ABSTRACT

Two monoclonal antibodies (6A10 and 12F5) were obtained after fusion of mouse P3X63-Ag.8.653 myeloma cells with spleen cells isolated from BALB/c mice immunized with imidazole ring-opened aflatoxin B1 (AFB1)-DNA and characterized by competitive enzyme-linked immunosorbent assays. Both antibodies are highly specific for imidazole ring opened AFB1-DNA and show some cross-reactivity with AFB1-DNA and no cross-reactivity with 8,9-dihydro-8-(7-guanil)-9-hydroxy-AFB1, AFB1 conjugated with bovine serum albumin, aflatoxin M1, or aflatoxin G1. Antibody 6A10 was further characterized and showed no cross-reactivity with DNA modified by several other carcinogens. It could detect adducts with 4-fold higher sensitivity in highly modified DNA (2.5 adducts/100 nucleotides) than in low modified DNA (4 adducts/100 nucleotides). With low modified DNA the limit of sensitivity is 5 adducts/100 nucleotides. Antibody 6A10 reliably detected adducts formed in vivo in rats and mice treated with AFB1. In a pilot study, AFB1 adducts were detected in liver tissues from individuals living in areas with suspected exposure to AFB1. Monitoring adduct levels in human tissue may provide information not only on carcinogen exposure but also on the relationship among infection with hepatitis B virus, dietary exposure to aflatoxin B1, and liver cancer.

INTRODUCTION

Epidemiological studies have demonstrated a strong association between exposure to AFB1, a known potent liver carcinogen, and human liver cancer incidence in Africa and Southeast Asia (1). When AFB1 is activated by monoxygenases in vivo or by peroxycids in vitro, the resulting 8,9-oxide binds covalently to N-7 of guanine in DNA (2, 3) resulting in the formation of AFB1-Gua as the major AFB1-DNA adduct. Conversion of AFB1-Gua to AFB1-FAPy (imidazole ring-opened guanine) adducts has been demonstrated in rat liver DNA in vivo (4, 5). Furthermore, accumulation of AFB1-FAPy adducts in rat liver DNA and a corresponding decrease in AFB1-Gua adducts was observed. It is possible that AFB1-FAPy, the persistent form of AFB1-Gua, may play an important role in hepatocarcinogenesis.

In recent years, it has become possible to quantify free carcinogens and their metabolites as well as carcinogen adducts on macromolecules by immunoassays (6-10). Monoclonal antibodies to AFB1-DNA, iro AFB1-DNA, and AFB1-protein have been developed and used to quantify adduct levels in animal models and human urine samples (11-14). However, pmol sensitivity and/or cross-reactivity to other aflatoxin metabolites limit the suitability of these antibodies for investigating AFB1-DNA levels in human liver tissues. Here we report the development and characterization of monoclonal antibodies specific to AFB1-FAPy adducts with fmol sensitivity. These antibodies have also been used to quantify AFB1-DNA levels in animal tissues as well as human liver tumor and adjacent-normal tissues from surgical patients.

MATERIALS AND METHODS

Chemicals. [3H]AFB1 (30 Ci/mmol) was obtained from Moravek Biochemicals, City of Industry, CA. Dichloroethane and m-chloroperbenzoic acid were purchased from Aldrich Chemical Company, Milwaukee, WI. AFB1, AFG1, AFB1-BSA conjugates, AFB1-BSA conjugates, calf thymus DNA, goat anti-mouse IgG and goat anti-rabbit IgG-alkaline phosphatase conjugates, and p-nitrophenyl phosphate (Sigma 104) were purchased from Sigma Chemical Co., St. Louis, MO. Rabbit anti-mouse IgG1, IgG2, and IgM were purchased from Litton Bionetics, Charleston, SC. Alkaline phosphatase-conjugated rabbit anti-mouse κ and λ chains were purchased from Western Biotechnology Associates, Inc., Birmingham, AL. Human smooth muscle actin (Sigma) was purchased from Sterile Systems, Logan, UT. BPDE I-modified DNA, AAF-DNA, 1-aminonaptyrene-modified DNA, and 8-MOP-DNA were prepared as described previously (15-18).

