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Production of Monoclonal Antibodies to the Mycotoxins Fumonisins B_1, B_2, and B_3

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Monoclonal antibodies were prepared against the fumonisins, a group of mycotoxins produced by *Fusarium moniliforme*. Splenic lymphocytes from mice immunized with a fumonisin B_1–cholera toxin conjugate were fused with NS-1 myeloma cells, and six hybridomas were selected. A direct competitive ELISA was devised whereby fumonisin B_1–peroxidase and free fumonisin B_1 competed for antibody binding. The detection limit for fumonisin B_1 in this assay was 50 ng/mL. Antibodies also cross-reacted with fumonisins B_2 and B_3. Mean concentrations of fumonisins B_1, B_2, and B_3 required to inhibit 50% antibody binding for the six clones were 630, 1800, and 2300 ng/mL, respectively. When the antibodies were applied to the direct ELISA in spiked (5–25 μg/g) feed, the average recovery was 103%, with mean intra- and interassay coefficients of variation of 11 and 15%, respectively. These antibodies should find wide usage in the ELISA screening of fumonisins in foods, feeds, and tissues.

INTRODUCTION

Fusarium moniliforme is a worldwide common fungus in corn that has been epidemiologically associated with equine leukoencephalomalacia (ELEM). Recently a new group of mycotoxins known as "fumonisins" have been isolated from cultures of *F. moniliforme* associated with ELEM (Gelderblom et al., 1988). The most abundant of these, fumonisin B_1 (FB_1), experimentally induces ELEM (Kellerman et al., 1990; Marasas et al., 1988; Wilson et al., 1990) and porcine pulmonary edema syndrome (Harrison et al., 1990), causes hepatic cancer in rats, and exhibits cancer-promoting activity (Gelderblom et al., 1988). The wide range of toxicological effects exerted by this mycotoxin along with the well-documented natural occurrence of fumonisins (Ross et al., 1991a,b; Sydenham et al., 1990a,b; Thiel et al., 1991; Voss et al., 1989; Wilson et al., 1990) suggests a need for screening and analytical methods to routinely monitor for the presence of fumonisins in the human and animal food supply.

Current methodology for fumonisins includes thin-layer chromatography (TLC) (Gelderblom et al., 1988; Plattner et al., 1990; Ross et al., 1991a), gas chromatography (GC) (Jackson and Bennett, 1990; Sydenham et al., 1990a), high-performance liquid chromatography (HPLC) (Alberts et al., 1990; Gelderblom et al., 1988; Ross et al., 1991a; Shephard et al., 1990; Wilson et al., 1990), liquid secondary ion mass spectrometry (LSIMS) (Bezuidenhout et al., 1988; Plattner et al., 1990; Voss et al., 1990), and gas chromatography–mass spectrometry (GC–MS) (Jackson and Bennett, 1990; Plattner et al., 1990; Voss et al., 1990; Wilson et al., 1990). Regardless of the detection limits, these methods require extraction, cleanup, and/or derivatization.

As an alternative to the above methods, immunoassays have already been proven to be useful in screening for other mycotoxins (Pestka, 1988). We have recently described the generation of mouse sera and ascites polyclonal antibodies against fumonisins following a novel immunization procedure that involves the use of a fumonisin B_1–cholera toxin (FB_1–CT) conjugate as immunogen (Azcona-Olivera et al., 1992). Here we report the development of stabilized hybridomas derived from mice immunized with FB_1–CT, as well as the production and characterization of fumonisin monoclonal antibodies. A competitive direct ELISA using these antibodies and its application are also described.

