Production and Characterization of Anti-idiotype and Anti-anti-idiotype Antibodies from a Monoclonal Antibody against Aflatoxin

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Anti-idiotype antibodies (anti-id, Ab2) for aflatoxin (AF) were generated by immunizing rabbits with affinity-purified mouse monoclonal antibody (mAb) 575G4H7 (Ab1) against the toxin. The affinity-purified polyclonal Ab2 antibodies (pAb2) were subjected to various anlayses and were used to generate polyclonal anti-anti-idiotype antibodies (pAb3) in the ascites of BALB/c mice. In a biotin–avidin-amplified ELISA, the concentration causing 50% inhibition (ID_{50}) of binding of Ab1 to the solid-phase AFB_1–bovine serum albumin (BSA) by pAb2 was found to be 0.76 μg/mL. Inhibition of binding of Ab1 to the solid-phase Ab2 by free AFB_1 (ID_{50} = 1.16 μg/mL) was less effective than in the AFB_1–BSA-based ELISA. Radioimmunoassay (RIA) revealed that ID_{50} values of binding of tritiated AFB_1 to Ab1 by free AFB_1 and pAb2 were 100 ng/mL and 100 μg/mL, respectively. Both indirect ELISA and RIA analysis showed that pAb3 has characteristics similar to those of other anti-AFB antibodies with high specificity and sensitivity for AFB_1 detection. In ELISA, the ID_{50} values of binding of pAb3 to the solid-phase AFB_1–BSA by aflatoxins B_1, B_2, G_1, G_2, and B_3 were found to be 0.38, 7.7, 4.42, 162, and 225 ng/mL, respectively. In RIA, the ID_{50} values of binding of pAb3 to the marker ligand [^{3}H]AFB_1 by aflatoxins B_1, B_2, G_1, G_2, and B_3 were found to be 0.88, 79, 34, 850, and 307 ng/mL, respectively. The overall analytical recoveries of AFB_1 (10–100 ppb) added to the white and yellow corn in the pAb3-based ELISA were found to be 84.1 and 85.4%, respectively.

Keywords: *Aflatoxin; antibody; anti-idiotype; immunoassay*

INTRODUCTION

Aflatoxins are a group of mycotoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Within this group, aflatoxin B_1 (AFB_1) has been found to be one of the most potent naturally occurring carcinogens and mutagens (Busby and Wogan, 1981; CAST, 1989; Eaton and Groopman, 1994). Agricultural commodities such as corn, cottonseed, and peanuts are frequently contaminated with aflatoxins (CAST, 1989; Jelinek et al., 1989). Due to the potential hazard of aflatoxins to human and animal health, extensive research has been conducted to search for efficient and accurate methods for toxin determination (Chu, 1991a). Such efforts have led to the development of immunochemical methods that are more versatile, sensitive, and specific than other approaches to mycotoxin analysis (Chu, 1989, 1991a,b; Morgan and Lee, 1990; Pestka, 1989). The immunochemical methods have gained wide application for mycotoxin detection during the past few years (CAST, 1989; Chu, 1991b, 1994), and hence there is a great demand for specific antibodies for the assay. Because aflatoxin is a low molecular weight, nonproteinaceous toxin, it is necessary to use an aflatoxin–protein conjugate for antibody preparation and specific marker (Chu, 1984, 1986, 1994), including radioactive aflatoxin and enzyme–aflatoxin conjugate, in various immunoassays. The need of preparation of these reagents always hinders its wide application. To overcome these problems, we have investigated whether anti-idiotype (Ab2) antibodies could be applied for these purposes. According to Jerne (1974), the variable domains of the idiotypic regions of an antibody (Ab1) could be in or around the antigen combining site. Thus, idiotypes are capable of behaving as immunogen to produce Ab2. However, Ab2 are heterogeneous and display various specificities (Bona and Kohler, 1984). Among three major populations, i.e., Ab2α, Ab2β, and Ab2γ, only Ab2β bears an idiotype that mimics the antigen epitope and resembles an internal image of antigen, which is capable of inhibiting the binding of antigen to Ab1. An important practical application of the development of Ab2 is selection of the Ab2β population that can be used in stimulating the production of anti-anti-idiotype antibodies (Ab3) with characteristics of Ab1.

Anti-idiotype antibodies for large molecules have been well developed and have gained wide application in diagnostic and therapeutic areas (Kennedy et al., 1983, 1987; Nisonoff and Lamoyi, 1981; Sacks et al., 1982). Within the past few years, numerous anti-idiotypes against small molecular weight haptens, including insecticides, herbicides (Spinks et al., 1993), hormones (Khole and Hegde, 1992; Madhok et al., 1992), and mycotoxins and phycotoxins (Chanh et al., 1989, 1990, 1992; Shestowsky et al., 1992, 1993), have been successfully generated. Some Ab2 has been shown to effectively mimic the biological functions of the hapten (Nisonoff, 1991). In the present study, mAb 575G4H7 against AFB_3–hemisuccinate (HS)–BSA conjugate, which has a wide cross-reactivity toward all four major aflatoxins, was chosen as Ab1 to generate Ab2 (Hefle and Chu, 1990). Details for the production and characterization of Ab2 and Ab3 are reported herein.

