Production and Characterization of Anti-idiotype and Anti-anti-idiotype Antibodies against Fumonisin B_1

Fun S. Chu,*,† Xuan Huang,† and Chris M. Maragos‡

Food Research Institute and Department of Food Microbiology and Toxicology, University of Wisconsin—Madison, Madison, Wisconsin 53706, and Mycotoxin Research Unit, National Center for Agricultural Utilization Research, U.S. Department of Agriculture, Peoria, Illinois 60614

Anti-idiotype antibodies (Ab2) for fumonisin B_1 (FmB1) were demonstrated in rabbits after immunization with purified monoclonal antibody (mAb1) against FmB1. Ab2 bound specifically to FmB1–mAb1. Indirect competitive ELISA revealed that the binding of to FmB1–ovalbumin (OVA) was inhibited by Ab2. Ab2 could also be used as FmB1–OVA surrogate in the ELISA for FmB1. In the FmB1–OVA-based ELISA, the concentrations causing 50% inhibition (ID_{50}) of binding of mAb1 to FmB1–OVA by FmB1 and FmB2 were found to be 0.14 and 0.15 μg/mL, respectively. In the Ab2-based ELISA, the ID_{50} values of binding of mAb1 to Ab2 by FmB1 and FmB2 were found to be 0.46 and 0.61 μg/mL, respectively. A good correlation was found between the data obtained from the FmB1–OVA-based and the Ab2-based ELISA for the analysis of FmB1 in corn. Using the affinity-purified Ab2 Fab fragment as immunogen, polyclonal anti-Ab2 antibodies (Ab3) were generated in BALB/c mice. The Ab3 was found to have characteristics similar to those of original mAb1. The ID_{50} values of binding of Ab3 to FmB1–OVA by FmB1 and FmB2 were found to be 0.19 and 0.26 μg/mL, respectively.

Keywords: *Fumonisin; antibodies; anti-idiotype; immunoassay*

INTRODUCTION

Fumonisins (Fm) are a group of toxic metabolites all containing propane-1,2,3-tricarboxylic acid diesters of an aminodimethyltetra- or pentahydroxyeicosane structure. These mycotoxins are produced primarily by *Fusarium moniliforme,* one of the most common fungi colonizing corn throughout the world (Gelderblom et al., 1988; Marasas, 1986; Nelson et al., 1993; Norred, 1993; Riley and Richard, 1992; Riley et al., 1993). Other than *F. moniliforme,* fungi in the *Fusarium* section of *Liseola,* including *F. proliferatum,* also produce these toxins (Nelson, 1992; Nelson et al., 1992, 1993; Ross et al., 1990). Fumonisin B_1 (FmB1), the most common naturally occurring Fm in this group of mycotoxins, has been found to be a potent cancer promoter as well as an etiological toxic agent responsible for the equine leukoencephalomalacia (ELEM) disease (Gelderblom et al., 1988; Marasas et al., 1984; Norred, 1993; Riley and Richard, 1992; Thiel et al., 1992) and for pulmonary edema in swine (Colvin and Harrison, 1992; Osweiler et al., 1992). Preliminary reports on the carcinogenicity of FmB1 in rats (Gelderblom et al., 1991; Jaskiewicz, 1987; Thiel et al., 1992) and recent reports (Chu and Li, 1994; Murphy et al., 1993; Norred, 1993; Ross et al., 1991; Thiel et al., 1992) on the worldwide occurrence of high concentrations of this group of mycotoxins in foods and feeds, generally at the parts per million level, have prompted an intensive study on the carcinogenic effects of this mycotoxin, currently being conducted in the U.S. Food and Drug Administration National Toxicology Center.

