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Production and Characterization of Antibody Against Aflatoxin Q_1

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Antibodies against aflatoxin Q_1 (AFQ_1) were obtained from rabbits after immunization of either AFQ_1-hemisuccinate or AFQ_{2a} conjugated to bovine serum albumin. Both radioimmunoassay and enzyme-linked immunosorbent assay (ELISA) were used for the determination of antibody titers and specificities. Antibodies obtained from rabbits after immunization with AFQ_1-hemisuccinate-bovine serum albumin had the highest affinity to aflatoxin B_1, whereas antibodies obtained from rabbits after immunization with AFQ_{2a}-bovine serum albumin bound most effectively with AFQ_{2a}. AFQ_{2a} antibody was selected for the subsequent direct and indirect ELISA for the detection of AFQ_1 in biological fluids. When AFQ_{2a}-peroxidase and AFQ_{2a} antibody were used, direct ELISA was able to detect as low as 2 ppb (ng/ml) of AFQ_1 spiked in the urine samples that had been subjected to a Sep-Pak cleanup treatment. In indirect ELISA in which the antigen (AFQ_{2a}-bovine serum albumin) was coated to the solid phase followed by reaction with rabbit antibody and goat anti-rabbit immunoglobulin G-peroxidase conjugate, 50-fold less antibody was used without sacrificing sensitivity. Recoveries of AFQ_1 added to urine samples (2 to 40 ppb) were 46.3 to 73% and 65.8 to 85.8% for direct and indirect ELISA, respectively.

Aflatoxin Q_1 (AFQ_1) is one of the major aflatoxin B_1 (AFB_1) metabolites found after incubation of AFB_1 with animal liver preparations (20, 21, 23, 25, 26, 29). It was first found in the urine after AFB_1 was orally administered to rhesus monkeys (8). In an in vitro metabolism study, Masri et al. (20) revealed that 19 to 52, 1 to 3, and 0.1 to 0.3% of AFB_1 were converted to AFQ_1 by microsomal preparations from monkey, rat, and chicken livers, respectively. With a fresh human liver extract (9,000 × *g*), as much as 30% (36.6 μmol) of AFB_1 was converted to AFQ_1 in 30 min (3). AFQ_1 is ca. 18 times less toxic than AFB_1 in the chicken embryo assay and is not mutagenic, with or without microsomal activation, in the Ames assay. Consequently, AFQ_1 has been considered to be a major detoxification metabolite of AFB_1 in primates (16).

Because AFQ_1 is one of the major AFB_1 metabolites, analysis of AFQ_1 in biological fluids could be used as an index for the intoxication of AFB_1. There is a need for a sensitive analytical method for the quantification of small quantities of AFQ_1. Thin-layer chromatography and high-pressure liquid chromatography are most commonly used for the analysis of AFQ_1 and other aflatoxins at present (3, 8, 14, 15, 17, 22, 23, 31). Although high-pressure liquid chromatography is sensitive, instrumentation is costly; it requires extensive cleanup for the samples to be analyzed, and only single samples can be analyzed at one time. More recently, specific antibodies against several mycotoxins including aflatoxins have been developed in our and other laboratories (5–7, 10, 11). Several immunological assays for the analysis of AFB_1 and AFM_1 in grains and milk have been developed (1, 9, 18, 19, 24, 25). In the present study, two approaches for the production of specific antibodies against AFQ_1 were tested. In addition, two enzyme-linked immunosorbent assays (ELISA) for the detection of AFQ_1 in urine were developed. The detection limits for AFQ_1 were 1 ng of pure toxin per ml and 2 ppb (ng/ml) of AFQ_1 added in the urine samples by either direct or indirect ELISA. In this paper, details for the production of antibodies, antibody specificity, and optimal conditions and protocols for ELISA of AFQ_1 are described.

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MATERIALS AND METHODS

Materials. All inorganic chemicals and organic solvents were of reagent grade quality or better. Bovine serum albumin (BSA; radioimmunoassay [RIA] grade), Tween 20, 2,2-azino-di-3-ethyl-benzthiazoline-6-sulfonate, hydrogen peroxide, and horseradish peroxidase were purchased from Sigma Chemical Co., St. Louis, Mo. Isobutyl chloroformate, thallous ethoxide, tetrahydrofuran, and triethylamine were products of Aldrich Chemical Co., Milwaukee, Wis. Tritiated AFB_1 (14 Ci/mmol) was purchased from Moravek Biochemicals, City of Industry, Calif. Thin-layer chromatography was used to check its purity and, if necessary, for its purification. Disposable Sep-Pak C-18 cartridges were purchased from Waters Associates Inc., Milford, Mass. AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, and aflatoxicol were prepared either directly from *Aspergillus* cultures or by chemical conversion as described previously (4, 13, 30). Complete and incomplete Freund adjuvants were obtained from Difco Laboratories, Detroit, Mich. Polyethylene glycol 6000 was purchased from J. T. Baker Chemical Co., Philipsburg, N.J. Albino rabbits were obtained from Klubertanz rabbit farm, Edgerton, Wis., and tested to verify that they were *Pasteurella* negative before use.