MATERIALS AND METHODS

Materials. Aflatoxins B_1, B_2, G_1, and G_2 were produced by *A. parasiticus* NRRL 2999 and were purified according to the

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method of Chu (1971). Aflatoxin B_3 was prepared from *A. parasiticus* culture according to the method of Heathcote and Dutton (1969). Tritiated AFB_1 was obtained from Moravek Biochemicals (City of Industry, CA) in 1981 with high specificity and further purified by Sep-Pak silica cartridge (Waters Associates, Milford, MA) before use in the radioimmunoassay (RIA). Briefly, 20 μL of [^{3}H]AFB_1 stock solution plus 1 mL of dry chloroform were loaded onto a Sep-Pak cartridge that had been washed with 5 mL of MeOH and 30 mL of dry chloroform subsequently. The cartridge was then washed with 10 mL of chloroform and 5 mL each of 5, 10, and 50% MeOH in chloroform and 100% MeOH. Radioactivity of each fraction was measured in a liquid scintillation counter (Beckman Instrument, Inc., Fullerton, CA). Aflatoxin in each fraction was determined by a direct ELISA. After purification, [^{3}H]-AFB_1 was associated with the 5% MeOH in chloroform wash and had a specific activity of 1.42 Ci/mmol.

Bovine serum albumin (BSA, RIA grade), *o*-phenylenediamine (OPD), dimethyl sulfoxide (DMSO), Tween 20, normal mouse IgG, and 2,6,10,14-tetramethylpentadecane (pristane, T-7640, lot 21H0869) were obtained from Sigma Chemical Co. (St. Louis, MO). Water-soluble carbodiimide [1-ethyl-[(3,3-dimethylamino)propyl]carbodiimide (EDPC)] was obtained from Aldrich Chemical Co. (Milwaukee, WI). Keyhole limpet hemocyanin (KLH), BCA protein assay kit, diaminodipropylamine agarose, *N*-hydroxysuccinimide (NHS)–biotin, goat anti-rabbit IgG–HRP conjugate, and goat anti-mouse IgG + IgM–peroxidase conjugate (ELISA grade) were obtained from Pierce Chemical Co. (Rockford, IL). Streptavidin–peroxidase conjugate was obtained from Boehringer Mannheim Co. (Indianapolis, IN). Bakerbond ABX was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). Activated CH-Sepharose 4B (Pharmacial Fine Chemicals, Inc., Uppsala, Sweden), Freund's complete adjuvant containing *Mycobacterium tuberculosis* (H37 Ra), and Freund's incomplete adjuvant were obtained from Difco Laboratories (Detroit, MI). Dulbecco modified Eagle's medium (DMEM) was obtained from GIBCO Laboratories (Grand Island, NY). Virus-free, 5–6-week-old, female BALB/c mice were obtained from Harlan Sprague Dawley (Madison, WI). *Pasteurella* negative New Zealand white rabbits were obtained from LSR Industries (Union Grove, WI). Hybridoma cell line 575G4H7 (IgG1,λ) against AFB_3–HS–BSA, obtained in a previous study (Hefle and Chu, 1990), was used to generate Ab1. The murine myeloma cell line P3/NS-1/1-AG4-1 was obtained from American Type Culture Collection (Rockville, MD). Aflatoxin B_1–carboxymethyloxime (AFB_1–CMO), AFB_2–CMO, AFB_1–CMO–BSA, and AFB_1–CMO–HRP were prepared according to the methods described previously (Chu et al., 1977, 1987). All other chemicals and organic solvents used were of reagent grade or better.

Production of mAb (Ab1) against Aflatoxin. Hybridoma (line 575G4H7), cultured in DMEM for a week (2×10^6 cells in 0.5 mL), were injected (ip) into each BALB/c mice that had been primed with 0.4 mL of pristane 10 days before the injection. On days 10–12 after injection, ascites fluids were tapped, collected, and centrifuged at 7000 rpm (5900*g*) for 5 min to remove cell debris. The supernatant fluid was used immediately or kept frozen at −20 °C. Preliminary purification of antibodies was achieved by precipitation of ascites fluids with $(NH_4)_2SO_4$ to a final saturation of 50%. The solution was left at 4 °C overnight and then centrifuged at 7000 rpm (5900 *g*) for 15 min at 4 °C. The pellet was resuspended in distilled water to its original volume and precipitated once again with $(NH_4)_2SO_4$ as above. The resulting pellet was resuspended in distilled water to half of its original volume and dialyzed against 2 L of KH_2PO_4 (10 mM, pH 6.0) at 4 °C overnight.

Purification of mAb (Ab1) against Aflatoxin. *1. ABX Column Purification.* The dialyzed antibody solution (pH was adjusted to 6.0) was loaded to an ABX column (1.7 × 15 cm, 10 g of ABX) that had been equilibrated with binding buffer (0.01 M of KH_2PO_4, pH 6.0) at room temperature. After the column was washed with the binding buffer to the absorbance (280 nm) fraction near zero, the antibodies were eluted from the column with 0.2 M KH_2PO_4, pH 6.8, at a flow rate of 0.5 mL/min. The eluates were collected in a fraction collector at 3 mL/fraction and analyzed for its binding ability with AFB_1 by RIA. Antibody-containing fractions were pooled, concentrated by a Centriprep 30 filtration device (30 000 molecular weight cutoff; Amicon Co., Danvers, MA), and frozen at −20 °C for further affinity chromatography.

2. Affinity Chromatography. Further purification of a specific antibody against aflatoxin was achieved by an affinity chromatography with a column armed with AFB_2–carboxymethyloxime (Chu et al., 1977). The affinity gel was prepared by reacting 2 mL of diaminodipropylamine agarose, which had been washed four times with distilled water, with 1.35 mg of AFB_2–CMO in 6 mL of 25% ethanol and 200 mg of EDPC in 2 mL of distilled water for 24 h at room temperature. After reaction, the gel was packed into a disposable polystyrene column (5 mL capacity) and washed with 300–400 mL of 0.5 M NaCl and then with 0.01 M sodium phosphate buffer containing 0.85% NaCl (PBS), pH 7.5. The column was stored in a sodium azide solution at 4 °C and allowed to come to room temperature and washed with PBS before use. For purification, 1 mL of Ab (containing 13 mg of protein) from the ABX column was applied to the column, recycled three times, and then allowed to stand for 30 min at room temperature. The column was then washed extensively with 0.01 M PBS, pH 7.5, until the absorbance at 280 nm of the eluant was near zero. Bound antibodies were eluted from the column with 1 M propionic acid, pH 2.0, at a flow rate of 0.5 mL/min. One milliliter fractions were collected and immediately neutralized by addition of the appropriate amount of 1.0 M Tris-HCl, pH 9.2, to each fraction. The specific antibody-containing fractions were pooled and dialyzed in 0.01 M PBS, pH 7.5, and then stored at −20 °C.