Synthesis of iro AFB1-DNA Adducts. Heat-denatured calf thymus DNA (10 mg) in 8 ml of 20 mM sodium phosphate (pH 7.4) was reacted with 2 µg of AFB1 and 2.5 µg of m-chloroperbenzoic acid dissolved in 8 ml of dichloroethane for 25 h at room temperature with stirring according to the procedure of Martin and Garner (19). DNA was then purified by extraction with chloroform five times, precipitated with 2 volumes of 95% ethanol, redissolved in 10 mM Tris-HCl (pH 7.0) and treated with 15 mM Na2CO3/30 mM NaHCO3 (pH 9.6) for 2 h to convert the adduct to the imidazole ring-opened form (AFB1-FAPy) as described previously (12). The modification level, quantitated by the absorbance at 260 nm (1 unit = 45 µg/ml) and 360 nm (ε = 1.8 x 104) for DNA and AFB1, respectively, was determined to be 2.5 adducts/100 nucleotides (20).

For the iro AFB1-d-DNA with low modification level, heat-denatured calf thymus DNA (10 mg) was reacted with 6 µg [3H]AFB1 (1.2 Ci/ mmol) and processed as described above. The modification level, quantitated by the absorbance at 260 nm for DNA and the radioactivity, was determined to be 4 adducts/100 nucleotides.

For the preparation of AFB1-d-DNA and AFB1-Gua, heat-denatured calf thymus DNA (10 mg) was reacted with 2 µg [3H]AFB1 (4 mCi/ mmol) and processed as stated above except without NaHCO3 treatment. The modification level was determined to be 7 adducts/100 nucleotides. Aliquots of DNA was hydrolyzed with 0.1 N HCl at 90°C for 30 min to depurinate. The AFB1-Gua was extracted with PBS saturated ethyl acetate, dried with a nitrogen stream, and redissolved in PBS. The concentration of AFB1-Gua was determined by radioactivity.

Immunization with iro AFB1-DNA, BALB/c mice (6-8 weeks old) (Charles River Breeding Laboratories, Wilmington, MA) were immunized as follows: week 1, i.p. injection of 100 µg highly modified iro AFB1-d-DNA conjugated with an equal amount of methylated keyhole limpet hemocyanin and emulsified with an equal volume of complete Freund's adjuvant; week 4, i.p. injection of 200 µg highly modified iro AFB1-d-DNA conjugated with an equal amount of methylated keyhole limpet hemocyanin and emulsified with an equal volume of complete Freund's adjuvant; week 6, subcutaneous injection of 10 mg of iro AFB1-d-DNA and 10 mg of iro AFB1-Gua.
AFLATOXIN B₁-DNA ADDUCTS

AFB₁-d-DNA-mKLH complex with incomplete Freund's adjuvant; week 7, tail vein i.v. injection of 100 μg highly modified iro AFB₁-d-DNA-mKLH complex without Freund's adjuvant.

During week 6, blood samples were removed from the tail and assayed by ELISA for antibody activity, as described below. Those animals showing the highest titer against highly modified iro AFB₁-d-DNA were selected for fusion.

Cell Fusion. Mouse spleen cells were fused with the myeloma cell line P3X63-AG.8.653 using polyethylene glycol 1000 essentially as described previously (18). The supernatant fluids were screened for the presence of specific antibodies by ELISA as described below. Cells from positive wells were subcloned in agarose plates containing an underlying monolayer of CREF cells.

Isolation of DNA. CD rats (Charles River Breeding Laboratory) were given i.p. injections of 0.5 and 1 mg/kg [3H]AFB₁ (90 mCi/mmol) in 0.2 ml corn oil and sacrificed 2 h after injection. BALB/c mice were given i.p. injections of 6 and 12 mg/kg [3H]AFB₁ (0.2 Ci/mmol) in 0.2 ml corn oil and sacrificed 4 h after injection. Livers and kidneys were excised for DNA purification.