Preparation of AFQ_1, AFQ_1-HS, and AFQ_{2a}. AFB_1 was prepared by the method of Chu (4). AFQ_1 was prepared from AFB_1 by the method of Büchi et al. (2) with some modifications. AFQ_1-hemisuccinate (HS) was prepared from AFQ_1 after acylation with succinic anhydride in the presence of *N*,*N*-dimethylaminopyridine and was purified by silica gel chromatography as previously described (19). AFQ_{2a} was prepared from AFQ_1 after acid hydration. In a typical experiment, 119 mg of AFQ_1 (363 μmol) was dissolved in 120 ml of acetone in a screw-topped Erlenmeyer flask, and 1.2 ml of 10% sulfuric acid aqueous solution was added to the flask. After reaction at 60°C for 5 h, two spots, one minor and one major corresponding to the R_f values of AFQ_1 (R_f = 0.88) and AFQ_{2a} (R_f = 0.59), respectively, were found in the thin-layer chromatography plate (Silica Gel G-60) developed in methanol-chloroform (1:9).

Further purification of AFQ_{2a} was carried out by a combination of solvent partition and silica gel chromatography.

After reaction, the mixture was first evaporated to 50 ml to which 50 ml of distilled water was added. After the volume was reduced to ca. 50 ml by rotary evaporation, the mixture was extracted with 50 ml of methylene chloride 10 times. The organic portions were combined and dried with anhydrous sodium sulfate overnight. After filtration and concentration, the mixture was mixed with a small amount of Silica Gel G-60 and then loaded on a Silica Gel G-60 column (2.5 by 25 cm). The column was first washed with 500 ml of methylene chloride, then with 1 liter of 10% acetone in methylene chloride, and then with 2.5 liters of 20% acetone in methylene chloride at a flow rate of 1.7 ml/min. The unreacted AFQ_1 was separated from AFQ_{2a} when the column was eluted with 20% acetone in methylene chloride. In this solvent system, AFQ_1 first eluted from the column, followed by AFQ_{2a}. A total of 74 mg (62.1%) of AFQ_{2a} was obtained, and 6.2 mg (5.2%) of AFQ_1 was recovered.

Preparation of AFQ_1-HS-BSA. A 200-μl volume of triethylamine in tetrahydrofuran (7.5 mg/ml) and 200 μl of isobutylchloroformate in tetrahydrofuran (10 mg/ml) were added to a cool solution (ice-salt mixture bath at −5°C) containing 4.2 mg of AFQ_1-HS. The solution was well mixed, kept at −5°C for 25 min, and then added to a solution containing 10 mg of BSA in 4.5 ml of 33% pyridine in water at 4°C. The coupling reaction was first carried out at 4 to 6°C for 30 min and then at room temperature overnight. The reaction mixture was dialyzed against 4 liters of distilled water for 96 h, with a change of water every 24 h. The amount of AFQ_1-HS conjugated to BSA was determined by a spectrophotometric method, using a molar absorptivity of 17,600 at 362 nm (19). The AFQ_1-HS-BSA conjugate contained 26 molecules of AFQ_1-HS per molecule of BSA.

Preparation of AFQ_{2a}-BSA, AFQ_{2a}-EDA-BSA, and AFQ_{2a}. AFQ_{2a} (25 mg; 72.2 μmol) in 4 ml of methanol-pyridine (1:1) was mixed with 250 mg of BSA in 25 ml of 0.05 M sodium phosphate buffer (pH 7.2) and incubated at 37°C. At 30 min after initiation of the reaction, 2.5 ml of 0.026 M sodium borohydride aqueous solution was added, and the mixture was incubated at 4°C for another 30 min. The unreacted sodium borohydride was decomposed by the addition of 2.5 ml of 0.1 N HCl. After reaction, the mixture was dialyzed against 4 liters of distilled water for 72 h, with a water change every 24 h. The same procedure was followed to prepare AFQ_{2a}-ethylene diamine (EDA)-BSA and AFQ_{2a}-peroxidase conjugate except 0.3 mg of AFQ_{2a} and 15.3 mg of peroxidase were used. The AFQ_{2a}-BSA conjugate contained 19 mol of AFQ_{2a} per mol. The molar ratio of AFQ_{2a} to EDA-BSA in the AFQ_{2a}-EDA-BSA conjugate was 36. In the AFQ_{2a}-peroxidase conjugate, 3 mol of AFQ_{2a} was coupled to 1 mol of enzyme.