Production and Purification of Anti-idiotype AB (Ab2). Two New Zealand white rabbits were immunized in 20 places on the back intradermally with a total of 0.5 mg of either affinity-purified Ab1 or purified Ab1-KLH conjugate that was prepared by water-soluble carbodiimide method in complete Freund's adjuvant as previously described (Chu and Ueno, 1977). The rabbits were boostered intramuscularly in both thighs every 3 weeks with the same amount of IgG in incomplete Freund's adjuvant over a period of 20 weeks. Preimmune serum was used as a control. Rabbits were bled weekly 4 weeks after the primary immunization.

Production and Purification of Anti-idiotype AB (Ab2). *1. $(NH_4)_2SO_4$ Precipitation.* The antisera were precipitated twice with $(NH_4)_2SO_4$ to a final saturation of 35% using a 100% saturated $(NH_4)_2SO_4$ solution. The precipitates were reconstituted with distilled water to half of the original volume, dialyzed against distilled water for 0.5–1 h, then against 0.01 M PBS, pH 7.5, overnight at 4 °C, and lyophilized.

2. Normal Mouse IgG and Purified Ab1 Affinity Columns. To eliminate anti-isotypic, antiallotypic, and pre-existing nonspecific rabbit antibodies, CH-Sepharose 4B columns armed with either normal mouse IgG or purified Ab1 were used. The columns were prepared by conjugation with either 4.8 mg of normal mouse IgG or 10 mg of purified Ab1 in 0.1 M $NaHCO_3$, pH 8.0, containing 0.5 M NaCl to 0.5 g of cyanogen bromide-activated CH-Sepharose 4B according to the manufacturer's instruction. The unreacted sites were blocked with 1 M ethanolamine, pH 8.0, at room temperature for 1 h. The column was washed alternately with 0.1 M Tris-HCl, pH 8.0, and 0.1 M acetate, pH 4.0, four or five times and then stored in azide at 4 °C. The efficiency of coupling was determined by measuring the protein concentration in washing with the BCA (Smith et al., 1985) protein assay kit (procedures are according to Pierce instruction booklet 23225X).

For Ab2 purification, 1 mL (equivalent to 20 mg of protein) of $(NH_4)_2SO_4$ precipitation-purified rabbit antiserum (Ab2, 8 week bleeding) was applied to the normal mouse IgG column. The column was then washed with 0.01 M PBS, pH 7.5, until the absorbance at 280 nm of the eluant reached zero. Unbound protein fractions from this column were further passed through an affinity column armed with purified Ab1 to remove nonspecific rabbit antibodies. The specific anti-idiotypic antibodies were eluted from the affinity column with 1 M propionic acid at pH 2.0 (1 mL per fraction) and immediately neutralized with an appropriate amount of 1 M Tris-HCl buffer, pH 9.2. The

purified Ab2 antibodies were dialyzed against 0.01 M PBS, pH 7.5, overnight and stored at −20 °C for further analysis and for production of anti-anti-idiotype antibodies.

Characterization of Anti-idiotype Antibody (Ab2). *1. Indirect Sandwich ELISA of Binding of Ab2 to Ab1.* An indirect sandwich ELISA was used to assess the specific binding of Ab2 to Ab1. Microtiter plate wells were coated with purified Ab1 (2 μg/mL in 0.01 M PBS, pH 7.5, 0.1 mL per well). After incubation at 4 °C overnight, the plate was washed five times with washing buffer (0.01 M PBS, 350 μL each, with 0.05% Tween 20, pH 7.4) (use "washing" afterward), and then 100 μL of blocking solution (0.1% gelatin in 0.01 M PBS, pH 7.4) was added to each well. After incubation for 60 min and the washing, 100 μL of various dilutions of purified Ab2, control (preimmune serum), or irrelevant mAb was added and incubated at 37 °C for 60 min. The plates were washed again, followed by addition of 100 μL of 1:10000 dilution of goat anti-rabbit IgG−HRP conjugate to each well and incubation at 37 °C for 60 min. After washing, 100 μL of substrate (5 mg of OPD and 10 μL of hydrogen peroxide in 12.5 mL of 0.05 M citrate buffer, pH 5.0) was added to each well and incubated at 37 °C for 10 min to develop color. The reaction was stopped by addition of 100 μL of 1 N HCl in each well. The absorbance at 490 nm was determined in an automatic ELISA reader (Thermomax microplate reader, Molecular Devices, Menlo Park, CA). All of the samples were run in triplicate.

2. Determination of Binding of Ab2 to Ab1 by a Biotin−Avidin-Amplified Competitive ELISA. The specific binding of Ab2 to Ab1 was analyzed by a biotin−streptavidin-amplified competitive ELISA. Biotin was covalently linked to antibodies (Ab1 or Ab2) with NHS−biotin according to the method of Goding (1986). Briefly, 0.12 mg of biotin succinimide ester solution (1 mg/mL of DMSO) was reacted with 1 mL of purified antibodies (1.0 mg of IgG/mL of 0.1 M $NaHCO_3$, pH 8.0) at room temperature for 2 h and then dialyzed against 0.01 M PBS with 0.1% NaN_3, pH 7.5, overnight. In this assay, microtiter plate wells were coated with AFB_1−BSA (2 μg/mL, in PBS, 0.1 mL). After overnight incubation and appropriate washing, 100 μL of Ab1−Ab2 mixture (50 μL of biotinylated Ab1 and 50 μL of purified Ab2 or control serum dilutions were preincubated together for 90 min) was added to each well and incubated for another 60 min. After washing, 100 μL of 1:10000 dilution of peroxidase-labeled streptavidin was added in each well and incubated for 60 min. All subsequent steps were the same as above.