Human liver tissues were obtained from patients undergoing surgery for liver cancer at National Taiwan University Hospital, Taiwan, Republic of China. Tumor and adjacent normal tissues were removed and immediately frozen in liquid nitrogen and kept at -70°C.

Frozen human liver tissues and fresh animal tissues were homogenized in 0.25 M sucrose, 2 mM CaCl₂, 0.1 M Tris-HCl (pH 7.0), and 5% Triton X-100. DNA was isolated from these crude nuclear preparations as described previously (2), treated with 15 mM NaOH and 30 mM NaHCO₃ (pH 9.6) for 2 h to convert any N-7 adduct to AFB₁ and then reprecipitated, dissolved in 1 x PBS, and denatured by boiling for 3 min and the AFB₁-FAPy adducts were quantitated by ELISA as described below.

ELISA. The hybridoma supernatants were screened as described previously (18). Polystyrene-U-bottomed microwell plates were coated with 20 ng highly modified iro AFB₁-d-DNA in PBS, by drying at 37°C overnight. After blocking nonspecific binding with 200 μl of 1% FCS for 1 h at 37°C, 100 μl of supernatant were added and the plates were incubated for 1.5 h. Plates were washed with PBS-Tween and incubated with 100 μl goat anti-mouse IgG alkaline phosphatase (1:500 dilution) for 1.5 h. The plates were washed, the substrate, p-nitrophenyl phosphate (1 mg/ml in 1 M diethanolamine, pH 8.6), was added and the color at 405 nm was measured with a Flow multiscan MC recorder.

Positive cultures were then rescreened by adding 50 μl of their supernatants to each of three wells, one coated with 50 ng highly modified iro AFB₁-d-DNA, one coated with 50 ng unmodified denatured DNA, and one blank, i.e., a well containing no DNA. This process eliminated nonspecific clones. Sera from the immunized mice were also titered by this procedure using three wells. Isotype determination was carried out as described previously (18).

For the competitive ELISA, wells were coated with 5 ng highly modified iro AFB₁-d-DNA. The hybridoma supernatant was diluted (1:16,000 for 6A10 and 1:20,000 for 12F5) and mixed with an equal volume of the competitor before adding 0.1 ml to the wells. Further steps were performed as described above.

DNA samples isolated from rat, mouse, and human tissues were assayed by competitive ELISA with antibody 6A10. For the standard curve, in vitro low modified iro AFB₁-d-DNA was serially diluted with nonmodified denatured calf thymus DNA such that 50 μl contained from 75 to 3000 fmol adduct and 50 μg DNA. These samples were mixed with an equal volume of diluted antibody (50 μl) and added to the plate. For the animal and human DNA samples, 50 μl 1 mg/ml denatured DNA were mixed with 50 μl diluted antibody before adding to the wells. Further steps were performed as described above. The percentage of inhibition for rat and mouse tissues was calculated by comparison to the absorbance values from controlled animals (animals without AFB₁, treatment). Assay values for calf thymus DNA and control animal DNA were essentially identical. The percentage of inhibition values for the human samples were calculated by comparison to the nonmodified denatured calf thymus DNA control. This procedure takes into account the cross-reactivity of antibodies with denatured unmodified DNA. Values below 20% inhibition were considered not detectable.

RESULTS

BALB/c mice were immunized with highly modified iro AFB₁-d-DNA (2.5 adducts/100 nucleotides) complexed to methylated keyhole limpet hemocyanin. Spleen cells were fused with mouse myeloma cells (P3X63-AG.8.653) and cultured in hypoxanthine-aminopterin-thymidine selection medium. Two stable clones producing antibody specific for iro AFB₁-d-DNA were isolated from two immunized animals and characterized by ELISA. Isotype classification showed that both antibodies are IgGl, κ.

