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Quantitation of Aflatoxin B_1 and Aflatoxin B_1 Antibody by an Enzyme-Linked Immunosorbent Microassay

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A specific microtest plate enzyme immunoassay has been developed for the rapid quantitation of aflatoxin B_1 at levels as low as 25 pg per assay. Multiple-site injection of rabbits with an aflatoxin B_1 carboxymethyloxime-bovine serum albumin conjugate was used for the production of hyperimmune sera. Dilutions of the purified antibody were air dried onto microplates previously treated with bovine serum albumin and glutaraldehyde and then incubated with an aflatoxin B_1 carboxymethyloxime-horseradish peroxidase conjugate. The amount of enzyme bound to antibody was determined by monitoring the change in absorbance at 414 nm after the addition of a substrate solution consisting of hydrogen peroxide and 2,2'-azino-di-3-ethyl-benzthiazoline-6-sulfonate. Antibody titers determined in this manner closely correlated with those determined by radioimmunoassay. Competition assays as performed by incubation of different aflatoxin analogs with the peroxidase conjugate showed that aflatoxins B_1 and B_2 and aflatoxicol caused the most inhibition of conjugate binding to antibody. Aflatoxins G_1 and G_2 inhibited the conjugate binding to a lesser degree, whereas aflatoxins M_1 and B_{2a} had no effect on the assay.

The aflatoxins represent a group of toxic metabolites produced by the fungal species *Aspergillus flavus* and *Aspergillus parasiticus*. The ability of aflatoxin B_1 (afla B_1) and its metabolites to function as potent carcinogens, mutagens, and teratogens has been well documented (2, 4). Since these compounds are known to occur naturally in agricultural commodities (2) such as peanuts, milk, corn, and animal feedstuffs, there has been increased interest in the development of rapid sensitive techniques for the detection of aflatoxin in food, feeds, and biological fluids. Although high-performance liquid chromatography might serve in this capacity (20), the instrumentation is costly, it requires extensive clean up procedures, and only single samples can be analyzed at a time.

Recent investigations have described the development of specific antibodies for afla B_1 (6, 14) and M_1 (10) as well as the efficacy of their use in radioimmunoassays (RIA) for quantitating the toxins in foods and clinical specimens (14, 21). However, these assays are expensive, necessitate radioactive reagents, and require overnight incubations.

Lawellin et al. (15) have reported an enzyme-linked immunosorbent assay (ELISA) which used antisera raised against an afla B_{2a}-bovine serum albumin conjugate. The assay was of limited usefulness because it yielded low maximal absorbance and had a high degree of nonspecific binding. Recently, a rapid microtest plate ELISA for afla B_1 has been developed in this laboratory (J. J. Pestka, P. K. Gaur, and F. S. Chu, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, P14, p. 191). The method is superior to previous immunoassays because it uses stable reagents, requires minimal exposure to aflatoxins and can detect levels of afla B_1 as low as 25 pg per assay. In this paper, details for the optimal conditions of the new aflatoxin ELISA are described.

MATERIALS AND METHODS

Chemicals. All inorganic chemicals and organic solvents were of reagent-grade quality or better. Horseradish peroxidase (type VI), bovine serum albumin (BSA; RIA grade), Tween 20, 1-ethyl-3,3-dimethylamino-propyl-carbodiimide, 2,2'-azino-di-3-ethyl-benzthiazoline-6-sulfonate, and hydrogen peroxide were purchased from Sigma Chemical Co. (St. Louis, Mo.). ^{3}H-labeled afla B_1 (13 Ci/mmol) was obtained from Moravek Biochemicals (City of Industry, Calif.). Sephadex G-25 fine was a product of Pharmacia (Uppsala, Sweden). Complete (Difco 3110-60-5) and incomplete (Difco 0639-60) Freund adjuvants were purchased from Difco Laboratories (Detroit, Mich.). Albino rabbits were supplied by Klubertanz's Rabbit Farm (Edgerton, Wis.) and tested to be *Pasteurella* negative before use. Afla B_1, B_2, B_{2a}, G_1, G_2, and M_1 and aflatoxicol were prepared either directly from *Aspergillus* cultures or by chemical conversions described previously (3, 13, 16, 19, 20).