3. ELISA of Inhibition of Binding between Ab2 and Ab1 by Aflatoxin. The ability of free aflatoxin to compete with the binding of Ab2 to Ab1 was determined by incubating 50 μL of various concentrations of AFB_1 together with 50 μL of biotinylated Ab1 (1 μg/mL) at 37 °C for 90 min and then adding them to Ab2 coated plate (2 μg/mL, 0.1 mL/well). All subsequent washing and incubation steps were the same as above. Likewise, the ability of AFB_1 to compete with the binding of Ab1 to Ab2 was also tested using the same protocol except that the biotinylated Ab2 and Ab1 coated plates were used.

4. Determination of Specific Activity of [^{3}H]AFB_1 by Direct ELISA. Direct ELISA was conducted as described by Chu et al. (1987). For antibody coating, 100 μL of rabbit anti-AFB_1−BSA antisera (1:10000 dilution in 0.01 M PBS, pH 7.5) was coated onto microtiter plates. After incubation at 4 °C overnight and washing, 100 μL of BSA solution (0.1% of BSA in 0.01 M PBS, pH 7.5) was added to each well. After incubation for 60 min and washing, 50 μL of AFB_1 standard or various dilutions of fraction with specific amounts of radioactivity (ranges from 10^1 to 10^5 dpm) and 50 μL of AFB_1−CMO−HRP conjugate were added and incubated at 37 °C for 60 min. All subsequent steps were the same as above in the indirect ELISA.

5. Determination of Binding of Ab2 to Ab1 in RIA. To determine the binding of Ab2 to Ab1, a radioimmunoassay was also used. In general, 50 μL of various concentrations of AFB_1 or purified Ab2 was incubated with a constant amount of 50 μL of [^{3}H]AFB_1 (10 000−14 000 dpm) and 50 μL of Ab1 (65 μg of IgG/mL) at 4 °C overnight. Separation of the bound and free ligand from the reaction mixture was achieved by precipitation with an equal volume of saturated ammonium sulfate solution at room temperature for 30 min and then centrifugation at 7000 rpm (5900*g*) for 15 min. Radioactivity of the free ligand was determined in a liquid scintillation counter (Beckman Instrument) after the supernatant solution was poured and mixed with 5 mL of Aquasol (New England Nuclear Co., Boston, MA). For determination of the inhibition of binding of [^{3}H]AFB_1 to Ab1 by either AFB_1 or Ab2, a competitive radioimmunoassay was used. The protocols were the same as above except that various concentrations of both free AFB_1 (0.01−1000 ng/mL) and anti-id (0.02−12.5 μg/mL) were present.

Production and Purification of Mouse Polyclonal Anti-anti-idiotype AB (Ab3). For generating a large amount of polyclonal anti-anti-id (Ab3) antibodies, female BALB/c mice (6−8 weeks of age) were injected with affinity-purified Ab2 according to the method of Kurpisz et al. (1988). In brief, six BALB/c mice were injected (ip) with 25 μg of purified Ab2, in 0.3 mL of PBS, on days 0, 14, and 28. The mice were primed with 0.3 mL of pristane on days 3 and 17. On day 31, 6×10^6 cells of a nonsecreting myeloma cell line NS-1 in 0.5 mL of DMEM was injected (ip) into each of the mice. The ascites fluid was collected and pooled on day 42. The ascites fluids were centrifuged at 7000 rpm (5900*g*) for 5 min to remove cell debris. The cleared ascites fluid was transferred and frozen at −20 °C for later purification. Purification of mouse Ab3 was achieved by precipitation with $(NH_4)_2SO_4$ and affinity chromatography with an AFB_2 column, the same way as for the purification of mAb (Ab1).

Characterization of Mouse Polyclonal Anti-anti-idiotype AB (Ab3). *1. Using Ab3 in a Competitive Indirect ELISA.* Competitive indirect ELISA with similar protocols for Ab2 was used to characterize Ab3. Briefly, 100 μL of AFB_1−BSA (2 μg/mL in PBS, pH 7.5) was coated to each of the microtiter plates. After overnight incubation at 4 °C, the plates were washed five times with washing buffer and incubated with 0.1% gelatin in PBS (0.01 M, pH 7.5) for 60 min. They were washed again and incubated with purified Ab3 (5 μg/well in PBS, 0.01 M, pH 7.5) together with 50 μL of different aflatoxin analogues at different concentrations (0.01−1000 ng/mL, also in PBS, pH 7.5) at 37 °C for 1 h. After washing, 100 μL of diluted (1:10000) goat anti-mouse IgG + IgM−peroxidase conjugate was added to each well and incubated at 37 °C for 60 min. Subsequent steps of addition of substrate and enzyme determination were the same as above.

For determination of inhibition of the binding of Ab1 to Ab2 by Ab3, the biotin−streptavidin-amplified competitive indirect ELISA with biotin−streptavidin amplification used in the detection of inhibition of binding between Ab2 and Ab1 by free AFB_1 was used with the exception that free AFB_1 was replaced by various concentrations of Ab3 (1 ng−100 μg/mL).