MATERIALS AND METHODS

Materials. All inorganic chemicals and organic solvents were of reagent grade or better. Fumonisins B_1, B_2, and B_3 (FB_1, FB_2, FB_3) (Figure 1) and the hydrolyzed backbone of fumonisin B_1 (HB-FB_1) were prepared from cultures of *F. moniliforme* grown on corn as reported (Plattner et al., 1990). Tricarballylic acid was obtained from Aldrich Chemical Co. (Milwaukee, WI). Ovalbumin (OA) (grade III; fraction VII), cholera toxin (CT), Tween 20, 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS), hydrogen peroxide, horseradish peroxidase (HRP) (fraction VI), sodium borohydride, glutaraldehyde, penicillin/streptomycin solution (pen/strep) (100 000 units/mL), sodium pyruvate, poly(ethylene glycol) (MW 1450) (PEG), hypoxanthine, aminopterin, thymidine, pristane, and dimethyl sulfoxide were obtained from Sigma Chemical Co. (St. Louis, MO). Goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase was obtained from Cappel Laboratories (West Chester, PA). Dulbecco's modified Eagle's medium (DMEM), NCTC supplemental medium, and fetal bovine serum (FBS) were from Gibco Laboratories (Grand Island, NY). Normal mouse serum (NMS) was purchased from Biocell Laboratories (Carson, CA). Tissue culture plasticware was purchased from Corning Laboratory Science Co. (Corning, NY), and microtiter plates (Immunolon 2 Removawells) were from Dynatech Laboratories (Alexandria, VA). The immunoglobulin subclass identification kit was purchased from Boehringer Mannheim Biochemical (Indianapolis, IN). The myeloma cell line P3/NS 1/1-Ag4-1 (NS-1) (ATCC TIB 18) was obtained from the American Type Culture Collection (Rockville, MD). Mice (BALB/c) were purchased from Charles River Laboratories (Wilmington, MA). Macrophage conditioned media (MCM) was prepared as described by Sugasawara et al. (1985).

Fumonisin B_1–Protein Conjugates. FB_1 was conjugated using glutaraldehyde to cholera toxin (FB_1–CT) for use as immunogen and to ovalbumin (fraction VII) (FB_1–OA) for use as a solid-phase antigen for the indirect ELISAs (Avrameas and

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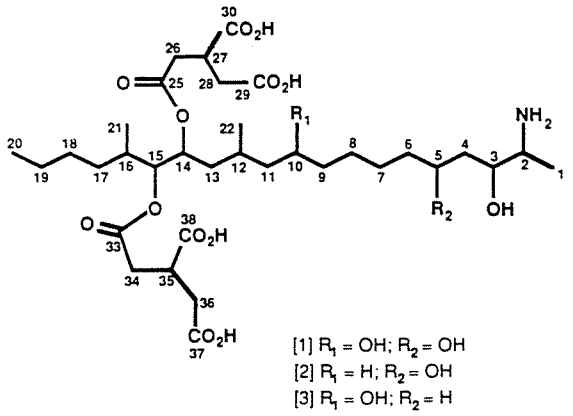

Figure 1. Structure of fumonisins: (1) fumonisin B_1; (2) fumonisin B_2; (3) fumonisin B_3.

Ternynck, 1969; Briand et al., 1985). Briefly, the coupling reaction was carried out at 10 °C in 0.01 M phosphate-buffered (pH 7.2) saline (PBS). FB_1 was added to a 1 mg/mL suspension of protein at molar ratios of toxin/protein of 50:1 for FB_1–CT and 20:1 for FB_1–OA, and an equal volume of glutaraldehyde (2% v/v) was then added dropwise with constant stirring. After 1 h, the reaction was stopped by the addition of sodium borohydride to a final concentration of 10 mg/mL. One hour later, the mixture was dialyzed for 72 h against PBS. OA conjugate was aliquoted in fractions of 1 mg (total protein), lyophilized, and stored at −20 °C. CT conjugate was diluted (five times in PBS, filter sterilized, dispensed aseptically in 1-mL fractions, and stored at 4 °C.

FB_1 was conjugated to horseradish peroxidase (FB_1–HRP) (1/5, w/w) for use in the competitive direct ELISA by the periodate method (Nakane and Kawaoi, 1974).

Immunization. Female BALB/c mice (6–8 weeks of age) were immunized with three doses of 7.5 μg of FB_1–CT (in 0.2 mL of PBS) given intravenously (iv) in the lateral tail vein at days 0, 10, and 16. At day 21 mice were bled, and serum titer and antibody specificity were determined by indirect ELISA. A 4-μg iv dose of immunogen was given 4 days prior to the fusion (3 weeks after the last dose).

Indirect ELISA. Wells of polystyrene microtiter plates were coated overnight (4 °C) with 100 μL of FB_1–OA (5 μg/mL) in 0.1 M sodium carbonate–bicarbonate buffer (pH 9.6). Plates were washed four times by filling each well with 300 μL of 0.02% (v/v) Tween 20 in PBS (PBS–Tween) and aspirating the contents. Nonspecific binding was decreased by incubating each well for 30 min at 37 °C with 300 μL of 1% ovalbumin (grade III) (w/v) in PBS (OA–PBS) followed by four washes of PBS–Tween. Next, 50 μL of serially diluted murine serum was added to each well and incubated for 1 h at 37 °C. Wells of serially diluted preimmune serum were used as control. Unbound antibody was removed by washing four times with PBS–Tween, and 100 μL of goat anti-mouse IgG peroxidase conjugate (2 μg/mL, in OA–PBS) was added to each well. Plates were incubated for 30 min at 37 °C and washed eight times with PBS–Tween, and bound peroxidase was determined with ABTS substrate as described previously (Pestka et al., 1982). Absorbance was read at 405 nm, and end-point titer of each serum was arbitrarily designed as the maximum dilution that yielded twice or greater the absorbance as the same dilution nonimmune control serum.