Immunizations. Three rabbits were used for each immunogen, and a multiple-site injection technique was used in the initial injection. A 2-ml volume of emulsion was prepared by mixing 0.5 ml of immunogen (0.5 mg of conjugate as calculated on the basis of the amount of BSA) and 1.5 ml of complete Freund adjuvant (1:3, vol/vol). Approximately 30 to 50 μl of emulsion was injected at each site on the shaved area. Each animal received ca. 30 to 40 injections intradermally. In addition, 0.2 to 0.25 ml of emulsion was injected subcutaneously into each shoulder. Starting at 3 or 4 weeks after the initial immunization, rabbits were bled on a weekly basis, and titers were determined. Booster injections were made intramuscularly with 0.5 mg of immunogen in incomplete Freund adjuvant emulsion (1:2, vol/vol) 6 to 8 weeks after the initial injection. The antibody was purified by the ammonium sulfate method (12).

RIA. For titer determination, 0.1 ml of the appropriate dilutions of antisera was incubated with 0.1 ml of tritiated AFB_1 (10,000 cpm) and analyzed by the protocols described below for RIA of AFQ_1 except that neither AFQ_1 nor other aflatoxins were added to the incubation mixture. The antibody titer was defined as the reciprocal of the amount (in milliliters) of antisera needed to give 50% binding of tritiated AFB_1 under stated conditions.

For RIA of AFQ_1, 50 μl of different concentrations of unlabeled standard AFQ_1 or other aflatoxins in 0.1 M sodium phosphate buffer (NaPB; pH 7.2) was incubated with a mixture of 50 μl of tritiated AFB_1 (ca. 10,000 cpm) and 0.05 ml of an appropriate dilution of antisera at room temperature for 30 min and overnight in a cold room in complete darkness. After incubation, saturated ammonium sulfate (in 0.1 M NaPB; pH 7.2) was added to each tube to a final concentration of 50% saturation. The mixture was allowed to stand for 1 h at room temperature in complete darkness and centrifuged at 5,000 rpm for 10 min. The supernatant fluid was collected in scintillation vials. The precipitates were washed with 0.5 ml of 35% ammonium sulfate in 0.1 M NaPB (pH 7.2) and centrifuged, and the supernatant fluid was collected. The radioactivity of the combined washings, which represented the free (not bound to antibody) tritiated AFB_1 radioactivity (F), was determined in a Beckman model LS-330 scintillation counter. The bound radioactivity (B), i.e., bound to antibody, was calculated from the total (T) radioactivity, i.e., $B = T - F$; thus, the B:F ratio of each analysis could be readily obtained.

Direct ELISA. For titer determination, 50 μl of the appropriate dilutions of antisera in sodium bicarbonate coating buffer (pH 9.6) was added to each well of a polystyrene microtiter plate (Immunlon 1 Removawell; Dynatech Laboratories, Alexandria, Va.) and incubated overnight at 5°C. After incubation, plates were washed twice with 0.1 ml of washing solution (0.1 M sodium phsophate-buffered normal saline (PBS) containing 0.1% Tween 20) per well, twice with 0.2 ml of washing solution per well, and three times with 0.32 ml of washing solution per well. To reduce nonspecific binding, 0.32 ml of BSA solution (0.1% of BSA in PBS [pH 7.5]) was added to each well and incubated at 37°C for 30 min to block the unbound sites on the plastic surface. After this treatment, the plate was washed three times with 0.32 ml of washing solution. Fifty microliters of the appropriate dilution of AFQ_{2a}-peroxidase conjugate in 0.1% BSA solution was added to each well and incubated for 1 h at 37°C. The plate was washed again with various volumes of washing solution in the same manner as the first wash. Peroxidase substrate 2,2-azino-di-3-ethyl-benzthiazoline-6-sulfonate (0.1 ml) was added to each well and incubated at 37°C for 20 min for color development. The reaction was terminated by adding 0.1 ml of hydrofluoric acid-EDTA stopping reagent (28), and absorbance at 410 nm was determined in a Dynatech minireader.

