Reactivity of Aflatoxin B$_{2a}$ Antibody with Aflatoxin B$_1$-Modified DNA and Related Metabolites

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Aflatoxin B$_{2a}$ (AFB$_{2a}$) antiserum has been previously used in an enzyme-linked immunosorbent assay (ELISA) for the quantitation of AFB$_1$ and AFB$_{2a}$. The present investigation examined the reactivity of the antiserum toward those adducts and metabolites of AFB$_1$ believed to play a major role in aflatoxicosis and carcinogenesis. 2,3-Dihydro-2-(N$_7$-guanyl)-3-hydroxyaflatoxin B$_1$ (AFB$_1$-N$_7$-Gua), the putative 2,3-(N$_5$-formyl-2',5',6'-triamino-4-oxo-N$_5$-pyrimidyl)-3-hydroxyaflatoxin B$_1$ (AFB$_1$-FAPyr), 2,3-dihydro-2,3-dihydroxyaflatoxin B$_1$ (AFB$_1$-diol), AFB$_1$-N$_7$-Gua-modified DNA, and AFB$_1$-FAPyr-modified DNA were prepared by in vitro incubation or chemical methods and subjected to competitive AFB$_{2a}$ ELISA. The antiserum showed significant reactivity with all five compounds, indicating that it had a high degree of specificity for both the cyclopentenone and the methoxy group of the parent aflatoxin molecule. Sensitivity for AFB$_1$-N$_7$-Gua-modified DNA, AFB$_1$-FAPyr-modified DNA, and AFB$_1$-diol by the ELISA method was 0.1 pmol per assay. To test the applicability of immunological detection of covalent binding of AFB$_1$ to DNA, the ELISA was compared with a conventional radioisotopic assay in two in vitro studies. The results showed that estimates of the kinetics and substrate dependence of covalent binding to calf thymus DNA in rat microsomal incubation mixtures by both methods were comparable. The broad specificity AFB$_{2a}$ antibody might be of considerable value in the detection of AFB$_1$ macromolecular adducts and related metabolites in epidemiological investigations or in the diagnosis of aflatoxicosis.

Aflatoxin B$_1$ (AFB$_1$) is a potent, naturally occurring hepatotoxin and hepatocarcinogen produced by the fungal species *Aspergillus flavus* and *A. parasiticus*. In view of its capacity for entry into the food supply via grain and nut products and epidemiological studies suggesting a potential role for it in human liver cancer (3, 4), extensive investigations on the metabolic fate of AFB$_1$ have been conducted (5, 13, 25). The AFB$_1$ metabolites capable of reacting with cellular macromolecules appear to be of particular significance. The reactions yielding these metabolites are summarized in Fig. 1. Metabolic activation of AFB$_1$ by a cytochrome P$_{450}$ to a reactive epoxide at the 2,3 position of the terminal furan and subsequent covalent binding to macromolecular nucleophiles have been suggested to be critical events in the carcinogenic process (24, 33). Hydrolysis of AFB$_1$-modified DNA has revealed the initial major nucleic acid adduct to be 2,3-dihydro-2-(N$_7$-guanyl)-3-hydroxy AFB$_1$ (AFB$_1$-N$_7$-Gua) (9, 20, 23). AFB$_1$-N$_7$-Gua has been determined to be a major urinary metabolite in rats exposed to AFB$_1$ (2). In vitro and in vivo kinetic studies have shown that because of a localized positive charge, the imidazole ring of AFB$_1$-N$_7$-Gua-modified DNA can spontaneously open to yield a more stable persistent DNA adduct (8, 35). The base adduct released after acid hydrolysis of this modified DNA has the putative structure 2,3-dihydro-2,3-(N$_5$-formyl-2',5',6'-triamino-4-oxo-N$_5$-pyrimidyl)-3-hydroxyaflatoxin B$_1$ (AFB$_1$-FAPyr) (8, 20). Covalent binding of the 2,3 epoxide to nucleophilic moieties in proteins is also likely, but specific characterization of these adducts has not yet been carried out. Another major metabolite, 2,3-dihydro-2,3-dihydroxyaflatoxin B$_1$ (AFB$_1$-diol), appears after spontaneous or enzymatic reaction of the 2,3 epoxide with water (19, 26, 27) and after the degradation of AFB$_1$-N$_7$-Gua-modified DNA (35). A functional role for AFB$_1$-diol in aflatoxicosis has been postulated on the basis that under neutral and alkaline
conditions, the metabolite becomes a dialdehydic phenolate ion which is capable of forming Schiff base adducts with amino groups of cellular proteins. Although early investigations suggested that AFB$_{2a}$, the hemiacetal of AFB$_1$, was a major reactive metabolite in vitro, Neal et al. (27) recently reported that the suspect metabolite in these cases probably was AFB$_1$-diol, the misidentification apparently arising from the similarity in the UV spectra of the two compounds.