A competitive indirect ELISA (CI-ELISA) was used to verify specificity of antibodies in mouse sera toward FB_1 during the course of immunization and to identify culture wells containing hybridomas secreting the desired antibody, following fusion and cloning. Briefly, microtiter plates were coated and blocked as described in the indirect titration procedure. Next, 50 μL of standard FB_1 (or analogues and metabolites) dissolved in PBS was simultaneously incubated with 50 μL of antiserum (diluted 1:100 in PBS) over the FB_1–OA solid phase for 1 h at 37 °C. Bound antibody was determined by addition of anti-mouse IgG peroxidase conjugate as described above. To identify cultures containing anti-FB_1 antibody in fusion and cloning wells, 50-μL aliquots of PBS were added as FB_1-free blanks to each of two FB_1–OA coated wells, and 50-μL aliquots of FB_1 (5 μg/mL in PBS) were added to two other FB_1–OA coated wells. To each of these four wells were added 50-μL aliquots of a single culture supernatant (or appropriate dilutions). The assay was then completed as described above.

Hybridoma Production. Spleen cells (1×10^8) from a hyperimmunized mouse were fused with NS-1 myeloma cells (1×10^7) using PEG (Galfre and Milstein, 1981). Fused cells were resuspended in complete medium [DMEM (supplemented with 1% NCTC, 10 mM sodium pyruvate, and 100 units/mL of pen/strep solution) containing 10% FBS, 5% NMS, and 10% MCM] and then distributed among 270 microculture wells and incubated for 24 h at 37 °C in a humid atmosphere of 7% CO_2 in air. Next, half of the supernatant from each well was removed and replaced with an equal volume of HAT medium (complete medium with hypoxanthine, aminopterin, and thymidine). This operation was repeated every 3 days for a period of 2 weeks, after which time HAT medium was eliminated by gradual replacement with HT medium (the same composition of HAT but without aminopterin). Supernatants of hybridoma cultures were tested for the presence of specific antibody by CI-ELISA. Cultures were successively scaled up and cloned by limiting dilution at 0.5–1 cell/well (Goding, 1980). Subclones yielding optimal antibody activity were then isotyped and stored in FBS–dimethyl sulfoxide (9:1) under liquid nitrogen. Mass production of fumonisin monoclonal antibodies was done by expansion of the selected subclones. Antibodies were purified and concentrated from cell-free culture supernatants by precipitation with 50% saturated ammonium sulfate (Hebert et al., 1973).

Competitive Direct ELISA (CD-ELISA). A CD-ELISA was developed and used to determine the sensitivity and specificity of the monoclonal antibodies secreted by the stabilized cell lines and to determine the recoveries in spiked samples. Plates were coated overnight by air-drying at 40 °C, with 125 μL of fumonisin monoclonal antibodies (50–150 μg/mL) in 0.1 M sodium carbonate–bicarbonate buffer (pH 9.6). After washing and blocking, 50 μL of mycotoxin standard (or sample) and 50 μL of FB_1–HRP (2 μg/mL, in OA–PBS) were added consecutively to each well. After 1 h of incubation at 37 °C, plates were washed and bound peroxidase was determined as described before.

A homogeneously ground corn-based feed sample containing approximately 1 ppm of FB_1 as determined by GC–MS and HPLC was used for the spiking studies. FB_1 dissolved in water (100 μg/mL) was added to 5 g of sample to give final concentrations of 5, 10, and 25 μg/g. Samples were extracted by shaking with 5 volumes (w/v) of 50% acetonitrile for 45 min. After centrifugation, 2 mL of supernatant was diluted with 6 mL of water and assayed by CD-ELISA.