In view of their potential hazard to human and animal health (Norred, 1993; Riley et al., 1993), extensive research has been conducted to develop more efficient methods for Fm determination. Several HPLC (Bennett and Richard, 1994; Holcomb et al., 1993; Murphy et al., 1993; Scott et al., 1992; Shephard et al., 1990; Stack and Eppley, 1992; Sydenham et al., 1992; Thiel et al., 1993; Ware et al., 1993) and TLC (Rottinghaus et al., 1992) methods with good sensitivity and accuracy have been developed. However, these methods generally require extensive sample cleanup and precolumn derivatization. With the availability of specific antibodies against FmB1 (Azcona-Olivera et al., 1992a,b; Fukuda et al., 1994; Usleber et al., 1994), more versatile immunochemical methods for Fms have been established (Azcona-Olivera et al., 1992a,b; Fukuda et al., 1994; Usleber et al., 1994). Such developments have led to a great demand for specific antibodies and related immunochemical reagents for the assay. An alternate approach to preparing immunochemical reagents is through generating anti-idiotype (anti-ID) antibodies (Bona and Kohler, 1984; Jerne, 1974; Nisonoff et al., 1991). Anti-idiotype antibodies (Ab2) for large molecules have been well-developed and have been applied to clinical diagnosis and immunotherapy (Kennedy et al., 1987; Nisonoff and Lamoyi, 1981; Sacks et al., 1982). Recent success in generating Ab2 against a number of small molecular weight haptens, including mycotoxins such as T-2 toxin (Chanh et al., 1989, 1990, 1992) and aflatoxin (Hsu and Chu, 1994), insecticides, herbicides (Spinks et al., 1993), hormones (Khole and Hegde, 1992; Madhok et al., 1992), and phycotoxins (Shestowsky et al., 1992, 1993), prompted our interest in generating anti-ID antibodies for FmB1. In the present study, mAb P2A5-3-F3 against FmB1–cholera toxin conjugate that has good cross-reactivity toward both FmB1 and FmB2 was chosen as the idiotype antibody (mAb1) for generating Ab2. Details for the production and characterization of Ab2 and anti-Ab2 (Ab3) and their use for immunoassay of FmB1 are reported herein.

* Address correspondence to this author at the Food Research Institute, University of Wisconsin, 1925 Willow Dr., Madison, WI 53706 [telephone (608) 263-6932; fax (608) 263-1114; e-mail fschu@macc.wisc.edu].

† University of Wisconsin.

‡ U.S. Department of Agriculture.

0021-8561/95/1443-0261$09.00/0

MATERIALS AND METHODS

Materials. A monoclonal antibody capable of binding both FmB1 and FmB2 was prepared from a hybridoma cell line designated P2A5-3-F3 developed jointly at the National Center for Agricultural Utilization Research, USDA (Peoria, IL), and the Cell Science Center Hybridoma Facility at the University of Illinois at Urbana/Champaign (Urbana, IL). Mice were immunized with FmB1 conjugated to glutaraldehyde-modified cholera toxin. Sera and hybridoma cell clones were screened using methods similar to those described previously (Azcona-Olivera et al., 1992b). Fumonisin B1–ovalbumin (FmB1–OVA) was also prepared by the cross-linking method with glutaraldehyde. Fumonisin B1(lot 102H0850), FmB2 (lot 100H05804), ovalbumin, *o*-phenylenediamine (OPD), Tween 20, normal mouse IgG, pristane (2,6,10,14- tetramethylpentadecane, T-7640, lot 21H0869), and gelatin were obtained from Sigma Chemical Co. (St. Louis, MO). FmB2, which had similar purity as the Sigma product, was also kindly supplied by Dr. Ronald Riley of the USDA (Athens, GA). Water soluble carbodiimide, i.e. 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDPC), was obtained from Aldrich Chemical Co. (Milwaukee, WI). Dithiothreitol (DTT) was obtained from United States Biochemicals (USB, Cleveland, OH). Goat anti-rabbit IgG–HRP, diaminodipropylamine agarose, immobilized papain gel (no. 20341), immobilized protein G (no. 20398, immunopure), and goat anti-mouse IgG+IgM–peroxidase conjugate (ELISA grade) were obtained from Pierce Chemical Co. (Rockford, IL). Activated CH-Sepharose 4B was obtained from Pharmacia 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). The murine myeloma cell line P3/NS-1/1-AG4-1 (NS-1) was obtained from American Type Culture Collection (Rockville, MD). *Pasteurella*-negative New Zealand white rabbits were obtained from LSR Industries (Union Grove, WI). All other chemicals and organic solvents used were of reagent grade or better.

Purification of mAb (Ab1) against Fumonisin B_1. Monoclonal antibody against FmB1 was first purified by ammonium sulfate precipitation (50% saturation for the final solution) and then subjected to an affinity column conjugated with FmB1. The column was prepared by conjugating 4 mg of FmB1 to 0.5 g of activated CH-Sepharose 4B in 0.1 M $NaHCO_3$ (pH 8.0) according to the manufacturer's instructions. After preparation, the column was equilibrated with 0.01 M sodium phosphate buffer, pH 7.5 (PB), containing 0.85% NaCl (PBS). For antibody purification, 1 mL of FmB1–mAb1 in 0.01 M PBS solution (prepared by dialyzing the ammonium-precipitated preparation against PBS) was added to the column and incubated at room temperature for 1 h. The column was then washed with 20 mL of 0.01 M PBS. Antibody was eluted from the column with 0.1 M glycine, pH 2.5. One-milliter fractions were collected and neutralized immediately by addition of an appropriate amount of 1.0 M Tris-HCl, pH 9.0, to each fraction. The specific antibody-containing fractions were pooled, dialyzed against 1 L of 0.01 M PB, pH 7.5 (two changes), overnight, and stored at −20 °C.