Minor modifications of the titration assay protocols were made for the competitive ELISA. Antibody (0.1 ml) at the appropriate dilution was added to each well instead of adding 50 μl as in the titration assay. Different concentrations of aflatoxins (50 μl each) diluted in PBS were incubated with 50 μl of the appropriate dilution of peroxidase conjugate diluted in 0.1% BSA solution containing 16% polyethylene glycol 6000 (molecular weight, 6,000 to 7,500; final concentration in each well, 8%) (27) for 1 h at 37°C.

Indirect ELISA. The optimal dilution of antigen precoated on the microtiter plate was determined by a checkerboard test. Fifty microliters of antigen (AFQ_{2a}-BSA; 1.0 μg/ml in

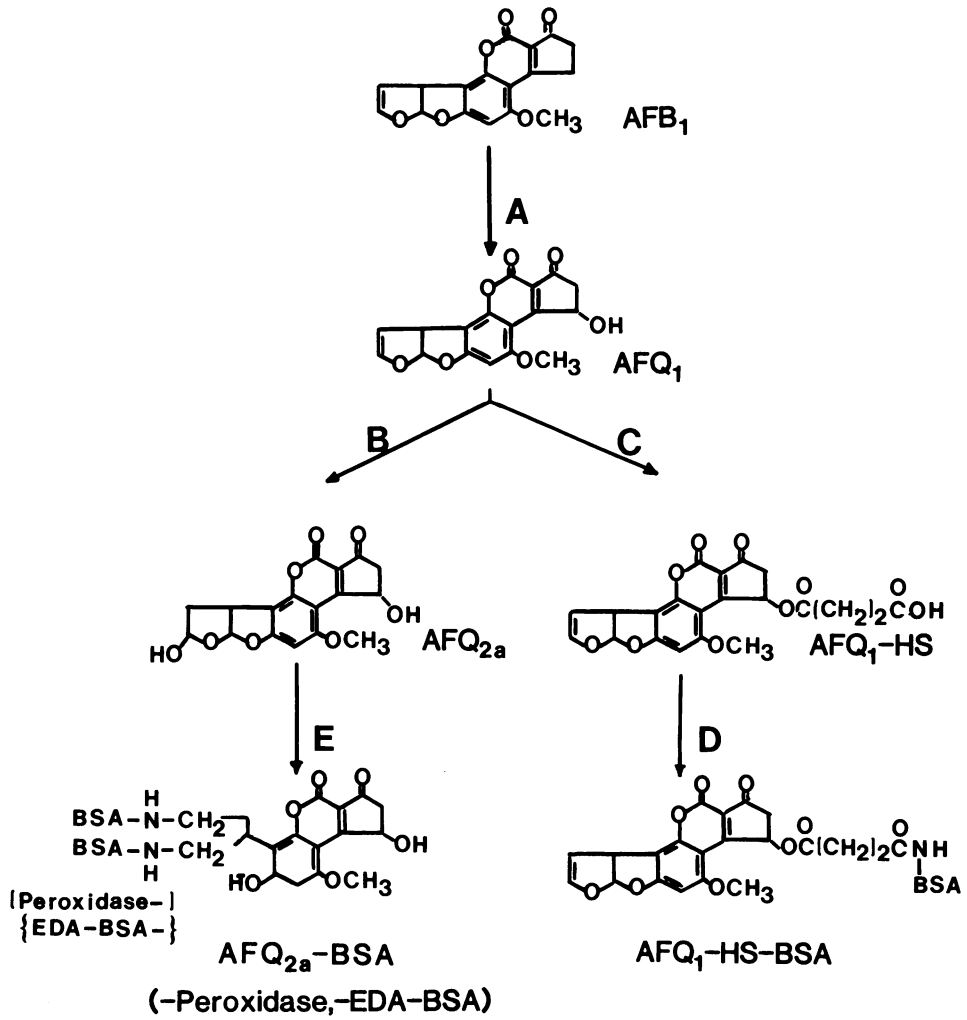

FIG. 1. Schematic diagram for the preparation of AFQ_1 antigens and peroxidase conjugates. AFQ_1 was prepared by oxidation of AFB_1 (A) in the presence of hydrogen peroxide-thallium ethoxide and then either converted to form AFQ_{2a} by acid hydration (B) or to form an HS (C) by the reaction with succinic anhydride in the presence of 4-*N*,*N*-dimethylaminopyridine. Conjugation of AFQ_{2a} to protein was achieved by a reductive alkylation method (E), whereas AFQ_1-HS was coupled to protein by a mixed anhydride method (D).