Until recently, the metabolism of carcinogens has been studied primarily by the use of radioisotopes and high-performance liquid chromatography (HPLC). In many situations, the expense and instability of radiolabeled carcinogens limit their use in long-term, low-dose feeding studies. The effectiveness of HPLC can be reduced by interference and sensitivity problems. A third means of studying carcinogen metabolism was initiated by Poirier et al. (32) when they quantitated N-2-acetyl aminofluorene adducts by radioimmunoassay (RIA). Since then, formation of these and other carcinogen adducts has been followed by RIA (18) as well as by enzyme-linked immunosorbent assay (ELISA) and ultrasensitive enzyme radioimmunoassay (USERIA) (14, 15).

Two general types of immunogens can be used for the production of carcinogen antibodies. In one approach, carcinogen-modified DNA is complexed electrostatically with methylated bovine serum albumin (BSA) and used for immunization (15). Alternatively, the carcinogen can be chemically modified and conjugated to a carrier protein such as BSA (32). Depending on the portion of the hapten molecule exposed distally from the immunogen, the resultant antibody will react in differing degrees with the parent carcinogen, its macromolecular adducts, and related metabolites. Using the first approach, Haugen et al. (11) immunized mice with AFB$_1$-N$'$-Gua-modified DNA and methylated BSA to prepare a highly specific monoclonal antibody that could be used in the USERIA quantitation of AFB$_1$-N$'$-Gua-modified DNA and AFB$_1$-FAPyr-modified DNA. The monoclonal antibody, however, required DNA for immune recognition and did cross-react with the base adducts or AFB$_1$-diol. Regarding the second approach, several methods for derivatization and conjugation of AFB$_1$ to BSA have been described by our laboratory and others (7, 10, 16, 17). In one of these methods, AFB$_{2a}$ is converted to its dialdehydic phenolate ion under alkaline conditions and allowed to react with BSA (1, 10). The resultant Schiff base adduct is then reduced to a stable conjugate by the addition of sodium borohydride. Immunoassays have revealed that antibody prepared against the AFB$_{2a}$-BSA conjugate has the following rank order of cross-reactivity with aflatoxin analogs: AFB$_{2a}$ = AFB$_1$ > AFB$_2$ > aflatoxicol > AFM$_1$ > AFG$_1$ > AFG$_2$. The cyclopentenone and methoxy groups thus have the immunodominant function in the immunogen. Since AFB$_1$ macromolecular adducts also have unmodified cyclopentenone and methoxy moieties (Fig. 1), they should
hypothesically react with AFB$_{2a}$ antibody. Reported herein are details on the specificity of AFB$_{2a}$ antibody towards AFB$_{1}$-N$^2$-Gua-modified DNA, AFB$_{1}$-FAPyr-modified DNA, AFB$_{1}$-N$^2$-Gua, AFB$_{1}$-FAPyr, and AFB$_{1}$-diol as determined by a competitive ELISA. The application of the AFB$_{2a}$ competitive ELISA to monitoring covalent binding of AFB$_{1}$ to DNA in vitro is also described. We believe that this broad specificity antibody will be widely applicable in future biochemical and histochemical studies of the metabolic fate of AFB$_{1}$.

(A preliminary report has been presented at the 1981 meeting of the American Society for Microbiology, Dallas, Tex.)

**MATERIALS AND METHODS**

**Materials.** All inorganic chemicals and organic solvents were of reagent-grade quality. Calf thymus DNA, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, dimethyl sulfoxide, RIA-grade fatty acid-free BSA, horseradish peroxidase (type VI), lysozyme, human immunoglobulin G (IgG), hydrogen peroxide, 2,2'azino-di-3-ethyl-benzthiazoline-6-sulfonate (ABTS), Tween 20, and NADP were purchased from Sigma Chemical Co. (St. Louis, Mo.). [H]$^3$AFB$_1$ (15 Ci/mmol) was obtained from Moravek Biochemicals (City of Industry, Calif.), and [U]$^4$C]AFB$_1$ (206 mCi/mmol) was supplied by Makor Chemicals Ltd. (Jerusalem, Israel). Purity of isotopes was verified by radiochromatography. Male Fischer white rats (100 to 150 g) were obtained from Sprague-Dawley (Indianapolis, Ind.). New Zealand white rabbits were purchased from Klubertanz’s Rabbit Farm (Edgerton, Wis.) and tested to be Pasteurella negative before use.