RESULTS AND DISCUSSION

Mouse Immunization and Hybridoma Production. Using a conjugate of fumonisin B_1–cholera toxin as immunogen, all treated animals (n = 10) exhibited specific FB_1 antibodies with serum titer values ranging from 3200 to 6400 5 days after the third iv immunization. The mean percent inhibitions (and ranges) of mouse antibody binding to the FB_1–OA solid phase in the presence of 0.1, 1, and 10 μg/mL free FB_1 were 13 (2–24), 45 (34–71), and 83% (71–88), respectively. Spleen cells (1×10^8) from a mouse producing antiserum with a high relative affinity for FB_1 were fused with NS-1 myeloma cells (1×10^7). The fusion efficiency (number of wells with growing colonies/number of wells seeded) was greater than 93% (253/270) 7 days after fusion. From 188 supernatants screened, 2 produced anti-FB_1 antibodies as determined by CI-ELISA. Upon further cloning and subcloning, six subclones (1D3, 1E11, 3G5, 3G7, 5D2, and 5G5) were selected for further characterization.

Characterization of Monoclonal Antibodies. The isotype of all six subclone immunoglobulins was IgG_1 with κ light chain. Specificity of the antibodies secreted by different clones was determined by direct ELISA (Figure 2; Table I). The concentration of mycotoxin required for a 50% inhibition of antibody binding for the different

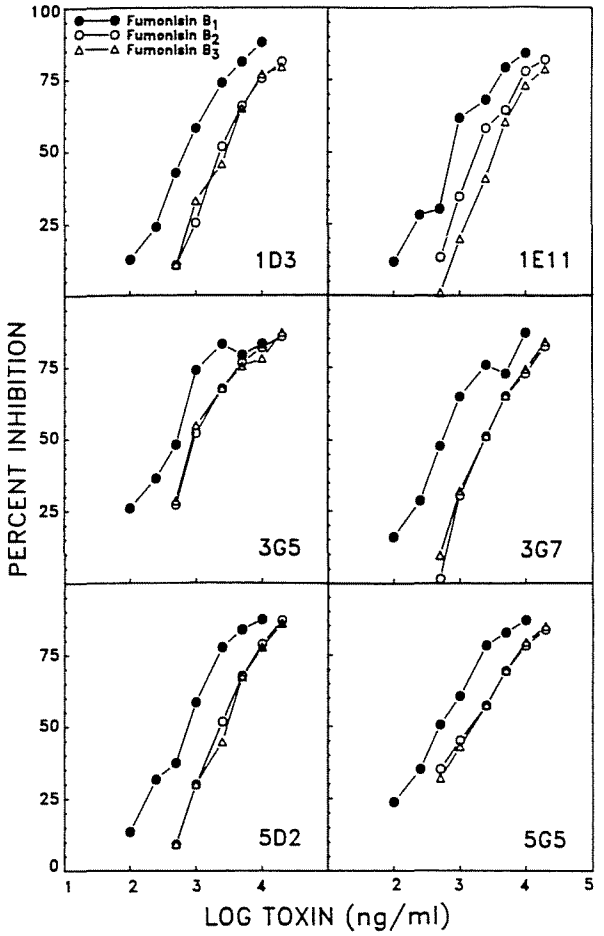

Figure 2. Specificity of fumonisin monoclonal antibodies produced by different clones as determined by competitive direct ELISA. Each data point represents the average value of triplicate determinations in a single microtiter plate.

Table I. Reactivity of Fumonisin Monoclonal Antibodies from Different Clones As Determined by Competitive Direct ELISA

	amount required for 50% inhibition[a] ng/mL		
clone	fumonisin B_1	fumonisin B_2	fumonisin B_3
1D3	680	2400 (28)[b]	2850 (24)
1E11	790	1950 (41)	3500 (23)
3G5	520	950 (55)	880 (59)
3G7	560	1900 (29)	2500 (22)
5D2	760	2300 (33)	2950 (26)
5G5	480	1090 (44)	1120 (43)

[a] Data from Figure 2. [b] Cross-reactivity defined as (ng/mL of fumonisin B_1 required for 50% inhibition)/(ng/mL of analogue required for 50% inhibition) × 100.

clones ranged from 480 to 790 ng/mL (mean, 630) for FB_1, from 950 to 2400 ng/mL (mean, 1800) for FB_2, and from 880 to 3500 ng/mL (mean, 2300) for FB_3. Mean cross-reactivities for FB_2 and FB_3 relative to FB_1 were 38 and 33%, respectively. Reactivity was not observed with the hydrolyzed backbone of FB_1 (HB-FB_1) (C_1–C_{20}) or the tricarballylic acid (present at positions C_{14} and C_{15}) (Figure 1) when concentrations up to 100 μg/mL were tested. This cross-reactivity was similar to that described using mouse polyclonal antibodies (Azcona-Olivera et al., 1992), although the values were higher for FB_2 (87%) and FB_3 (40%). Reactivity toward FB_2 and FB_3 but not toward HB-FB_1 and tricarballylic acid suggests that the immunodominant region of the immunogen existed near the union of the tricarballylic acids to the C_{11}–C_{20} positions