bicarbonate buffer; pH 9.6) was added to each well and incubated at 4°C overnight. The plate was washed with various amounts of washing solution (0.1 M PBS containing 0.1% Tween 20 [pH 7.5]), i.e., twice with 0.1 ml, twice with 0.2 ml, and three times with 0.32 ml for each well. To eliminate the nonspecific binding, 320 μl of 0.1% gelatin in PBS was added to each well to block the unbound sites on the plastic surface. After incubation at 37°C for 30 to 60 min, each well was washed four times with 0.32 ml of washing solution; next, 50 μl of different dilutions of antibody was added to each well. After incubation at 37°C for 1 h, the plate was washed again to remove the free antibody in the same manner as the first wash. Fifty microliters of goat anti-rabbit immunoglobulin G-peroxidase conjugate (1:500) in PBS containing 0.1% BSA was added to each well and incubated at 37°C for 1 h. The plate was washed as previously described. The peroxidase substrate (0.1 ml of 2,2-azino-di-3-ethylbenzthiazoline-6-sulfonate in citrate buffer [pH 4.0] per well) was added. After incubation at 37°C for 20 min, the reaction was terminated by the addition of 0.1 ml of hydrofluoric acid-EDTA stopping reagent. Absorbance at 410 nm was determined in a Dynatech minireader. For the competitive displacement assay, 0.1 ml of antigen was used, and 50 μl of purified AFQ_1 and 50 μl of antibody at the appropriate dilution were incubated together. The amount of bound antibody was determined by adding 0.1 ml of goat anti-rabbit immunoglobulin G-peroxidase conjugate (1:500 dilution; batch no. 72F-8830).

Preparation of urine samples. Urine samples were subjected to a simple Sep-Pak C-18 cartridge treatment. The cartridge was prewashed sequentially with 5 ml each of methanol, 50% methanol in water, and water. Urine (50 ml) was passed through the prewashed cartridge and then sequentially washed with 10 ml of water and 10 ml of 20% methanol in water. AFQ_1 was eluted from the cartridge with 9 ml of 50% methanol in water, and immediately 1 ml of PBS (0.1 M [pH 7.4]) was mixed to maintain the neutral pH.

RESULTS

Production of antibodies against AFQ_1. Three AFQ-BSA conjugates were used as immunogens for the production of antibodies against AFQ_1. Approaches for the preparation of such conjugates are summarized in Fig. 1. In the initial

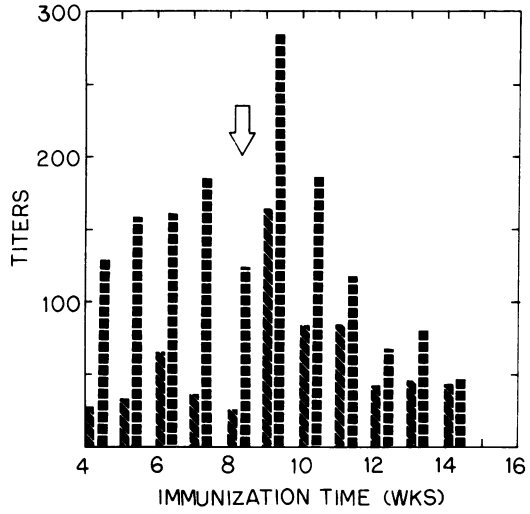

FIG. 2. Production of AFQ_1 antibody. Each rabbit was initially immunized with 500 μg of AFQ_1-HS-BSA and boosted with another 500 μg of the same immunogen at week 8. Antibody titers are defined as the reciprocal of serum dilution required for 50% binding of 10,000 cpm of tritiated AFB_1. Bars represent the responses of two individual rabbits.

studies, AFQ_1 was conjugated to BSA via the carboxylic acid residue in the AFQ_1-HS molecule. The antibody obtained from rabbits after immunization with AFQ_1-HS-BSA (designated as AFQ_1 antibody) showed a strong affinity to AFB_1; thus, RIA was used both for the antibody titer determinations and for subsequent analysis of antibody specificity. Tritiated AFB_1 was used as a marker ligand in such studies. Results for the antibody titers of two representative rabbits after immunization with AFQ_1-HS-BSA over a period of 14 weeks are shown in Fig. 2. Antibody titers of ca. 180 were obtained 5 weeks after immunization and reached a maximum at week 9 after one booster injection.