**Preparation of AFB$_1$, AFB$_{2a}$, AFB$_{1}$DNA adducts, and related metabolites.** AFB$_1$ was purified from Aspergillus cultures directly (6), and AFB$_{2a}$ and AFB$_1$-diol were prepared according to the respective procedures of Pohland et al. (31) and Swenson et al. (34). AFB$_1$-N$^2$-Gua-modified DNA was prepared by a modification of the in vitro method of Lin et al. (20). Microsomes were prepared from male Fischer rats and adjusted to 1.0 g of original liver wet weight per ml of Tris-hydrochloride (pH 7.0) containing 0.25 M sucrose. The following were then added to a 250-ml Erlenmeyer flask: 0.14 M KC1, 1.0 ml; 60 mM MgCl$_2$, 1.0 ml; 2.0 mM EDTA, 1.0 ml; 0.1 M glucose-6-phosphate, 1.0 ml; 1.0 M Tris-hydrochloride (pH 7.0), 2.0 ml; 10 mM NADP, 1.0 ml; calf thymus DNA (2 mg per ml of 10 mM sodium acetate, pH 5.0), 10 ml; glucose-6-phosphate dehydrogenase, 20 U; and microsomal preparation, 4.0 ml. The solution was mixed for 15 min at 37°C, 4.0 mg of AFB$_1$ (20 µCi of [H]$^3$AFB$_1$) dissolved in 0.25 ml of dimethyl sulfoxide was added, and the flask was shaken for 1 h at 37°C. Modified DNA was extracted with phenol-chloroform, precipitated onto glass rods, and dried (9). AFB$_1$-DNA adducts were dissolved in 15 mM sodium chloride-1.5 mM sodium citrate (pH 6.5). Total bound [H]$^3$AFB$_1$ was measured in Aquasol (New England Nuclear Corp., Boston, Mass.) on a Beckman LS-335 liquid scintillation system (Beckman Instruments, Inc., Fullerton, Calif.) using the channel ratio method for quench correction. DNA concentration was determined by UV spectroscopy using a molar absorbancy of 6,600 at 260 nm (22). AFB$_1$-FAPyr-modified DNA was prepared by incubation of AFB$_1$-modified DNA in 0.1 M carbonate-bicarbonate buffer (pH 9.6) for 1 h at 37°C and then was reprecipitated and redissolved in sodium citrate buffer.

For preparation of AFB$_1$-N$^2$-Gua and AFB$_1$-FAPyr, modified DNA was isolated according to the above procedure, except that [H]$^3$AFB$_1$ (1.5 µCi/mg) was included instead of [H]$^3$AFB$_1$. The base adducts were prepared by hydrolyzing AFB$_1$-N$^2$-Gua-modified or AFB$_1$-FAPyr-modified DNA in 0.15 M HCl (20) and purified by semipreparative HPLC on a Waters (Milford, Mass.) µ Bondapak Porasil A reversed-phase column (0.78 by 122 cm) at room temperature according to Essigmann et al. (9). Crude hydrolysates were pumped onto the column with a Glencore HPLC pumping system (Glencore Scientific, Houston, Tex.), and an ISCO model UA-4 absorbance monitor (Instrumentation Specialties Co., Lincoln, Neb.) was used to follow the stepwise isocratic elution of adducts by methanol-water at 254 nm. Concentrations of the two base adducts were then determined by quantitating $^{14}$C present in purified preparations. Estimates of AFB$_1$-N$^2$-Gua made by UV absorbance at 360 nm (20) were found to be identical to those made with radiolabel. The purity of AFB$_1$, AFB$_{2a}$, AFB$_1$-diol, AFB$_1$-N$^2$-Gua, and AFB$_1$-FAPyr was routinely tested on a Waters analytical HPLC equipped with a µ Bondapak C$_{18}$ reversed-phase column. The compounds were eluted with ethanol-water-acetic acid (5:15:0.001) and monitored at 360 nm on an LDC Spectrometer II (Laboratory Data Control Co., Riviera Beach, Fla.) according to Lin et al. (20). Further purification was performed when necessary by repeated analytical HPLC with the same solvent system. AFB$_1$ solutions were handled with polyvinyl gloves in a fume hood. Contaminated glassware was detoxified by soaking in 5% sodium hypochlorite overnight.