2. Using Ab3 in a Competitive Direct ELISA. Competitive direct ELISA for AFB_1 was carried out under the same conditions as described previously (Chu et al., 1987) with the exception that the purified Ab3 at a concentration of 9.6 μg/well (in 0.1 mL) was coated to the microtiter plate wells. The AFB_1−HRP conjugate was used as the marker enzyme in this assay.

3. Using Ab3 in a Competitive RIA. To further characterize the Ab3, a competitive RIA was also used. In general, 50 μL of different aflatoxin analogues at different concentrations (0.001−1000 ng/mL, in 0.01 M PBS, pH 7.5, was incubated with a constant amount of 50 μL of [^{3}H]AFB_1 (10 000−14 000 dpm) and 50 μL of Ab3 (10 μg of IgG/mL) at 4 °C overnight. Subsequent steps of separation of the bound and free ligand and determination of radioactivity were the same as above (RIA for Ab2).

Anti-anti-idiotype Antibody (Ab3)-Based Indirect ELISA of AFB_1 in Corn Meal Sample. An analytical recovery study was carried out to test the efficacy of an Ab3-based indirect ELISA for the analysis of AFB_1 in white or yellow cornmeal. In this study, 5 g of each cornmeal was spiked with AFB_1 at concentrations ranging from 10 to 100 ppb. A control sample with no toxin added was used to serve as the blank. The spiked samples were kept at room temperature in the dark for 1 day, and then each of the samples was homogenized with 25 mL of sample extract solvent (70%

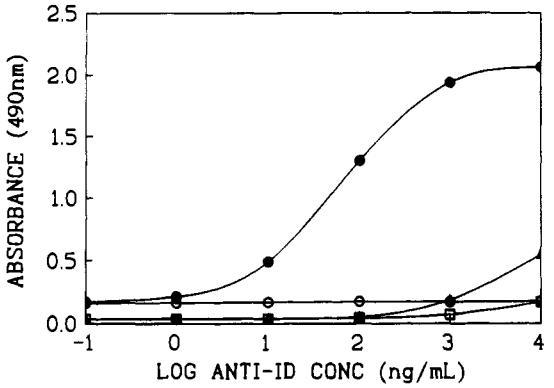

Figure 1. Specific binding of anti-idiotype antibodies with aflatoxin mAb. Microtiter plate wells were coated with either AFB–mAB (Ab1, ●) or control antibodies [including T-2 mAb (△) and normal mouse IgG (NMIg) (□)] at a concentration of 0.2 μg/well. The binding of purified anti-id (Ab2) or nonrelated Ig (NRIg) to the solid-phase antigens was determined with goat anti-rabbit IgG–peroxidase conjugate [NRIg on mAb1 (○)].

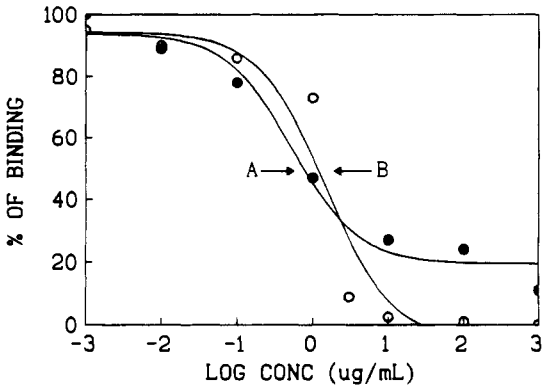

Figure 2. Effect of anti-id (Ab2) on the binding of mAb1 to solid-phase AFB_1–BSA (curve A) and the binding of free AFB_1 to mAb1 (curve B). Curve A was generated from an experiment in which biotinylated mAb1 and Ab2 were preincubated and then transfered onto the AFB_1–BSA (0.2 μg/well) coated plates. Curve B was generated from an experiment in which biotinylated mAb1 and various concentrations of AFB_1 were preincubated and then transferred onto the Ab2 (0.1 μg/well) coated plates. In both experiments, the binding of unreacted mAb1 to the solid-phase AFB_1–BSA or Ab2 was detected with HRP-labeled streptavidin (1:10000).

methanol, 29% water, and 1% DMF) for 3 min at high speed and filtered through Whatman No. 1 filter paper. The sample extract was then diluted with PBS and ready for indirect competitive ELISA by using anti-anti-id antibodies (Ab3).

RESULTS

Production of Anti-idiotype Antibodies. Polyclonal anti-idiotype antibodies for AFB_1 were generated from rabbits by immunization with either purified mAb Ab1 alone or purified mAb Ab1 conjugated to KLH. Both groups of rabbits exhibited anti-Ab1 titer in ELISA 4 weeks after immunization. The antibody titers peaked at the eighth week after initial immunization with one booster injection (seventh week). Rabbits immunized with Ab1 alone showed higher titer than those immunized with Ab1 conjugated to KLH (data omitted). Serum from the eighth week bleeding was selected for further purification and characterization. The majority of anti-isotypic, anti-allotypic, and pre-existing nonspecific antibodies in the serum were removed after ammonium sulfate precipitation, normal mouse IgG–Sepharose 4B column, and purified Ab1–Sepharose 4B affinity purification. The specific binding of the purified polyclonal anti-id Ab to the solid-phase monoclonal Ab1 is shown in Figure 1. A dose-dependent binding of Ab2 to Ab1 was found. The 50% endpoint for the binding of Ab2 to Ab1 was approximately 40 ng/mL (equal to $1:2\times10^6$ dilution). Control rabbit serum did not react with Ab1-coated effectively even at much higher concentration ($1:10^2$ dilution). The specific binding of Ab2 to Ab1 also could be seen from the results that no significant binding occurred between rabbit Ab2 and solid-phase normal mouse immunoglobulin or the mAb specific for T-2 toxin.