RIA was not used for the determination of antibody titers

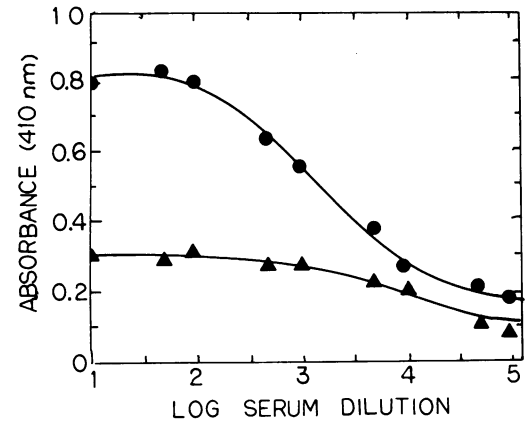

FIG. 3. Determination of antibody titers by direct ELISA method. The antiserum used in the assay was obtained from a rabbit that had been immunized with AFQ_{2a}-BSA. Serum dilutions were plotted on a logarithmic scale. Symbols: ●, serum obtained from the immunized rabbits; ▲, serum obtained from the same rabbit before immunization. The antibody titer was arbitrarily defined as the highest antiserum dilution that gave an absorbance 0.1 U above the preimmune serum at the same dilution.

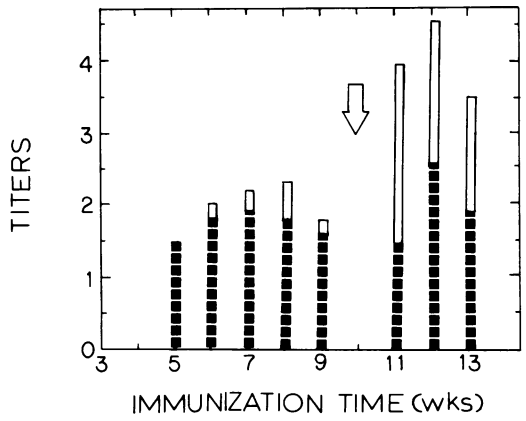

FIG. 4. Production of AFQ_{2a} antibody. Each rabbit was initially immunized with 500 μg of AFQ_{2a}-EDA-BSA and then boosted at week 10 with 500 μg of AFQ_{2a}-BSA. Antibody titers were in logarithmic scale (Y axis) and were determined by the method described in the text, using the definition described in the legend for Fig. 3. Open bars on top of the square bars represent the better response of another rabbit.

for antisera obtained from rabbits that had been immunized with AFQ_{2a}-EDA-BSA and AFQ_{2a}-BSA (designated as AFQ_{2a} antibody), because the AFQ_{2a} antibody bound radioactive AFB_1 poorly. Both direct and indirect ELISA were used for characterization of these antibodies. In the competitive direct ELISA, AFQ_{2a}-peroxidase conjugate was used as a marker enzyme, whereas in indirect ELISA, AFQ_{2a}-BSA was coated to the plate. A typical titration curve of antibody titer as determined by direct ELISA is shown in Fig. 3. The titration was carried out by incubating a serial dilution of antisera with AFQ_{2a}-peroxidase conjugate, followed by determining the total enzyme bound to the antisera. By using the arbitrary definition as described in the legend of Fig. 3, the antibody titer for this antiserum was found to be ca. 10,000. Results for the antibody titers of two representative rabbits after immunizing with AFQ_{2a} conjugates are presented in Fig. 4. Poor titers were obtained from both rabbits when AFQ_{2a}-EDA-BSA was used in the initial injections. At week 10 after the initial injection, these rabbits were boosted with AFQ_{2a}-BSA at a dose of 0.5 mg each. The antibody titers increased considerably (10- to 100-fold) after the booster injection.

In indirect ELISA, a preliminary test was done to ensure that the antibody produced against protein carrier was removed during the washing by dilution of the antibody in 0.1% BSA in PBS. The optimal antibody concentration precoated on the plate was determined by a checkerboard-fashion assay. AFQ_{2a}-BSA at a concentration of 1.0 μg/ml (50 μl per well or 50 ng per well; equivalent to 4.83 ng of AFQ_{2a} per well) was chosen. Compared with direct ELISA, indirect ELISA generally required 50 times less antibody than did the direct assay to obtain the same absorbance even in the presence of 8% polyethylene glycol 6000.

Determination of antibody specificity. Specificity of antibodies obtained from rabbits after immunization with AFQ_1-HS-BSA (AFQ_1-HS antibody) was determined by a competitive RIA, using tritiated AFB_1 as the ligand. Results indicate that the antibodies have a higher affinity toward AFB_1 than toward AFQ_1 (Fig. 5). Concentrations required to displace 50% of the radioactive AFB_1 by the unlabeled ligands were 2.95, 7.76, 100, 209, and 2,951 ng/ml for AFB_1, AFL, AFQ_1,