Production of afla B_1 antisera. Afla B_1 was first

converted to afla B_1-*o*-carboxymethyloxime (afla B_1-oxime) by the method of Chu et al. (5). The oxime derivative was then conjugated to BSA in the presence of 1-ethyl-3,3-diethylamino-propyl-carbodiimide (6), yielding a ratio of 5 mol of B_1 per mol of BSA. Antiserum against afla B_1 was prepared by a modification of the method of Chu and Ueno (6). Initially, multiple-site intradermal injections of rabbits were made with a 2-ml emulsion containing 1 volume of afla B_1-BSA (300 to 1,000 μg) in normal saline and 3 volumes of Freund complete adjuvant. Subsequent booster injections in thigh muscles were carried out with 1 to 2 ml of an emulsion prepared by mixing 1 volume of the antigen (250 to 400 μg) with 2 volumes of incomplete adjuvant. The immunoglobulin G fraction of the rabbit blood was purified by the ammonium sulfate precipitation method of Herbert et al. (11), and antibody titers were determined by RIA (10).

Peroxidase assay. Horseradish peroxidase activity (17, 18) was determined by adding the following components to a test tube (13 by 100 mm) in a total volume of 1.0 ml: 50 mM citrate buffer (pH 4.0), 0.4 mM 2,2′-azino-di-3-ethyl-benzthiazoline-6-sulfonate, and 1.2 mM hydrogen peroxide. Diluted enzyme was added in 25-μl aliquots, and the mixture was incubated at room temperature for 5 min. The reaction was terminated by adding 1.0 ml of hydrofluoric acid-edetic acid stopping reagent (17), and absorbance at 414 nm was determined.

Preparation of afla B_1-peroxidase conjugates. A modification of the method used for the conjugation of afla B_1 to BSA (6) was used for peroxidase. Afla B_1-oxime (600 μg) was dissolved in 2.5 ml of ethanol, and a 7.5-ml volume of distilled water was added slowly with gentle mixing. This was followed by the addition of, first, 188 mg of 1-ethyl-3,3-diethylamino-propyl-carbodiimide and then 5.0 mg of peroxidase previously dissolved in 1.0 ml of 25% (vol/vol) ethanol in water. The mixture was slowly mixed at room temperature in the dark for 30 min and an additional 188 mg of 1-ethyl-3,3-diethylamino-propyl-carbodiimide was added. The preparation was then stirred at 4°C for another 16 h. The resultant conjugate was centrifuged to remove precipitated material and passed through a column of Sephadex G-25 (fine) for the removal of unreacted afla B_1-oxime. Phosphate buffer (0.01 M, pH 7.5) was used as the eluant, and the combined fractions of purified peroxidase contained 0.1 mg of protein per ml. A ratio of 1 mol of afla B_1 per mol of peroxidase was determined spectrophotometrically for the conjugate (6).

ELISA. The enzyme immunoassay was based on the method developed by Saunders et al. (17, 18) for the detection of staphylococcal enterotoxin A. Polystyrene microtissue culture plates (Falcon 3040) were prepared by air drying 50 μl of a BSA solution (0.2 mg/ml of distilled water) in each well. The wells were then reacted with 50 μl of 0.2% (vol/vol) glutaraldehyde in 0.1 M phosphate-buffered (pH 7.5) normal saline (PBS) for 30 min, washed exhaustively with distilled water, and air dried overnight. Purified antisera were diluted in 1.0 M saline, and 50-μl aliquots were added to each well. The plates were then air dried under a current of forced air at room temperature. Microplates prepared in this manner could be stored over a desiccant for at least 2 weeks at room temperature. Plates were washed three times with PBS containing 0.05% (vol/vol) Tween 20 (PBS-Tween) before use.

For determination of antibody titer, afla B_1-oxime–peroxidase conjugate was diluted (1:20) in PBS containing 1.0% BSA, and 50-μl aliquots were added to the microplate wells previously treated with appropriate dilutions of antisera. The plates were incubated for 2 h at 37°C, washed four times in PBS-Tween, and then reacted for 5.0 min with 0.1 ml of the 2,2′-azino-di-3-ethyl-benzthiazoline-6-sulfonate–hydrogen peroxide substrate described above. The reaction was terminated by the addition of 0.1 ml of hydrofluoric acid-edetic acid stopping reagent. Absorbance at 414 nm was determined with a Beckman DU spectrophotometer equipped with a flow cell (Altex model 154), peristaltic pump (Pharmacia P-3), and strip chart recorder (Heath-Schlumberger model EU-205-11). Direct chart recordings could be made by pumping the reaction product through the flow cell and concurrently monitoring the absorbance. Individual readings were separated by allowing a volume of air to pass through the flow cell.