Competitive ELISAs were used to determine the sensitivity of the antibodies and to obtain additional information about their specificity. Antibody 6A10 has higher reactivity with highly modified iro AFB₁-d-DNA (50% inhibition at 160 fmol) than with low modified iro AFB₁-d-DNA (50% inhibition at 500 fmol) or with highly modified AFB₁-d-DNA (50% inhibition at 1300 fmol) (Fig. 1A; Table 1). While there is no cross-reactivity with unmodified native calf thymus DNA, there is a low cross-reactivity with unmodified denatured DNA (50 μg caused 35 ± 5% inhibition). Antibody 12F5 also showed better reactivity with highly modified iro AFB₁-d-DAN (50% inhibition at 200 fmol) than highly modified AFB₁-d-DNA (50% inhibition at 9000 fmol) (Fig. 1B; Table 1).

Table 1 also compares the two antibodies with respect to

![Graph 1](https://example.com/graph1)

Fig. 1. Competitive inhibition of monoclonal antibody (A) 6A10 and (B) 12F5 binding to iro AFB₁-d-DNA. The competitors were highly modified iro AFB₁-d-DNA (2.5 adducts/100 nucleotides) (△), highly modified AFB₁-d-DNA (7 adducts/100 nucleotides) (▼), and low modified iro AFB₁-d-DNA (4 adducts/100 nucleotides) (○).
samples were tested in a competitive ELISA using a monoclonal antibody (8G1) recognizing 8-MOP-DNA adducts (18). These patients have not been treated with 8-MOP nor is there environmental exposure. The level of inhibition of all samples was less than 20% indicating there was no nonspecific inhibition of antibody binding by the DNA samples (data not shown).

**DISCUSSION**

The monoclonal antibodies described here are highly specific for AFB1-modified DNA containing AFB1-FAPy covalent adducts. Studies with competitive ELISA indicate that these antibodies react about 10-fold better with iro AFB1-d-DNA than AFB1-d-DNA which contains both the ring-opened and ring-closed forms of adduct. They do not cross-react with AFB1, AFG1, AFB1-Gua, AFB1-BSA, AFB1, AAF-DNA, 1-aminopyrene-modified-DNA, 8-MOP-DNA, or BPDE-I-DNA even when tested at high concentrations. These highly specific monoclonal antibodies to iro AFB1-d-DNA have allowed the detection and quantitation of AFB1-FAPy adducts in various biological samples. The limit of sensitivity of detection of adducts in vivo, based on assaying 50 μg DNA/well and >20% inhibition, is 0.5 μmol/mol DNA. Antibody 6A10 can detect AFB1-FAPy adducts formed in vivo in rats and mice treated i.p. with AFB1. High performance liquid chromatography studies have indicated that AFB1-Gua represented 90 and 60% of the total AFB1-DNA adducts in male F344 rat liver and male CD1 Swiss mouse liver, respectively (21, 22). The remainder are unidentified. Before analysis with antibody 6A10, DNA samples were tested in a competitive ELISA using a monoclonal antibody (8G1) recognizing 8-MOP-DNA adducts (18). These patients have not been treated with 8-MOP nor is there environmental exposure. The level of inhibition of all samples was less than 20% indicating there was no nonspecific inhibition of antibody binding by the DNA samples (data not shown).

<table>
<thead>
<tr>
<th>Animal, dose, organ, and time at sacrifice</th>
<th>AFB1-DNA adducts by[^H] (μmol/mol DNA)</th>
<th>AFB1-FAPy adducts by ELISA[^A] (μmol/mol DNA)</th>
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</thead>
<tbody>
<tr>
<td>Rat, 0.5 mg/kg, 2 h</td>
<td>0.8</td>
<td>ND[^p]</td>
</tr>
<tr>
<td>Liver</td>
<td>ND</td>
<td>ND[^p]</td>
</tr>
<tr>
<td>Kidney</td>
<td>ND</td>
<td>ND[^p]</td>
</tr>
<tr>
<td>Rat, 1 mg/kg, 2 h</td>
<td>115.4</td>
<td>62.7</td>
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<tr>
<td>Liver</td>
<td>5.4</td>
<td>ND[^p]</td>
</tr>
<tr>
<td>Kidney</td>
<td>ND</td>
<td>ND[^p]</td>
</tr>
<tr>
<td>Mouse, 6 mg/kg, 4 h</td>
<td>1.0</td>
<td>ND[^p]</td>
</tr>
<tr>
<td>Liver</td>
<td>0.2</td>
<td>ND[^p]</td>
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<tr>
<td>Kidney</td>
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<td>ND[^p]</td>
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<tr>
<td>Mouse, 12 mg/kg, 4 h</td>
<td>65.1</td>
<td>41.7</td>
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<tr>
<td>Liver</td>
<td>2.3</td>
<td>ND[^p]</td>
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<tr>
<td>Kidney</td>
<td>ND</td>
<td>ND[^p]</td>
</tr>
</tbody>
</table>