Production of Anti-idiotype AB (Ab2). Immunization schedule and methods of immunization were essentially the same as those described previously (Chu and Ueno, 1977). In the initial immunization, two rabbits were each injected intradermally with 0.45 mg of affinity-purified FmB1–mAb1 in 1.0 mL of PBS (0.01 M, pH 7.5) emulsified with 1.0 mL of complete Freund's adjuvant by a multiple injection method (20 sites/rabbit). Booster injections were made at the 6th and 23rd weeks with 0.3 mg of immunogen in 1.0 mL of PBS and 1.0 mL of incomplete Freund's adjuvant at two sites in both thighs intramuscularly.

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 h and then against 0.01 M PB, pH 7.5, overnight at 4 °C, and lyophilized.

2. Normal Mouse IgG Affinity Column Chromatography. A CH-Sepharose 4B column conjugated with normal mouse IgG was used to remove nonspecific antibodies. The column was prepared by conjugation of 4.8 mg of normal mouse IgG 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 as described previously (Hsu and Chu,1994). For Ab2 purification, 1 mL of $(NH_4)_2SO_4$ precipitation purified rabbit antiserum (Ab2, 7th–9th week bleedings prepared as described above), equivalent to 10.5 mg of protein as analyzed by the method of Smith et al. (1985), was applied to the normal mouse IgG column. The column was washed with 0.01 M PBS, pH 7.5, until the absorbance at 280 nm of the eluant reached zero. The unbound protein fractions, which contained Ab2 antibodies, were pooled, dialyzed against 0.01 M PBS, pH 7.5, overnight, and stored at −20 °C for further analyses.

Characterization of Anti-idiotype Antibody (Ab2). *1. Monitoring of FmB1–Ab2 Titers by Indirect ELISA.* An indirect ELISA was used to monitor the Ab2 antibody titers after rabbits were immunized with Ab1. Microtiter plate (Nunc plate 2-69620; Nunc, Roskilde, Denmark) wells were each coated with 100 μL of (27 μg/mL). After overnight incubation at 4 °C, the wells were washed four times (0.35 mL/well) with PBS–Tween buffer (0.01 M PBS, pH 7.5, with 0.5% Tween 20). This was followed by incubation with 0.17 mL of 0.1% gelatin (blocking agent) in 0.01 M PBS at 37 °C for 30 min. The plate was washed four times with 0.35 mL of PBS–Tween to remove the excess blocking agent. To each well, 0.1 mL of various dilutions of antiserum or purified Ab2 or control (preimmune serum) or irrelevant mAb was added, gently mixed, and incubated at 37 °C for 1 h. The plate was washed with 0.35 mL of PBS–Tween four times, and 0.1 mL of goat anti-rabbit IgG–HRP (1:7500 dilution in 0.01 M PBS) was added to each well. After incubation at 37 °C for 1 h, the plate was washed, and 0.1 mL of freshly prepared OPD substrate solution [10 mg of OPD plus 13 μL of 30% hydrogen peroxide in 25 mL of 0.05 M citrate–phosphate buffer (4.8 g of citric acid and 7.1 g of Na_2HPO_4 in 500 mL of distilled water with pH adjusted to 5.0)] was added. Ten minutes after incubation at room temperature in the dark, the reaction was terminated by adding 0.1 mL of 1 N HCl. The absorbance at 490 nm was determined in an automatic ELISA reader (THERMOmax microplate reader, Molecular Devices Co., Menlo Park, CA).

2. Characterization of Ab2 by Competitive Indirect ELISA.
a. Regular Indirect Competitive ELISA Using FmB1–OVA. A regular indirect competitive ELISA was conducted to characterize mAb1 as well as to serve as a control for comparison purposes. In this assay, FmB1–ovalbumin (OVA) conjugate (1 μg/mL) was coated to the wells of ELISA plate similar to those described above for monitoring antibody titers. Subsequent incubation and washing steps were the same as above except that a constant amount of mAb, i.e. FmB1–mAb1 (50 μL/well, 10 μg/mL), plus 50 μL of FmB1or FmB2 at different concentrations was added to the FmB1–OVA-coated microplate well. Thus, free FmB1or FmB2 competed with the solid-phase FmB1–OVA for the binding of FmB1–mAb1 in solution.

b. Anti-ID-Based Indirect Competitive ELISA. For characterization of Ab2, an Ab2-based competitive indirect ELISA was used. In this assay, 100 μL of purified Ab2 for FmB1 (1:10000 dilution) instead of FmB1–OVA was coated to each well of the ELISA plate. Subsequent incubation and washing steps were the same as the regular indirect competitive ELISA as described above. Thus, free FmB1 or FmB2 in solution competes with the solid-phase Ab2 for binding with the FmB1–mAb1.