For competition assays, 25 μl of standard aflatoxin in PBS was added to microplate wells which had been coated with a 1:400 dilution of antibody. This was followed by the addition of 25 μl of a 1:10 dilution of afla B_1-peroxidase conjugate in PBS containing 1.0% (vol/vol) BSA. Plates were incubated and bound enzyme was determined as described above.

RESULTS

Effect of afla B_1 on horseradish peroxidase. Development of an enzyme immunoassay for afla B_1 required the use of an enzyme that was not inhibited by the toxin. Incubation of horseradish peroxidase (5 ng/ml) with concentrations of afla B_1 ranging from 0 to 100 ng/ml showed no loss of peroxidase activity. It was therefore concluded that afla B_1 did not have a deleterious effect on the enzyme.

ELISA titration of afla B_1 antibody. Antisera specific for afla B_1 were obtained from three rabbits (R-1, R-2, and R-3) after immunizing with afla B_1-BSA conjugate. Antibody titers were determined throughout the immunization and booster procedures by RIA (10). An ELISA antibody titration was performed by incubating dilution series of the three antisera with the afla B_1-oxime–peroxidase conjugate and then determining total bound enzyme. The last well in a dilution scheme to give color visually distinct from the preimmune serum control was designated as the titer endpoint (9). These values approximated about twice the absorbance measured spectrophotometrically for the preimmune control sera. Results for the ELISA titration and controls are indicated in Fig. 1. Dilutions of the preimmune control sera showed negligible binding of the afla B_1-peroxidase conjugate. When dilution series of pooled rabbit antisera R-1, R-

2, and R-3 were incubated with control peroxidase (unconjugated), only a low degree of nonspecific binding was detected. The three antisera exhibited distinct titration endpoints as well as different maximal absorbance plateaus at the low dilutions of antibody. Titers determined for the three antisera by the ELISA and RIA showed close correlation (Table 1). The results suggested that the afla B_1-oxime-peroxidase bound specifically to the antibody and that the method described here could be used for the rapid titration of afla B_1 antibody.

Competitive ELISA for afla B_1. In establishing a competitive ELISA for afla B_1 quantitation, it was necessary to determine the dilution of antibody which would yield the highest degree of absorbance and still be sensitive to low concentrations of the toxins. The effect of incubating the afla B_1-oxime-peroxidase conjugate with afla B_1 was therefore determined at four dilutions of antiserum R-3 and plotted as a function of percent maximal absorbance (Fig. 2). The results indicated that dilution of the antibody increased the sensitivity of the competitive assay. Similar results were obtained for rabbit antisera R-1 and R-2. High antibody concentrations might require significantly more afla B_1 for the displacement of the afla B_1-oxime-peroxidase conjugate, thus lowering assay sensitivity. A second possibility, first offered by Engvall et al. (8), was that at high concentrations the antibody is less efficiently bound and detaches during the incubation procedure. Since free antibody would compete for the hapten more effectively than bound antibody there would be a resultant loss in sensitivity. A 1:400 dilution was the most sensitive antibody concentration of those tested, detecting between 25 and 2,500 pg per assay, and it was therefore used for further competitive assays.

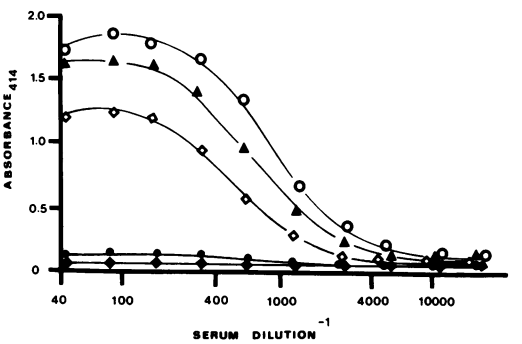

FIG. 1. *ELISA titration of afla B_1 antisera. Titrations were performed as described in the text. Serum dilutions were plotted on a logarithmic scale. Symbols: (○) R-1 antiserum; (▲) R-3 antiserum; (◇) R-2 antiserum; (●) pooled R-1, R-2, and R-3 preimmune sera; (◆) pooled R-1, R-2, and R-3 antisera assayed with control horseradish peroxidase (unconjugated).*