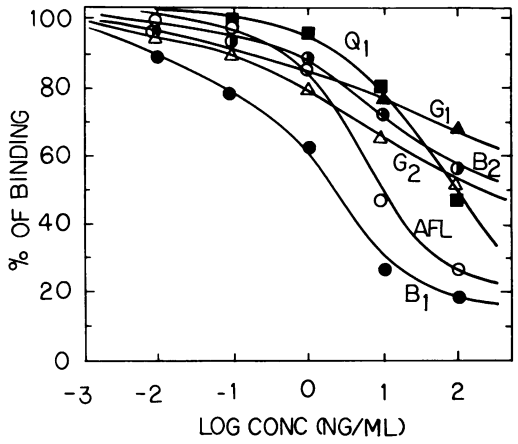

FIG. 5. Specificity of AFQ_1 antibody as determined by RIA. The antiserum used in the assay was obtained from a rabbit that had been immunized with AFQ_1-HS-BSA. The relative inhibition of binding of ^3H-AFB_1 with the antibody by different concentrations of unlabeled aflatoxin is shown. All values have been normalized to 100% binding. Concentrations of different unlabeled aflatoxins required to displace 50% of ^3H-AFB_1 were 2.95, 7.76, 100, 209, and 2,951 ng/ml for AFB_1 (●), AFL (○), AFQ_1 (■), AFG_2 (△) and AFB_2 (◑), and AFG_1 (▲), respectively.

AFG_2 and AFB_2, and AFG_1, respectively. Thus, AFQ_1 is ca. 34 times less effective than AFB_1 for the displacement of bound AFB_1 from AFQ_1 antibody.

To determine the specificity of antibodies obtained from rabbits after immunization with the AFQ_{2a}-BSA conjugate, competitive direct ELISA was used. Results indicate that the antibodies have highest affinity to AFQ_{2a} (Fig. 6). Concentrations required to decrease absorbance by 50% (or to displace the bound AFQ_{2a}-enzyme) in the presence of different ligands were 1.0, 90, 100, and >350 ng/ml for AFQ_{2a}, AFB_{2a}, AFQ_1, and AFB_1, respectively.

Specificity of the antibodies obtained from rabbits after immunization with AFQ_{2a}-BSA also was determined by competitive indirect ELISA (Fig. 7). Concentrations that

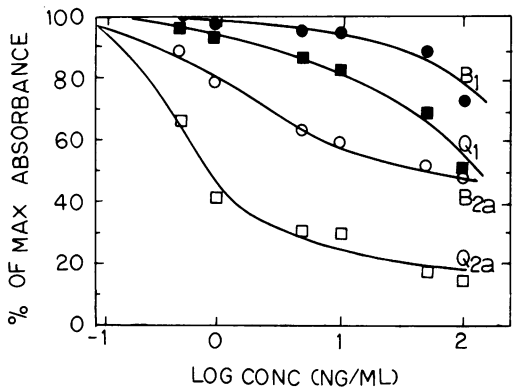

FIG. 6. Specificity of AFQ_{2a} antibody as determined by competitive direct ELISA. The antiserum used in the assay was obtained from a rabbit that had been immunized with AFQ_{2a}-BSA. The absorbance in the assay without free aflatoxins was used as the maximal value (100%). Concentrations required to decrease 50% of the maximal absorbance by different aflatoxins were 1.0, 90, 100, and >350 ng/ml for AFQ_{2a} (□), AFB_{2a} (○), AFQ_1 (■), and AFB_1 (●), respectively.

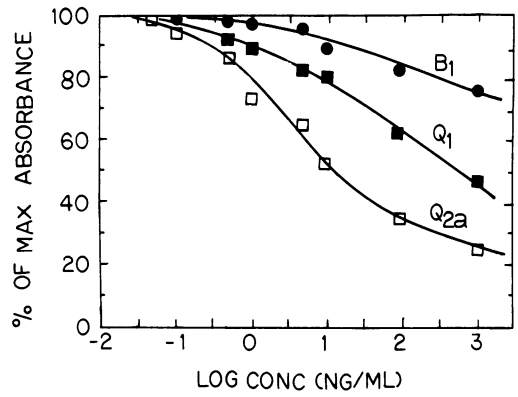

FIG. 7. Specificity of AFQ_{2a} antibody as determined by a competitive indirect ELISA. The antiserum used in the assay was the same as that described in the legend for Fig. 6. AFQ_{2a}-BSA was coated to the plate as the solid phase for binding with the antibody. The absorbance in the assay without free aflatoxins was used as the maximal value (100%). Concentrations required to decrease 50% of the maximal absorbance by different aflatoxins were 10, 900, and >1,000 ng/ml for AFQ_{2a} (□), AFQ_1 (■), and AFB_1 (●), respectively.

gave a 50% decrease in absorbance were 10, 900, and >1,000 ng/ml for AFQ_{2a}, AFQ_1, and AFB_1, respectively. The results suggest that both ELISAs are more sensitive for the detection of AFQ_1 than is RIA.