**Production of AFB$_{2a}$ antibody.** Adult male rats were exposed to fatty acid-free BSA by a reductive alkylation method, and the resultant immunogen was injected into multiple sites of rabbits as previously described (10). The rabbits were bled via the marginal ear vein at weekly intervals, and the IgG was purified by the method of Herbert et al. (12). IgG concentration was determined spectrophotometrically (21), and titers were determined by ELISA (28).

**Competitive ELISA.** The competitive ELISA was based on that devised for AFB$_1$-O-carboxymethyl oxime-BSA antibody by Pesti et al. (28). Polystyrene microtiter plates (Falcon 3040) were first pretreated with BSA and glutaraldehyde. AFB$_{2a}$-antiseraum (6 mg/ml), with a titer of 5,120, was diluted 1:500 in phosphate-buffered saline (PBS) (0.1 M sodium phosphate buffer containing 0.15 M saline, pH 7.5), and 50µl aliquots were added to each well. The plates were then dried under a current of warm (ca. 40°C) air. Plates prepared in this manner could be stored over silica gel for up to 3 months. Before use, the plates were washed three times in PBS-Tween (0.05% Tween 20 in PBS) on Dynatech Miniwash (Dynatech Laboratories, Inc., Alexandria, Va.), preincubated with 0.2 ml of PBS-BSA (1% RIA-grade BSA in PBS) at 37°C for 1 h, and washed three more times. For the competitive ELISA, 25 µl of standard or sample in PBS was added to appropriate wells followed by the addition of
25 μl of the AFB₂a-peroxidase conjugate (5 μg/ml) (10) diluted in PBS-BSA. The plates were incubated for 1 h at 37°C, washed five times, and then reacted for 30 min with 0.1 ml of substrate consisting of 50 mM citrate buffer (pH 4.0), 0.4 mM 2,2'-azino-di-3-ethyl-benzthiazoline-6-sulfonate, and 1.2 mM hydrogen peroxide. The reaction was terminated by the addition of 0.1 ml of hydrofluoric acid-EDTA stopping reagent, and absorbance at 410 nm was measured on a Dynatech Microelisa Minireader.

RESULTS

Reactivity of AFB₂a antibody. The various AFB₁-DNA adducts and related metabolites were prepared chemically or by in vitro incubation in a rat microsomal metabolic activation system, and their molar concentrations were determined by radiolabel or UV spectroscopy. Figure 2A and B indicates the high degree of cross-reactivity of the AFB₂a antiserum towards these compounds as determined by a competitive ELISA using AFB₂a-peroxidase as the marker ligand. Minimal detection levels for AFB₂a, AFB₁-diol, AFB₁, AFB₁-N²-Gua-modified DNA, and AFB₁-FAPyr-modified DNA were approximately 100 fmol per assay. The relative immunoreactivity of the AFB₂a antiserum for the aflatoxin analogs, as defined by the number of picomoles required for 50% inhibition of maximal binding, was as follows: AFB₂a; 0.8; AFB₁-diol, 1.0; AFB₁, 1.4; AFB₁-N²-Gua-modified DNA, 1.7; AFB₁-FAPyr-modified DNA, 2.0; AFB₁-FAPyr, 3.5; and AFB₁-N²-Gua, 4.5. The results support previous contentions that the cyclopentenone and methoxy of the parent AFB₁ molecule were critical in determining the specificity of the antiserum and suggest that the latter could be utilized in detecting AFB₁-modified DNA as well as the metabolites AFB₁-diol, AFB₁-N²-Gua, and AFB₁-FAPyr. Apparently, an additional hydroxyl at the 3 position of the terminal furan of AFB₁-diol was not a specificity factor, since AFB₂a antibody reacted almost identically with AFB₂a and AFB₁-diol. Competitive assays conducted with AFB₂a and AFB₁-diol after a 1-h preincubation (37°C) at pH 7.5 with BSA (10 mg/ml), human IgG (10 mg/ml), or lysozyme (10 mg/ml) all showed comparable competition patterns to those found for the compounds without the protein preincubation. Since under these incubation conditions, AFB₂a and AFB₁-diol form Schiff base adducts with proteins (1, 26), the AFB₂a antiserum might also be useful for detecting such adducts.