Preparation of Fab Fragment of Ab2. For generating polyclonal anti-Ab2 antibodies (Ab3), Fab fragment of Ab2 was prepared by digesting the antibody with an immobilized papain gel according to the supplier's instruction (Pierce). In

a typical experiment, 0.5 mL of papain gel was equilibrated and washed with 4 mL of digestion buffer [(DB), 20 mM NaH_2PO_4 + 20 mM DTT (instead of 20 mM cysteine hydrochloride) + 10 mM EDTA-Na_4, pH 7.0] twice. After centrifugation (2000 rpm) for 2 min and decanting, the gel was resuspended in 0.5 mL of DB. The affinity-purified Ab2 (4.9 mg) obtained above, in 0.5 mL of DB, was then added to the gel, incubated at 37 °C in a shaker. Five hours after incubation, the reaction was terminated by addition of 1.5 mL of 0.01 M Tris-HCl, pH 7.5, and centrifuged. The supernatant solution was dialyzed against 2 L of 0.01 M PB overnight with two exchanges of buffer and then lyophilized.

Further purification of Fab of Ab2 was achieved by passing the digested solution through a protein G column (2 mL size) according to the manufacturer's instruction. Typically, the dialyzed and lyophilized preparation (3.7 mg) was redissolved in 1 mL of 0.1 M acetate buffer, pH 5.0, and applied to the prepacked column and incubated at room temperature for 1 h. The Fab fragment (ca 1.8 mg) was eluted from the column by washing the column with 15 mL of acetate buffer (0.1 M, pH 5.0) after the incubation.

Production and Purification of Mouse Polyclonal Anti-anti-idiotype AB (Ab3). For generating polyclonal Ab3, female BALB/c mice (6–8 weeks of age) were immunized with the Fab fragment of Ab2 according to the protocols described by Kurpisz et al. (1988). Briefly, 13 BALB/c mice were each injected (ip) with 25 μg of Fab of Ab2, in 0.15 mL of PBS plus 0.15 mL of complete Freund's adjuvant. Booster injections were made on days 14 and 28 with Ab2 (25 μg each) in PBS. The mice were primed with 0.3 mL of pristane on days 3 and 17. On the 31st day after the initial injection, 6×10^6 cells of a nonsecreting myeloma cell line NS-1 in 0.3 mL of 0.01 M DMEM were injected (ip) into each mouse. The ascites fluid was collected and pooled 2 weeks after the last booster injection (day 42). The ascites fluid was 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 a FmB1 column, the same way as for the purification of mAb (Ab1).

Characterization of Mouse Polyclonal Anti-anti-idiotype AB (Ab3). A competitive indirect ELISA similar to the FmB1–OVA based regular ELISA was used to characterize Ab3. Briefly, 100 μL of FmB1–OVA (1 μg/mL in PBS, pH 7.5) was coated to each well 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 30 min. The plates were washed again and incubated with purified Ab3 (50 μL/well, 18 μg/mL in PBS, 0.01 M, pH 7.5) instead of FmB1–mAb1 together with 50 μL of FmB1 or FmB2 at different concentrations (0.01–10 μg/mL, also in PBS, pH 7.5) at 37 °C for 1 h. After washing, 100 μL of diluted (1:7500) 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.

Analysis of FmB1 with Idiotype (Ab1) and Anti-idiotype (Ab2) Antibody-Based ELISA. To test the efficacy of Ab2-based indirect ELISA for FmB1 in corn, two sets of corn samples that have been previously analyzed with regular ELISA or by a HPLC method (Ross et al., 1991) in Dr. Frank Ross's laboratory were subjected to Ab2-based ELISA. All of the samples were subjected to a cleanup before ELISA. Briefly, ground samples (4 g each) were shaken with 40 mL of CH_3CN/H_2O (1/1 v/v) in a cool room (6 °C) overnight. The suspension was allowed to settle, and 3 mL of the solution was aspirated into a 15 mL test tube to which 9 mL of distilled water , pH 4.0, was added. The mixture was then added to a C_{18} reversed-phase Sep-Pak cartridge that had been washed with 2 mL of CH_3CN and 4 mL of distilled water in sequence. The cartridge was then washed with 4 mL of acidified distilled water, pH 4.0, followed by 4 mL of CH_3CN/H_2O (15/85 v/v). Fms were eluted from the column with 4 mL of CH_3CN/H_2O (7/3 v/v) and then diluted with PBS for ELISA (generally more than 100 times dilutions for samples contaminated with high levels of FmB1). For low levels of Fms (less than 1 ppm), the acetonitrile extracts were evaporated in a rotary evaporator to dryness and redissolved in 1 mL of PBS before ELISA.