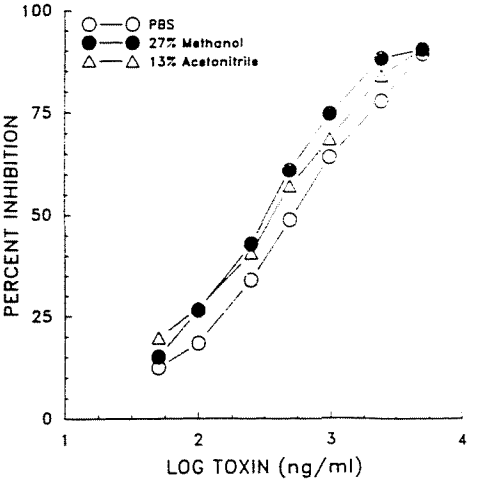

Figure 3. Competitive direct ELISA standard curve of fumonisin B_1 in PBS, methanol, and acetonitrile. Each data point represents the average value of triplicate determinations in a single microtiter plate. Absorbance values at 100% for PBS, methanol, and acetonitrile were 1.2, 1.3, and 1.3, respectively.

Table II. Recovery of Fumonisin B_1 from Spiked Feed by Competitive Direct ELISA

fumonisin B_1 added, μg/g	sample[a]	recovery[b,c] μg/g	recovery[b,c] %	CV,[d,e] %
5	A	6.0 ± 1.6	120	27.2
5	B	5.9 ± 0.4	117	7.0
5	C	8.4 ± 0.6	168	7.1
10	A	10.0 ± 2.8	100	28.3
10	B	8.6 ± 0.6	86	6.6
10	C	11.4 ± 1.0	114	8.8
25	A	20.9 ± 0.2	83	0.9
25	B	14.5 ± 2.3	58	16.2
25	C	20.6 ± 0.2	82	1.0

[a] Triplicates of each sample spiked separately were assayed after extraction on separate days (A–C). [b] Mean recoveries for samples containing 5, 10, and 25 μg/g of fumonisin B_1. [c] Interassay coefficients of variation (n = 3) for 5, 10, and 25 μg/g were 17.1, 11.4, and 15.8%, respectively. Mean interassay CV was 14.8%. [d] Intraassay coefficient of variation. [e] Mean intraassay CV was 11.5%.

of the backbone which are commonly present in the FB_1, FB_2, and FB_3 molecules.

Competitive ELISA standard curves for FB_1 in different solvents obtained after simultaneous incubation of FB_1 and FB_1–HRP over a monoclonal antibody (pool of different clones) solid phase are shown on Figure 3. Response range for the curves was from 50 to 5000 ng/mL. Competition curves prepared in 27% methanol and 13% acetonitrile approximated those in PBS, suggesting that samples can be screened for fumonisins in ELISA by direct comparison to standard in extractant. When competition by extracts of spiked samples was compared directly to standard FB_1 competition curves in 13% acetonitrile (Table II), recoveries for spiked samples containing 5, 10, and 25 μg/g were 135, 100, and 74%, respectively. The mean recovery was 103%. The mean intra- and interassay coefficients of variation were 11.5 and 14.8%, respectively. Besides the ELISA, possible sources of variability are sample spiking and assay extraction efficiency. These recovery values as well as the intra- and interassay coefficients of variation reported here are similar to others previously described for mycotoxins (Azcona et al., 1990; Dixon et al., 1987; Warner et al., 1986).

Summary. Although minimum safe levels for fumonisins have not been established, Ross et al. (1991b) evaluated FB_1 concentrations in feeds from 45 confirmed cases of ELEM and reported that ≥10 μg/g (10 ppm) of

FB_1 can induce this disease. Thus, the monoclonal antibody based ELISA described here should be useful in the rapid analysis of suspected samples since its detection limit for FB_1 is 50 ng/mL (50 ppb). Supporting this are the results of the spiking studies where the recoveries obtained in the range 5–25 μg/g should be sufficient for screening of samples exceeding 5 ppm of FB_1. Reactivity with FB_1, FB_2, and FB_3 is particularly desirable since it enables simultaneous detection of all three mycotoxins. Other possible applications of these antibodies are in the detection of fumonisins and its metabolites in tissues and biological samples in exposed animals, in immunoaffinity chromatography for rapid sample concentration, and as a cleanup step prior to analysis by GC–MS and HPLC.

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