[^a] Mean value of three determinations.
[^p] ND, not detectable. The limit of detection is 0.5 μmol/mol DNA.

the reactivity with free aflatoxins, aflatoxin-protein conjugates, and AFB1-Gua. Neither antibody showed cross-reactivity with up to 10^5 fmol AFB1 or AFG1, 5 x 10^4 fmol AFB1-Gua, 1.8 x 10^5 fmol AFB1-BSA, or 1.4 x 10^5 fmol AFM1-BSA. Antibody 6A10 was further characterized for its cross-reactivity with up to 10^5 fmol AAF-DNA, 1-aminopyrene-modified DNA, or BPDE-I-DNA (Table 1).

AFB1-Gua is the major DNA adduct formed when rats and mice are treated with AFB1 (21). Thus, to further validate the immunoassay, antibody 6A10 was used to quantitate AFB1-FAPy adducts formed when CD rats and BALB/c mice were treated with[^H]AFB1. Table 2 compares the levels of covalent modification of DNA in liver and kidney of rats and mice measured by scintillation counting and competitive ELISA using the low modification level in vitro iro AFB1-d-DNA in the standard curve. In agreement with previous studies, both rat and mouse livers have much higher total AFB1-DNA adduct levels than kidney. ELISA detection of AFB1-FAPy adducts is about 55-65% of total AFB1-DNA adducts by radioactivity in both rat and mouse liver.

To determine whether AFB1-DNA adducts could be detected in human liver, tissues were obtained from liver cancer patients at the National Taiwan University Hospital, Taiwan, Republic of China. Antibody 6A10 was used to quantitate AFB1-FAPy adducts formed in tumor and adjacent-normal tissues. AFB1-FAPy adducts were detectable in 2 of 8 adjacent-normal and 7 of 7 tumor tissues (Table 3). To further validate these results,
AFB<sub>1</sub>-Gua, the major adduct formed in vivo, is unstable and disappears rapidly from DNA by spontaneous hydrolysis of the glycosyl bond and is excreted in urine (25). In recent years, AFB<sub>1</sub>-Gua adducts have been detected in urine samples from individuals living in areas with suspected exposure to AFB<sub>1</sub> (13, 26). Thus, it is not surprising to detect AFB<sub>1</sub>-FAPy adducts in human liver using an antibody against aflatoxin itself. Liver samples from hepatocellular carcinoma patients in Czechoslovakia had DNA adducts ranging from nondetectable to 3.51 pmol/mg (1.1 µmol/mol DNA) comparable to those reported here. Our data showed that AFB<sub>1</sub>-FAPy adducts were detectable in only 2 of 8 adjacent-normal but in 7 of 7 tumor liver tissues. However, due to the small sample size these results may not be significant evidence for higher adduct levels in tumor tissues.

The AFB<sub>1</sub>-DNA-specific antibodies described here can be used to monitor large numbers of human samples. These studies can provide information not only on carcinogen exposure but also on the relationship between infection with hepatitis B virus, dietary exposure to aflatoxin B<sub>1</sub>, and liver cancer.

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Immunological Detection of Aflatoxin B₁-DNA Adducts Formed in Vivo

Ling-Ling Hsieh, Shang-Wei Hsu, Ding-Shinn Chen, et al.


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