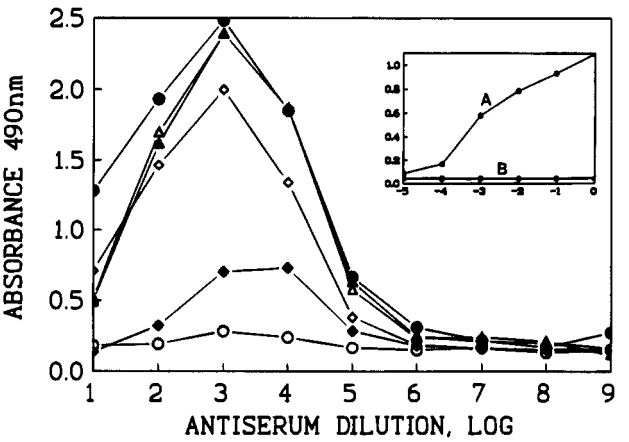

Figure 1. Production of fumonisin anti-ID antibodies in rabbits afte the animal was immunized with purified FmB1–mAb1. For the determination of antibody titers, microtiter plate wells were coated with FmB1–mAb1 (27 μg/mL, 100 μL/well) and various dilutions of rabbit antiserum at 0 (○), 5 (■), 7 (□), 8 (▲), 9 (△), and 10 (●) week bleedings were tested. The binding of rabbit anti-ID (Ab2) to the solid-phase FmB1–mAb1 was determined with goat-anti-rabbit IgG–peroxidase conjugate. The inset shows the binding of FmB1–mAb1 (curve A) and controls (curve B, including normal mouse IgG and mAbs against cyclopiazonic acid and aflatoxin) with purified Ab2 (9 week bleeding) coated on the microtiter wells (0.4 μg/mL, 100 μL/well). The numbers on the *x* axis on the inset represent the $\log_{10}$ of the concentrations of various mAbs used in the test (μg/mL). The binding of different mouse mAbs to the solid-phase rabbit anti-ID (Ab2) or nonrelated IgG was determined with goat-anti-mouse IgG+IgM–peroxidase conjugate.

RESULTS

Production of Anti-ID Antibodies (Ab2). Polyclonal Ab2 for FmB1 were generated in rabbits after immunization with FmB1–mAb1. Results of typical ELISA titration curves of the antibody titers of a rabbit over a 10-week period after immunization are shown in Figure 1. The response of a second rabbit was similar. The rabbit exhibited anti-mAb1 titer 5 weeks after immunization. The antibody titers peaked at the 10th week after initial immunization with one booster injection (6th week). Purified serum from the 9th week bleeding was selected for further characterization. Data in the inset of Figure 1 show a typical dose-related specific binding FmB1–mAb1 (curve A) with the solid-phase anti-ID (Ab2), while no binding occurred between Ab2 with normal mouse IgG and irrelevent mAbs such as mAb for cyclopiazonic acid and aflatoxin (curve B).

Characterization of Ab2 Antibodies. Several approaches were used to characterize Ab2 antibodies. In an initial study, the effectiveness of Ab2 in competing with the solid-phase FmB1–OVA for the binding of FmB1–mAb1 in solution was examined, and the results are shown in Figure 2. It is clear that while competition for binding did occur, a large amount of Ab2 was required. The concentrations causing 50% inhibition of binding of the mAb1 to the solid-phase FmB1–OVA by free FmB1 and Ab2 were found to be 0.135 μg/mL (1.9×10^{-7} mmol/mL) and 176 μg/mL (1.1×10^{-6} mmol/mL), respectively.

The second analysis involved a typical competitive indirect ELISA in which either Ab2 or FmB1–OVA was coated to the wells of the ELISA plate to serve as a solid-phase antigen. Results as shown in Figure 3 indicate that free FmB1 and FmB2 effectively compete with the