TABLE 1. *Comparison of the microplate ELISA and RIA methods for the titration of afla B_1 antibody*

Rabbit antiserum	Titer by:	
	ELISA	RIA
R-1	5,020	4,762
R-2	1,280	2,857
R-3	2,560	3,333

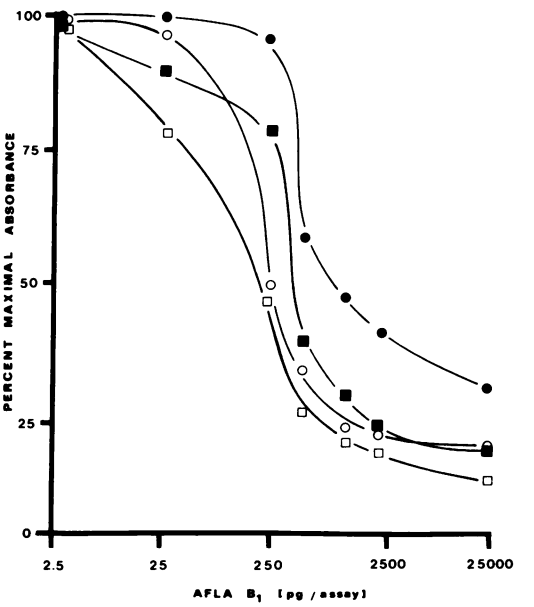

FIG. 2. *Effect of antibody dilution on competitive afla B_1 ELISA. Antiserum R-3 was used. Symbols: (●) 1:100; (■) 1:200; (□) 1:400; (○) 1:800 antibody dilutions.*

Two-step ELISA for afla B_1. Since applications of the afla B_1 ELISA might require incubation of sample extracts which could inhibit peroxidase, a second method of afla B_1 quantitation was attempted. The procedure was a modification of that used by Lawellin et al. (15) and used separate incubation steps for afla B_1 and the peroxidase conjugate (Fig. 3). In the first step, 50 μl of afla B_1 was incubated in each well for 16 h at 4°C. The plates were washed in PBS-Tween four times and then incubated with 50 μl of afla B_1-oxime-peroxidase conjugate for 15, 60, and 120 min. The two-step assay could detect between 25 and 250 pg of afla B_1 per assay, but higher levels of toxin could not be detected because of elevated background absorbancy. This was probably due to displacement of bound afla B_1 by afla B_1 peroxidase. Whereas decreasing peroxidase conjugate incubation times de-

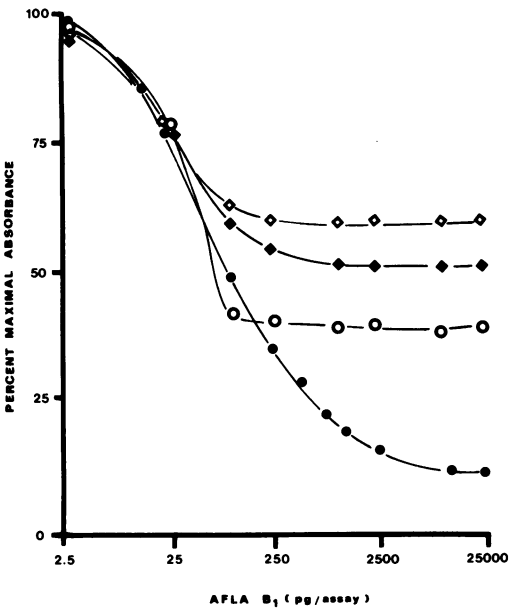

FIG. 3. *Comparison of competitive and two-step ELISAs for afla B_1 quantitation. In the two-step ELISA, a 1:400 dilution of antiserum R-3 was first incubated with 50 μl of afla B_1 followed by incubation with afla B_1-peroxidase for 15* (○), *60* (◆), *and 120* (◇) *min.* (●) *Standard competitive assay.*