Characterization of Anti-idiotype Antibodies. *1. Indirect Sandwich ELISA of Binding of Ab2 to Ab1.* To determine whether the rabbit Ab2 has an epitope that resembles AFB_1 for its binding to Ab1, a biotin–avidin-amplified ELISA involving either biotin-labeled Ab1 or biotin-labeled Ab2 was used. Results of the competition binding of Ab1 to the solid-phase immunogen (AFB_1–BSA) by Ab2 and of the binding of Ab2 to the solid-phase Ab1 by free AFB_1 are shown in Figure 2. The rabbit Ab2 effectively competed with the binding of Ab1 with AFB_1–BSA, with an ID_{50} of 0.76 μg/mL. In the biotin–avidin-amplified competitive ELISA, AFB_1 at a concentration of 1.16 μg/mL inhibited approximately 50% of the binding of rabbit Ab2 to Ab1. Aflatoxin B_1 also inhibited the binding of Ab1 to rabbit Ab2 coated plates when biotin–Ab1 was used. However, as much as 3 μg of AFB_1/mL was needed to cause a decrease in A_{490nm} from 1.73 to 1.24.

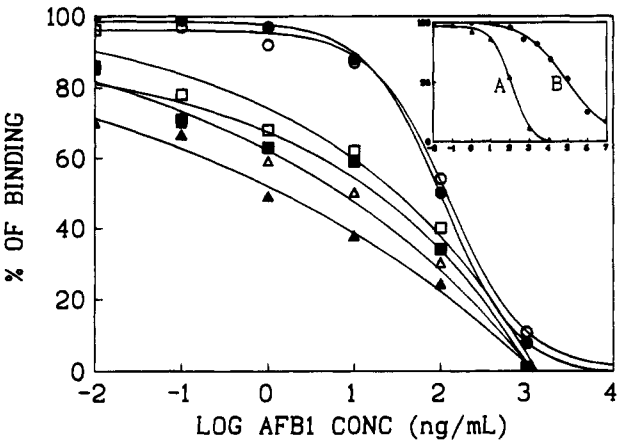

Figure 3. Effect of anti-id (Ab2) on the binding of the [^{3}H]-AFB_1 to mAb1 in RIA. In this assay, 50 μL of [^{3}H]AFB_1 and 50 μL of mAb1 (65 μg/mL) were incubated with various concentrations of free AFB_1 (0.01–1000 ng/mL) in the presence of 0 (○), 0.02 (●), 0.1 (□), 0.5 (■), 2.5 (△), and 12.5 (▲) μg of Ab2/mL. The inset represents the inhibition of the binding of the [^{3}H]AFB_1 to mAb1 by either AFB_1 (curve A) or Ab2 (curve 2). On a molar basis, the concentrations causing 50% inhibition by AFB_1 and Ab2 were 2.9×10^{-7} and 3.3×10^{-7}, respectively.

2. Competitive RIA. The specific interaction between rabbit Ab2 and mAb Ab1 was further demonstrated in a competitive radioimmunoassay (RIA). Data in Figure 3 indicate that the binding of [^{3}H]AFB_1 to Ab1 was inhibited by either unlabeled AFB_1 or rabbit Ab2. The ID_{50} values of binding of the [^{3}H]AFB_1 to Ab1 for AFB_1 and rabbit Ab2 were approximately 10^2 and 10^5 ng/mL, respectively. On a molar basis, the ID_{50} values of binding of [^{3}H]AFB_1 to Ab1 for AFB_1 and rabbit Ab2 are similar (2.9×10^{-7} and 3.3×10^{-7} mmol). A concentration-dependent inhibition of Ab2 with AFB_1 is also shown in Figure 3. At concentrations of 2.5 and 12.5 μg of Ab2/mL, the ID_{50} values for AFB_1 were found

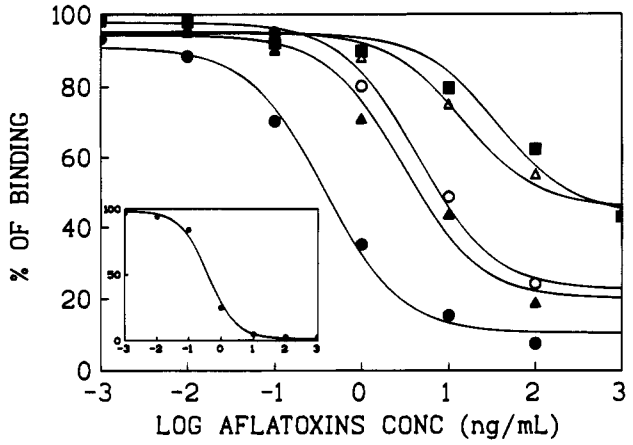

Figure 4. Effect of different aflatoxins (●, AFB_1; ○, AFB_2; ▲, AFG_1; △, AFG_2; ■, AFB_3) on the binding of mouse polyclonal anti-anti-id (Ab3) to solid-phase AFB_1–BSA in an indirect ELISA. The inset shows the effect of AFB_1 on the binding of AFB_1–HRP to solid-phase Ab3 by a direct ELISA analysis.