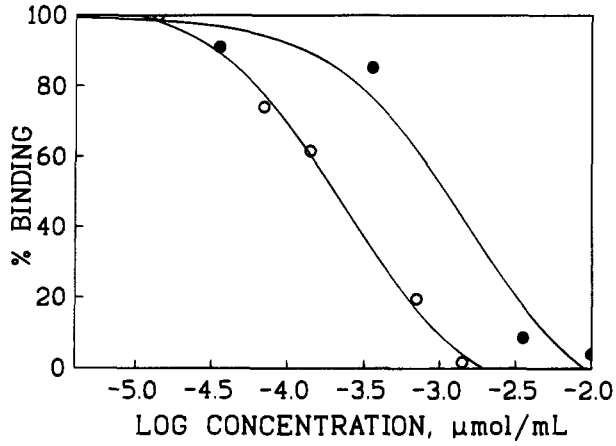

Figure 2. Effect of anti-ID (●) and free FmB1(○) on the binding of mAb1 to solid-phase FmB1–OVA. In this assay, microtiter plate wells were coated with 100 μL of FmB1–OVA (1 μg/mL) and a constant amount of FmB1–mAb1 (10 μg/mL, 50 μL/well) plus various concentrations of Ab2 (●, 5.7 μg to 1.14 mg/mL) or free FmB1 (○, 0.01–10 μg/mL) were present. On a molar basis, the concentrations causing 50% inhibition by free FmB1 and Ab2 were 1.9×10^{-7} and 1.1×10^{-6} mmol (0.135 and 176 μg/mL), respectively.

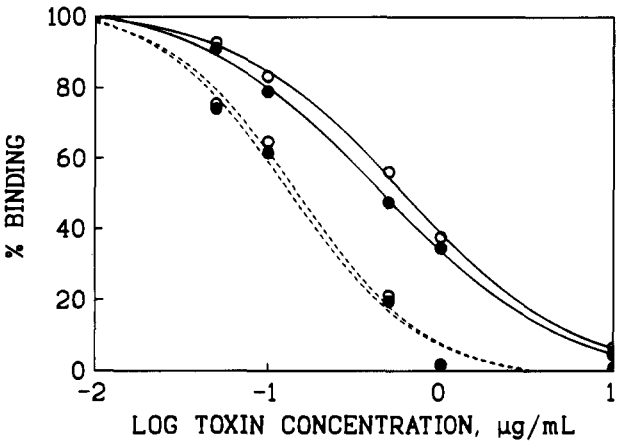

Figure 3. Effect of FmB1 (●) and FmB2 (○) on the binding of FmB1–mAb1 (10 μg/well) with the solid-phase antigens Ab2 (solid lines) and FmB1–OVA (dashed lines). The microtiter plate wells were coated with 100 μL of either Ab2 (0.57 μg/mL) or FmB1–OVA (1 μg/mL). The binding of mAb to the solid-phase antigens was determined by goat anti-mouse-HRP conjugate (1:7500 dilution for FmB1–OVA coated plate or 1:10000 dilution for the Ab2 coated plate; all at 100 μL/well).

solid-phase antigen in both systems for their binding with the monoclonal antibody (mAb1) against FmB1. In the FmB1–OVA-based-ELISA, the concentrations causing 50% inhibition of binding of mAb1 to the solid-phase FmB1–OVA by free FmB1 and FmB2 were found to be 0.14 and 0.15 μg/mL, respectively. In contrast, the concentrations causing 50% inhibition of binding of mAb1 to the solid-phase Ab2 by free FmB1 and FmB2 in the Ab2-based ELISA were found to be 0.46 and 0.61 μg/mL (23 and 30.5 ng/assay), respectively. The Ab2-based ELISA was only slightly less sensitive than the FmB1–OVA-based ELISA.

Analysis of FmB1 in Moldy Corn by Ab2-Based and FmB1–OVA-Based Indirect ELISA. To test the efficacy of Ab2-based indirect ELISA for FmB1, 29 corn samples obtained from China in a previous study (Chu and Li, 1994) were subjected to both Ab2-based and FmB1–OVA-based indirect ELISAs. Results of these analyses are shown in Figure 4. A good correlation was found between the data obtained from Ab2-based and

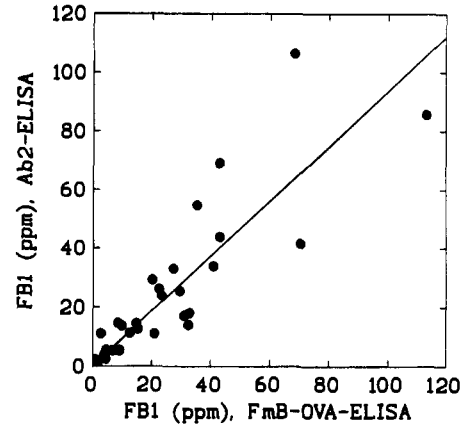

Figure 4. Correlation of FmB1 in 29 corn samples as determined by Ab2-based and FmB1–OVA-based indirect competitive ELISA.

