recognized by the antibody, a finding consistent with the specificity found earlier for this monoclonal antibody (10). The retained aflatoxins were eluted from the column with 50% Me2SO/Pi/NaCl and analyzed by analytical reversed-phase HPLC. Representative HPLC chromatograms of UV and radioactivity profiles in the rat urine are shown in Figs. 2 and 3. The predominant metabolite was AFM1, which accounted for 41–50% of the recovered 14C. AFP1 and AFB1 were also detected, and together they accounted for <10% of the radioactivity and/or UV absorbance. Another major metabolite detected was the 2,3-dihydro-2-(N7-guanyl)-3-hydroxyl-AFB (AFB1-N7-Gua) adduct, which accounted for 16% of the applied radioactivity. The level of AFB1-N7-Gua adduct in the urine agreed well with the amount calculated from the pharmacokinetic data of Moss et al. (12). Overall recovery of the radioactive aflatoxins applied to the HPLC column was >95%. Material not retained by the affinity column (Pi/NaCl washes) was analyzed by preparative HPLC procedures. All of the radiolabeled material contained in this fraction chromatographed with properties of polar derivatives of aflatoxin. Thus, the major metabolites isolated from urine of rats dosed in vivo were AFM1, AFP1, and AFB1-N7-Gua. These findings suggest that it should be
possible to quantify other urinary aflatoxin derivatives such as the oxidative conjugates by using monoclonal antibodies having different specificities.

**Analysis of Human Urine Samples from People Environmentally Exposed to AFB₁.** Competitive RIAs were run on eluates from the monoclonal antibody affinity column through which urine samples had been passed (as described above). The AFB₁ equivalent content in 100 µl of the 50% Me₂SO eluate measured by RIA ranged from 2.5 to 16.0 pmol. These data indicated that the aflatoxin equivalent concentration in the urine as collected was in the range of 0.1–10 ng/ml, calculated by using a linear extrapolation of the RIA data. These estimates are consistent with the estimated intake data, but further experiments need to be performed to verify that linear extrapolation is properly applied to the pharmacokinetics of aflatoxin excretion into urine in humans. Urine samples from individuals who had been exposed to the highest dose (87 µg) on the previous day were passed through an antibody affinity column and column eluates were analyzed by analytical HPLC. A chromatogram of one of these samples, depicted in Fig. 4, clearly shows the presence of the major aflatoxin–DNA adduct AFB₁-N⁷-Gua in the urine, at a

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**Fig. 3.** HPLC profile of [¹⁴C]aflatoxin metabolites in rat urine isolated by the monoclonal antibody affinity column. This is the radioactivity profile that corresponds to the UV tracing in Fig. 2. UNK, unknown.

**Fig. 4.** HPLC UV absorbance (340 nm) profile of a human urine sample isolated by the monoclonal antibody affinity column from an individual whose estimated AFB₁ intake had been 87 µg on the previous day.
level of 7–10 ng. Thus, these findings indicate that monoclonal antibody columns coupled with HPLC can detect and quantify levels of aflatoxin–DNA adducts in human samples obtained from people exposed to the carcinogen under ambient conditions.

DISCUSSION

We have been applying monoclonal antibody technology to produce antibodies that recognize aflatoxins and that can be used in conjunction with other chemical analytical techniques to develop noninvasive screening methodologies to monitor human exposure to environmentally occurring carcinogens. Useful screening methodologies require that monoclonal antibodies be selected that recognize aflatoxins and their metabolites, including DNA adducts, in readily accessible compartments, such as serum and urine. An equally important attribute of such procedures is that they should require limited time for completion in order to be applicable to large numbers of samples. Earlier studies on aflatoxin derivatives in human samples have used techniques such as TLC, HPLC, and immunological assays to prepare and analyze aflatoxin contaminated samples. These methods have been reported to detect the presence of AFM\(_1\) (5, 6), AFB\(_1\) (4), and AFB\(_2\)-N\(^2\)-Gua (7) in human urine and serum samples. A disadvantage of many of these procedures was the lengthy preparation time required before the sample could be submitted to analytical analysis. Methods outlined here require <1 hr for completion and are therefore applicable to large-scale epidemiological studies to measure aflatoxin exposure in human populations. Furthermore, they permit resolution of aflatoxin mixtures by HPLC into their individual components. For some components of interest, such as the DNA adducts, it will be possible to increase the limit of detectability by further processing. For example, we reported earlier on the method to prepare a chemical procedure to quantitate AFB\(_1\)-N\(^2\)-Gua in urine (13). This method was based upon the isolation of aflatoxin–DNA adducts from urine by preparative and analytical liquid chromatography, with subsequent radiometric postlabeling to achieve a limit of detectability in samples of purified standards of 1 pg of AFB\(_1\)-N\(^2\)-Gua per ml of urine. However, we found that when attempting to apply this method to urine samples, the nonspecific interfering materials present often prevented the attainment of this level of sensitivity. This problem may now be avoided by using the preparative affinity column procedure described here.

The development of a reusable monoclonal antibody affinity chromatographic column first required the production of high-affinity monoclonal antibodies recognizing aflatoxins. Our initial endeavors to produce monoclonal antibodies recognizing aflatoxins culminated in the production of antibodies specific for aflatoxin B\(_1\)–DNA adducts. In a competitive ELISA using these initial antibodies a limit of detectability of one AFB\(_1\) residue per 1,355,000 nucleotides was obtained (8, 9). However, these antibodies did not crossreact with the aflatoxin derivatives expected to be found in human urine and serum, and we continued attempts to produce high-affinity monoclonal antibodies with the desired properties. One of these, an IgM (2B11) that was found to have a particularly high affinity toward the aflatoxins (10), was used in studies described in this and a previous report. In the competitive RIA, a 50% inhibition value of about 3 pmol is routinely obtained with this antibody, with a lower limit of detectability of about 300 fmol for AFB\(_1\), aflatoxin B\(_2\), and AFM\(_1\). We also have found that when this antibody is bound to a solid-phase matrix, a reusable column can be prepared that selectively isolates aflatoxins from complex mixtures, such as urine, serum, and milk. Isolates from this column chromatographed by HPLC show very little contamination by non-aflatoxin derivatives. Recently, we have also produced monoclonal antibodies specific for AFM\(_1\) and AFQ\(_1\); a monoclonal antibody with higher affinity for the AFB\(_1\)-N\(^2\)-Gua adduct is yet to be produced. Use of these antibodies could lead to further development of monoclonal antibody affinity chromatography procedures and subsequent immunological analysis for quantifying these metabolites in human samples. On the basis of this experience, the methodological approach to the detection of additional environmental carcinogens and mutagens may be extended.

We gratefully acknowledge the assistance of Dr. M. Wang of the Institute of Health, China NCMP, and the research scientists in the Institute for Liver Cancer Prevention and Treatment, Guangxi Province, who helped with sample collection and determination of AFB\(_1\) content in corn. This research was supported in part by Grant 5 F01 ES00597 from the National Institutes of Health and Grant 1529-C-1 from the American Cancer Society, Massachusetts Division.