Table 1. Comparison of ID_{50} of Binding Different Aflatoxin Analogues to Anti-AFB (Ab1) and Anti-anti-id (Ab3) Using ELISA Method

aflatoxin analogue	ID_{50} values of different types of antibodies (ng/mL)		
	AFB_1–mAb Ab1[a]	AFB_3–pAb Ab1	mouse pAb3
B_1	13.4 (100)[b]	2.6 (100)	0.4 (100)
B_2	352.0 (3.8)	17.8 (14.6)	7.7 (4.9)
G_1	13.6 (99)	3.4 (76)	4.4 (8.6)
G_2	317.0 (4.2)	22.4 (8.6)	162.0 (0.2)
B_3	50.8 (26)	2.8 (93)	225.0 (0.2)

[a] AFB_1–mAb/Ab1, AFB_3–pAb/Ab1, and mouse pAb3 are mAb against AFB_1–BSA conjugate (provided by Dr. J. J. Pestka of Michigan State University), rabbit pAb against AFB_3–HS–BSA conjugate, and mouse polyclonal anti-anti-idiotype antibodies (Ab3, present study), respectively. [b] Values in parentheses are relative cross-reactivity (%) using aflatoxin B_1 as 100%.

to be approximately 10 and 1.8 ng/mL, respectively. In contrast, the ID_{50} of the control (absent of Ab2) was 100 ng/mL; the values of ID_{50} decrease with the amount of Ab2 added.

Production and Characterization of the Mouse Polyclonal Anti-anti-id (Ab3). Anti-anti-idiotype antibodies (Ab3) were produced in the ascites of BALB/c mice immunized with affinity-purified rabbit Ab2. The Ab3 were further purified by ammonium sulfate precipitation and AFB_2-oxime affinity purification steps and then analyzed by competitive ELISA and RIA. ELISA data showed (Figure 4) that Ab3 have similar characteristics as various anti-AFB antibodies with high specificity for AFB_1. The ID_{50} values of binding of Ab3 to the solid-phase AFB_1–BSA by aflatoxins B_1, B_2, G_1, G_2, and B_3 were found to be 0.38, 7.7, 4.42, 162, and 225 ng/mL, respectively. The ID_{50} values and cross-reactivities (in parentheses) of various aflatoxin analogues by ELISA for various anti-AFB antibodies are shown in Table 1. The Ab3-based ELISA for the AFB_1 system was 10 times higher than in the Ab1-based systems. Results of the direct competitive ELISA are shown in the inset of Figure 4. In the direct competitive ELISA, only AFB_1 was tested. The ID_{50} of binding of AFB_1–HRP to the solid-phase Ab3 by AFB_1 is found to be 0.39 ng/mL.

Data for the cross-reactivity of Ab3 with different aflatoxins as analyzed by RIA were similar to those by ELISA. Figure 5 shows that Ab3 also have high specificity for AFB_1; the ID_{50} values for the binding of Ab3 to the [^{3}H]AFB_1 by aflatoxins B_1, B_2, G_1, G_2, and

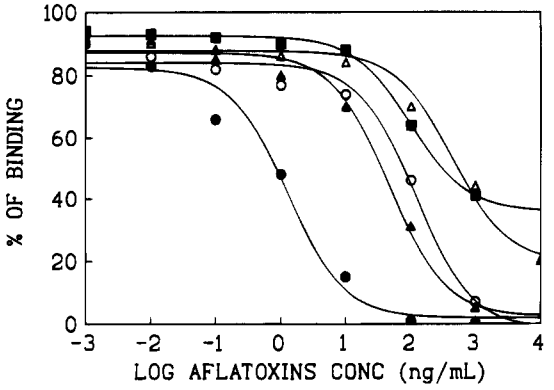

Figure 5. Effect of different aflatoxins (●, AFB_1; ○, AFB_2; ▲, AFG_1; △, AFG_2; ■, AFB_3) on the binding of [^{3}H]AFB_1 to mouse polyclonal anti-anti-id (Ab3).

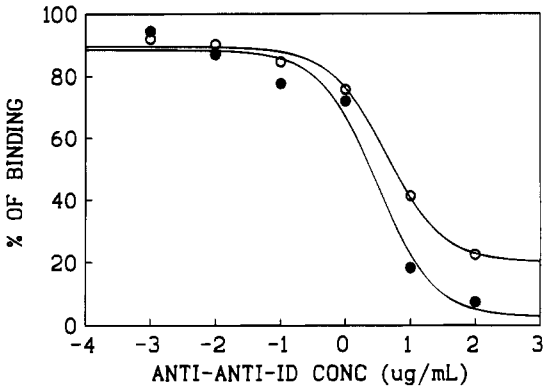

Figure 6. Effect of anti-anti-id (Ab3) on the binding of anti-id (Ab2) to AFB–mAb (Ab1). Biotinylated mAb1 (50 μL, 0.5 μg/mL) and various concentrations of Ab3 (50 μL/well) were incubated in the microtiter wells coated with Ab2 (0.1 μg/well) for 1 h. Either purified (●) or nonpurified (○) Ab2 and Ab3 were tested. The binding of unreacted mAb1 to the solid-phase Ab2 was detected using streptavidin–peroxidase conjugate (1:10000).

B_3 were found to be 0.88, 79, 34, 850, and 307 ng/mL, respectively. Data for the inhibition binding of Ab1 to Ab2 by Ab3 are shown in Figure 6. Affinity-purified Ab3 at a concentration of 2.37 μg/mL inhibits by 50% the binding of affinity-purified Ab2 to Ab1. When nonpurified Ab2 and Ab3 were used in the ELISA system, the Ab3-based ELISA was about 2 times less sensitive (ID_{50} = 5.5 μg/mL) than the purified antibodies.

Analytical Recovery of AFB_1 Added to Cornmeals by the Ab3-Based ELISA. Results of the analytical recovery of AFB_1 added to white or yellow cornmeal by Ab3-based indirect competitive ELISA are presented in Table 2. The amounts of AFB_1 in the spiked corn sample were in the range 10–100 ng/g. The analytical recovery ranged from 77.5 to 88.7% with an overall recovery of 84.1% (CV of 4.96%) and ranged from 76.1 to 91.7% with an overall recovery of 85.4% (CV of 21.61%) for the white corn or yellow cornmeal, respectively.