Table 1. Ab2-Based Indirect ELISA of FmB1 in Corn[a]

sample no.	Ab2-based ELISA[b]	LC[c]
1	0.9	0.7
2	3.7	1.9
3	1.4	0.9
4	0.7	0.9
5	2.0	0.9
6	3.1	0.7
7	1.3	1.2
8	0.9	ND[d]
9	ND	0.6
10	1.5	1.4
11	3.5	1.7
12	1.6	1.0
13	2.6	2.6
14	1.2	0.5
15	1.3	1.0
16	1.2	0.8
17	1.2	0.5
18	2.5	2.8
19	1.3	2.3
20	ND	ND
21	2.0	5.0
av	1.83 ± 0.89	1.53 ± 1.26

[a] Concentration of FmB1 in corn, μg/g. [b] ELISA plates coated with anti-idiotype antibody (Ab2). [c] LC data supplied by Frank Ross and Larry Rice of the National Veterinary Service Laboratories of USDA, Ames, IA. [d] ND, not detected.

FmB1–OVA-based ELISA. The correlation coefficient between these two methods was found to be 0.86 with a p value of less than 0.0001 (linear regression with data force to zero, Figure 4).

In addition to the above analyses, 21 samples, which were supplied by Dr. C. S. Yang of Rutgers University in a recent collection from China (1993 fall), were subjected to the Ab2-based ELISA. Those samples were also analyzed for FmB1 with HPLC (Ross et al., 1991) at the National Veterinary Service Laboratories of the USDA, Ames, IA. Results of these analyses are shown in Table 1. Although the overall means for the data obtained were very similar for this set of samples, linear regression analysis revealed that correlation was poor. The correlation coefficient was found to be 0.31 with y (HPLC data) intercepting at 0.72 ppm and x (ELISA data) intercepting at −1.65 ppm.

Production and Characterization of Polyclonal Anti-anti-ID (Ab3) Antibodies in Mouse. Anti-anti-idiotype antibodies (Ab3) were produced in the ascites of BALB/c mice immunized with affinity-purified Fab fragment of rabbit Ab2. The Ab3 were further purified by ammonium sulfate precipitation and FmB1 affinity

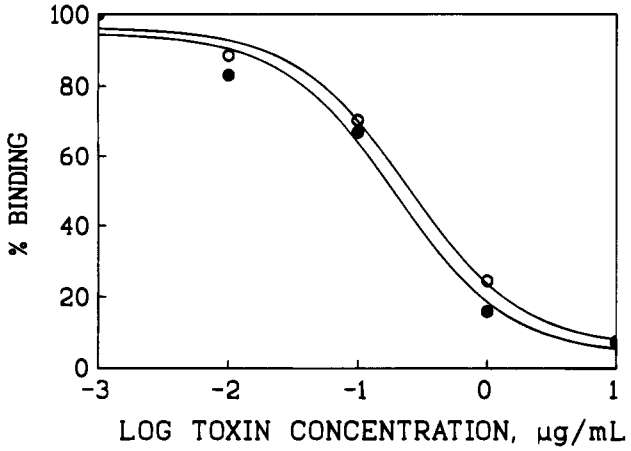

Figure 5. Anti-anti-ID (Ab3)-based ELISA for the analysis of FmB1 (●) and FmB2 (○). The microtiter plate wells were each coated with 100 μL of FmB1–OVA (1 μg/mL). The binding of Ab3 (18 μg/mL, 50 μL/well) to the solid-phase FmB1–OVA in the presence various concentrations of FmB1 and FmB2 (50 μL/well) was determined by goat anti-mouse–HRP conjugate (1:7500 dilution, 100 μL/well).

purification steps and then analyzed by competitive ELISA. ELISA data showed (Figure 5) that Ab3 have characteristics similar to those of the original FmB1–mAb1 with high specificity for FmB1. The ID_{50} values of binding of Ab3 to the solid-phase FmB1–OVA by free FmB1 and FmB2 were found to be 0.19 and 0.26 μg/mL, respectively. Attempts to use Ab2-coated ELISA plate and Ab3 as the antibody for ELISA of either fumonisin were unsuccessful.

DISCUSSION

Immunoassays have been used widely for mycotoxin analysis (Chu, 1986, 1990, 1992; Pestka, 1989; Morgan and Lee, 1990), including fumonisin B_1 (Azcona-Olivera et al., 1992a,b; Fukuda et al., 1994; Usleber et al., 1994), in recent years. Thus, there is a demand for immunochemical reagents for such assays. Anti-idiotype antibodies provide an alternative approach to obtaining these reagents. In the present study, we have demonstrated that anti-idiotype antibodies for FmB1 were elicited in rabbits by immunization with affinity-purified mAb1 against FmB1. Indirect ELISA revealed that the polyclonal anti-idiotype antibodies bound specifically to mAb1 against FmB1 but not to IgG from mAb with irrelevant specificity or to normal mouse immunoglobulins. Data obtained from ELISA revealed that the Ab2 effectively inhibited the binding of FmB1 to idiotype antibodies, and the concentrations causing 50% inhibition of the binding of FmB1–mAb1 by FmB1 and anti-ID (Ab2) were similar in order when expressed on a molar basis (1.9×10^{-7} vs 1.1×10^{-6} mmol/mL). When the purified Ab2 was coated on the wells of the ELISA plate as the solid-phase antigen, both FmB1 and FmB2 were effective in competing with the binding of FmB1–mAb1. These data suggest that the generated, purified Ab2 has an internal image of FmB1. This conclusion was further sustained by the fact that anti-anti-idiotype antibodies (Ab3) with high specificity for FmB1 were generated in mice after immunization of the animals with purified Ab2.