Determination of AFQ_1 in urine by direct and indirect ELISA. Various levels of AFQ_1 were added to urine followed by a Sep-Pak cleanup, and concentration then was determined by ELISA. Results indicate that 54.2 to 73% and 65.8 to 85.8% of AFQ_1 added in the range of 2.0 to 40 ppb to urine were recovered by direct ELISA and indirect ELISA, respectively (Table 1).

DISCUSSION

Among three AFQ-BSA conjugates tested in the present study, two of them, i.e., AFQ_1-HS-BSA and AFQ_{2a}-BSA, were found to be good immunogens. AFQ_{2a}-EDA-BSA, which has more than 30 mol of AFQ_{2a} per mol of BSA, was found to be less satisfactory for antibody production. Although there are a number of factors which may affect the immunogenicity of a compound, the following two factors may directly contribute to the poor immunogenic properties of this conjugate: (i) the lyophilized AFQ_{2a}-EDA-BSA was

TABLE 1. Recovery of AFQ_1 in urine by direct and indirect ELISA[a]

AFQ_1 added (ppb)	AFQ_1 recovered by:			
	Direct ELISA		Indirect ELISA	
	ppb	%	ppb	%
2	1.46 ± 0.18	73.0 ± 9.0	1.60 ± 0.7	80.0 ± 35
5	3.06 ± 1.26	61.2 ± 25	3.50 ± 1.06	70.0 ± 21
10	5.48 ± 2.96	54.8 ± 29	6.58 ± 1.86	65.8 ± 18.6
20	9.26 ± 2.36	46.3 ± 11.8	14.48 ± 4.0	72.4 ± 20
40	18.6 ± 4.34	54.2 ± 10.8	34.32 ± 4.0	85.8 ± 10

[a] The average percent recovery was 57.9 for direct ELISA and 74.8 for indirect ELISA. The standard deviation was 17.1 for direct ELISA and 20.9 for indirect ELISA. The coefficient variation was 30.0 for direct ELISA and 28.0 for indirect ELISA. Replicate assays were 24 for direct ELISA and 20 for indirect ELISA.

not very soluble in normal saline as compared with AFQ_{2a}-BSA which had excellent solubility in the solution; the poor solubility of this conjugate may prevent the immunogen from reaching the antibody-forming cells; and (ii) the AFQ_{2a}-EDA-BSA preparation had a very high molar ratio of AFQ_{2a} to the protein carrier (36 mol/mol) compared with AFQ_{2a}-BSA which had a relatively lower epitope density (19 mol/mol); the presence of a large amount of AFQ_{2a} may inhibit the immune response.

Antibodies obtained from the rabbits after immunization with AFQ_1-HS-BSA and AFQ_{2a}-BSA have distinct specificity. The former immunogen elicited antibodies that had specificity toward AFB_1, whereas the latter immunogen produced antibodies that strongly reacted with AFQ_{2a}, reacted less with AFQ_1, and reacted weakly with AFB_1. Such specificities were not surprising because the HS of AFQ_1-HS was conjugated to the protein carrier; the hydroxyl group, which is located on the cyclopentenone ring of the AFQ_1 molecule, was blocked by the succinyl residues. The molecular structure exposed to the immune system is similar to the structure of AFB_1. However, the hydroxyl group in the AFQ_{2a}-BSA conjugate is completely exposed; the antibody produced is capable of recognizing the beta OH residue in the cyclopentenone ring.

Although AFQ_1-HS-BSA antibodies have shown specificity only toward AFB_1 and AFL and cannot be used for immunoassay of AFQ_1, such antisera could be used for RIA of AFB_1 and AFL. Additional efforts in our laboratory were then concentrated on the use of AFQ_{2a}-BSA antibody for monitoring AFQ_1 in biological fluids. To avoid the use of radioactive ligands, further experiments were also directed to the ELISA studies. Our results clearly demonstrated that both direct and indirect ELISA could be used for monitoring AFQ_1 in urine after the sample was subjected to Sep-Pak cleanup treatment. We found that the sensitivity of ELISA of AFQ_1 (1 to 10 ng/ml or 20 to 200 pg per assay) was relatively high compared with the AFB_1 system (0.1 to 1 ng/ml); a combination of cleanup and concentration steps was necessary. However, ELISA is more sensitive than high-pressure liquid chromatographic and thin-layer chromatographic analyses (16, 23). In conclusion, the two approaches used in the present study produced antibodies that were useful in the analysis of AFB_1, AFQ_1, and AFQ_{2a}.

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