DISCUSSION

Anti-idiotype antibody for aflatoxin was demonstrated in rabbits by immunizing affinity-purified mAb against aflatoxin alone or purified mAb–KLH conjugate. Indirect ELISA revealed that the polyclonal anti-idiotype antibodies bound specifically to mAb1 against aflatoxin but not to IgG from mAb with irrelevant specificity or

Table 2. Analytical Recovery of AFB_1 Added to Corn by Ab3-Based Indirect ELISA

	analytical recovery							
	white corn				yellow corn			
amount added (ng/g)	ng/g	%	SD	CV%	ng/g	%	SD	CV%
10	8.37	83.7	0.32	3.77	7.61	76.1	2.80	36.78
10	26.60	88.7	1.20	4.51	25.12	83.7	2.47	9.82
50	43.25	86.5	0.45	1.04	45.83	91.7	12.13	26.71
100	77.45	77.5	8.10	10.50	90.15	90.2	11.82	13.12
overall		84.1		4.96		85.4		21.6

to normal mouse Igs. Data obtained from both ELISA and RIA revealed that Ab2 effectively inhibited the binding of AFB_1 to idiotype antibodies. More quantitative information was obtained from RIA analysis. On a molar basis, we found that the concentrations causing 50% inhibition of the binding of [^{3}H]AFB_1 to the monoclonal Ab1 by the AFB_1 and anti-id (Ab2) were similar (2.9×10^{-7} vs 3.3×10^{-7} mmol). These data suggest that (a) AFB_1 and Ab2 may exhibit an affinity similar to that of Ab1 and (b) the purified Ab2 molecule may retain one epitope that could mimic AFB_1. The ELISA and RIA data clearly suggest that the generated, purified Ab2 has an internal image of AFB_1. This conclusion was further sustained by the fact that anti-anti-idiotype antibodies (Ab3) with high specificity for AFB_1 were generated in mice after immunization of the animals with purified Ab2.

Although Ab2 effectively competes with AFB_1 by various immunochemical analyses, the concentration of Ab2 needed to cause such inhibition was high. Results from indirect ELISA in which Ab2 was coated to the ELISA plate instead of AFB_1–BSA conjugates and in the RIA showed that these systems were not as sensitive as most other ELISA systems that have been commonly used for immunoassay of AFB in recent years (Chu, 1994). These observations are similar to data obtained for the anti-idiotype antibodies of some other hapten-type toxins and insecticides. For example, the ID_{50} for quantitation of T-2 toxin (Chanh et al., 1990), okadaic acid (Shestowsky et al., 1992), and pyrethroid insecticides and herbicide paraquat (Spinks et al., 1993) in the idiotype–anti-idiotype ELISA system ranged from 40 ng to 5 μg/mL. The need for higher concentrations of free toxin for the displacement of Ab2 from their binding to the Ab1 is understandable from the observation of similarity of the affinity of Ab2 and free toxin to the idiotype antibodies. Thus, although the ID_{50} values are similar on a molar basis, there is a big difference for their mass.

Whereas Ab2 appear to have little use for the immunoassay of aflatoxins at present, Ab2 have been found to be good immunogens in generating the Ab3. The Ab3-based ELISA and RIA have been shown to have better sensitivity than the Ab1-based immunoassays. Because affinity chromatography was used extensively in purifying Ab1, Ab2, and Ab3, the Ab3 generated in the present study was also found to be highly specific to AFB_1. In the Ab3-based immunoassays, as low as 5 pg of AFB_1 can be determined. Because good analytical recovery was obtained in all of the tested ranges for AFB_1 for the white or yellow corn samples, Ab3-based ELISA could be applied to the analysis of aflatoxin in agricultural commodities. This is the first demonstration of using anti-anti-idiotype (Ab3) as immunochemical reagents for aflatoxin analysis. One of the factors that may limit the wide application of the Ab3-based ELISA is the production of large amounts of polyclonal Ab3 in mice. This problem could be overcome by developing monoclonal Ab3, which is currently under study in our laboratory.

In conclusion, polyclonal anti-idiotype and anti-anti-idiotype antibodies for AFB_1 have been generated in rabbits and mice, respectively. The purified Ab2 has an epitope with a mirror image of AFB_1 and has been shown to be an effective immunogen for generating Ab3 with similar characteristics as the idiotype antibody, i.e. Ab1, against AFB_1. Thus, Ab2 has the potential to be developed for a vaccine (Chanh et al., 1992; Nisonoff, 1991). Various studies of the Ab3 revealed that these antibodies could be used in the immunoassay for aflatoxin. Thus, a new generation of immunochemical reagents is now available for various purposes in aflatoxin analysis and other toxicological work. There are a number of advantages of using the anti-id antibody approach for mycotoxin analysis and diagnosis of mycotoxicoses: (a) it is not necessary to use a toxin–carrier conjugate for antibody production, thus alleviating the toxicity and metabolism problem encountered for the toxin–protein carrier; (b) it is not necessary to use a toxin–carrier or toxin–enzyme conjugate in the immunoassay system, thus avoiding the concerns about the toxicity and stability of toxin–enzyme conjugate in the assay kits; (c) it is simpler and more economical and versatile to produce these antibodies and to prepare Ab2 markers for immunoassay; (d) improved efficacy of immunoassay could be made through modification of Ab structures and through selecting mAb; (e) Ab2 could serve as a competitor for the toxin binding site, thus alleviating the toxicity of the toxin in vivo; (f) Ab2 could be used as an effective and safe vaccine for animals; and (g) modification of Ab2 molecules through chemical, enzymic, and genetic methods may yield alternate Ig molecules that could be used as immunogen to yield Ab3 with diversified ability for binding with toxin and toxin homologues. The present study is only the first step that could lead to these advantages and possibilities in mycotoxin research.

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