Results from the Ab2-based indirect ELISA showed that this system was slightly less sensitive than the FmB1–OVA-based ELISA (Figure 3). These observations are similar to data obtained from the anti-idiotype antibodies of some other hapten-type toxins. In the anti-ID system for T-2 toxin, Chanh et al. (1990) found that free T-2 toxin at a concentration as high as 200 μg/mL was unable to inhibit the binding of anti-ID antibody to the solid-phase mAb against T-2 toxin. Similar observations were obtained in the anti-ID system for aflatoxin in our laboratory (Hsu and Chu, 1994), although both types of Ab2 were able to generate anti-anti-ID bodies against these two mycotoxins. Nevertheless, Ab2 for FmB1 has been found to be useful for the analysis of FmB1 in the corn sample. As low as 0.9 ppm of FmB1 was detected by the Ab2-based ELISA in some of the corn samples naturally contaminated with FmB1. Data obtained from the Ab2-based ELISA were consistent with those obtained from the FmB1–OVA-based ELISA. Although the correlations between the Ab2-based ELISA and HPLC data were poor, data for the overall average of these two methods (Table) were similar. Recognizing that the analyses were conducted independently in two separate laboratories where different reagents, toxin standards, and completely different methods were used, we could attribute the poor correlation to the inherent problems existing for both methods (Pestka et al., 1994) because the toxin levels in several samples were near the detection limits of both methods.

In addition to using Ab2 as testing antigen in the ELISA, we found that purified Fab of Ab2 was also a good immunogen in generating the Ab3. The Ab3-based ELISA has sensitivity similar to that of the mAb1-based immunoassay. Such results suggest that the Ab3-based ELISA system could also be used for FmB1 analysis. However, because only limited amounts of Ab3 are produced in the ascites, this may limit the wide application of the Ab3-based ELISA. 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 FmB1 have been generated in rabbits and mice, respectively. The purified Ab2 that has an epitope with an internal image of FmB1 has been shown to be an effective immunogen for generating Ab3 with similar characteristics as the idiotype antibody, i.e. mAb1, against FmB1. Both Ab2 and Ab3 could be used separately in the ELISA for FmB1. In addition, Ab2 has the potential to be used as a vaccine (Chanh et al., 1992; Nisonoff, 1991). Thus, a new generation of immunochemical reagents is now available for various purposes in FmB1 analysis and other toxicological work. The major advantages of using the anti-ID antibody approach for mycotoxin analysis and diagnosis of mycotoxicoses are twofold: (a) it is not necessary to use a toxin–protein/enzyme conjugate for antibody production and immunoassays, thus alleviating the toxicity, toxin metabolism, and stability problem encountered for such conjugates; (b) it is simpler and more versatile to produce these antibodies and to prepare Ab2 markers for immunoassay. Improvement of the efficacy of the immunoassay could also be made through modification of Ab2 structures and by selecting new monoclonal antibodies. From the toxicological point of view, Ab2 could serve as a competitor for the toxin binding site in vivo, thus alleviating/modulating the toxic effect in addition to its effect in serving as an effective and safe vaccine in animals. The present study is only the first step that could lead to these advantages, and further work for generating monoclonal Ab2 and Ab3 and to improve the Ab2- and Ab2-based immunoassys is warranted.

ACKNOWLEDGMENT

This work was supported by Grant NC-129 from the College of Agricultural and Life Sciences, University of Wisconsin—Madison, Public Health Service Grant CA-15064, from the National Cancer Institute, and funds from food industries. We thank C. S. Yang of Rutgers University for providing corn samples that he collected from the People's Republic of China, Frank Ross and Larry Rice of the National Veterinary Services Laboratories of the USDA (Ames, IA) for HPLC analysis of fumonisin of these samples, and Ms. Carole Ayres for help in preparing the manuscript.

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Received for review May 31, 1994. Accepted October 17, 1994.[⊗]

JF940281S

[⊗] Abstract published in *Advance ACS Abstracts,* November 15, 1994.