ELISA of AFB₁-DNA adducts formed in vitro. To test the applicability of ELISA detection of covalent binding of AFB₁ to DNA, the technique was compared to a conventional radioisotopic assay in two in vitro studies. The standard in vitro assay system consisted of rat liver microsomes, [³H]AFBI, calf thymus DNA, and an NADPH-generating system. Covalent binding of AFB₁ was determined on modified, dehydrolyzed DNA by both the competitive ELISA and direct measurement of [³H]AFBI. Figure 3 reveals that the two estimates for microsomal activation as a function of time were nearly identical. Both methods showed that a maximal binding of 8 μg of AFB₁ per mg of DNA occurred after 60-min incubation. Figure 4 illustrates that the two methods were also comparable when the effect of AFB₁ concentration was examined. Under the conditions employed, 400 μg of AFB₁ per assay was not sufficient to saturate the microsomal enzyme present. The results indicated the AFB₂a competitive ELISA to be a sensitive and reliable method for determining covalent binding of AFB₁ to DNA.

DISCUSSION

Recent investigations in our laboratory have
toxin in various agricultural commodities (28–30). Specific immunoglobulins can also serve as useful probes in monitoring the metabolic fate of mycotoxins. It is of particular significance that AFB$_{2a}$ antibody has specificity for those adducts and metabolites believed to play a major role in AFB$_1$-induced toxicosis and carcinogenesis, namely, AFB$_1$-dil, AFB$_1$-N$_2$-Gua-modified DNA, AFB$_1$-FAPyr-modified DNA, AFB$_1$-N$_2$-Gua, and AFB$_1$-FAPyr. Table 1 compares the cross-reactivity of AFB$_{2a}$ antiserum with AFB$_1$-DNA adducts and related metabolites to that previously determined for free aflatoxin analogs (10). Modification of the cyclopentenone such as occurs with aflatoxicol, AFG$_1$, and AFG$_2$ or hydroxylation of the 4 position of the difuran as occurs with AFM greatly decreases the ability of this antibody to react with the aflatoxin molecule. However, all of the remaining AFB adducts and metabolites, which had unmodified cyclopentenone and methoxy moieties, were nearly as cross-reactive as AFB$_{2a}$. We found these general relationships to exist for several AFB$_{2a}$ antiserum preparations obtained from different rabbits and at different post-immunization time intervals.

Haugen et al. (11) described a monoclonal antibody that was specific for AFB$_1$-N$_2$-Gua-modified DNA and AFB$_1$-FAPyr-modified DNA. The hybridoma antibody had an absolute requirement for the DNA molecule in its immune recognition of these adducts and, hence, did not react with AFB$_1$, AFB$_{2a}$, AFB$_1$-dil, AFB$_1$-N$_2$-Gua, or AFB$_1$-FAPyr. Whereas the competitive USERIA performed with this antibody was inhibited 50% by 1 pmol of AFB$_1$-DNA adducts, a nearly equivalent sensitivity of 50% inhibition by 1.7 pmol could be achieved by

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<th>Aflatoxin analog</th>
<th>Amt (pmol) for 50% inhibition</th>
<th>Analog/ AFB$_{2a}$ ratio</th>
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* Determined from Fig. 2A and B, except the data for AFB$_2$, AFG$_1$, AFG$_2$, AFM$_1$, and aflatoxicol, which were taken from our previous study (10).
the competitive ELISA described here. Furthermore, the competitive AFB\textsubscript{2a} ELISA was simpler to perform, was subject to a much lower signal-to-noise ratio than the USERIA, and did not require use of radioisotopes. Depending on the specific investigation being undertaken, therefore, the relative advantages and disadvantages of the broad specificity AFB\textsubscript{2a} rabbit antibody and the specific monoclonal AFB\textsubscript{2a}-DNA antibody must be considered.

In this report, we have found that AFB\textsubscript{2a} antibody can cross-react with a number of important aflatoxin metabolites and aflatoxin macromolecular adducts. The efficacy of monitoring covalent binding of aflatoxin in vitro by the competitive ELISA using AFB\textsubscript{2a} antibody was well demonstrated. Thus, the antibody should be extremely useful for the detection of aflatoxin metabolites and adducts in long-term feeding studies and in epidemiological investigations. It might also be used as an immunosorbent for the separation and identification of aflatoxin-bound cellular macromolecules by an affinity chromatography technique.

ACKNOWLEDGMENTS

This work was supported by the College of Agricultural and Life Sciences, the University of Wisconsin-Madison, by Public Health Service research grant CA15064 from the National Cancer Institute, and by Public Health Service training grant T32 ES07015 from the National Institute of Environmental Health Sciences.

We thank Philip Lau for technical assistance.

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