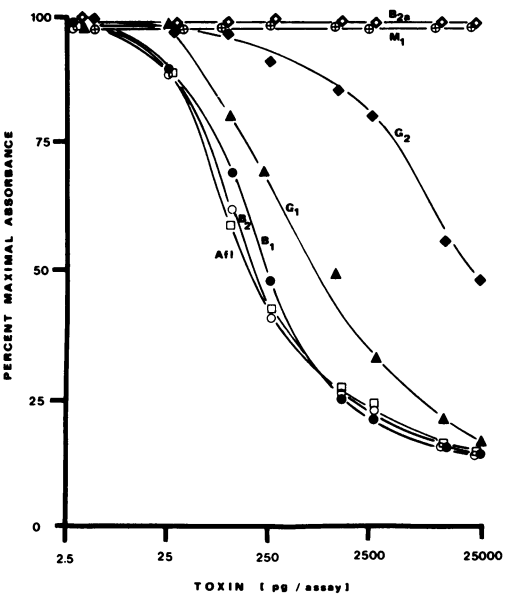

FIG. 4. *Cross-reactivity of aflatoxin analogs with afla B_1 antibody in competitive ELISA. A 1:400 dilution of R-3 antiserum was used. Toxins symbols:* (●) *afla B_1;* (○) *afla B_2;* (□) *aflatoxicol;* (▲) *afla G_1;* (◆) *afla G_2;* (⊗) *afla M_1;* (◇) *afla B_{2a}. The concentrations causing 50% inhibition of binding of the peroxidase conjugate with antibody were 0.25, 0.19, 0.14, 0.8, and 19 ng for afla B_1, afla B_2, aflatoxicol, afla G_1, and afla G_2, respectively.*

creased the background in the two-step method, the competitive assay appeared to be a more satisfactory method for the quantitation of afla B_1. Similar results were obtained for the two-step assay when afla B_1 was first incubated for 2 h at 37°C rather than 16 h (data not shown).

Specificity of the competitive ELISA. Competitive assays were conducted to determine the specificity of the afla B_1 antibody for various aflatoxin analogs (Fig. 4). The relative cross-reactivities (analog:B_1) of the different aflatoxins with antibody as estimated from the amount of toxin necessary to cause 50% inhibition of maximal conjugate binding were found to be 1.0, 0.8, 0.6, 3.2, and 76 for afla B_1 and B_2, aflatoxicol, and afla G_1 and G_2, respectively. Thus, the antiserum cross-reacted with afla B_2 and aflaxtoxicol to the same degree as with afla B_1. Cross-reactivity with afla G_1 and G_2 was weaker and afla M_1 and B_{2a} had no effect on the assay. The results suggest that the difuran moiety of aflatoxin was critical to the specificity of antibody raised against afla B_1-oxime–BSA conjugates. Modifications like the hydroxyl groups occurring in the difurans of afla M_1 and B_{2a} prevented binding of the antibody to the hapten. The cyclopentenone ring played a secondary role in determining specificity as evidenced by the cross-reactivity with aflatoxicol and lesser binding of afla G_1 and G_2.

DISCUSSION

Previous reports have established the efficacy of the use of RIA for the analysis of aflatoxin in peanut butter, corn, wheat. serum, and urine (14, 21). However, these methods are lengthy and require the use of radioactive reagents. The simplicity of the microplate afla B_1 ELISA makes it a more convenient method for the routine screening of food and clinical samples. Both the antibody and peroxidase conjugate can be stored in the lyophilized form, and the antibody-coated plates could be used 2 weeks after preparation with no loss in activity. Although the afla B_1 competitive assay was routinely conducted with a 2-h incubation, identical competition curves of percent maximal absorbance were obtained by incubating for only 15 min. Since the microplate afla B_1 ELISA cross-reacts with afla B_2 and aflatoxicol, and to a lesser degree with afla G_1 and G_2, definitive identification of a toxin might require the use of a second antibody which is specific for the other types of aflatoxins or use of a second technique such as high-performance liquid chromatography.

Lawellin et al. (15) have described a polystyrene tube enzyme immunoassay for afla B_1 in which both BSA and peroxidase were conjugated to the dihydrofuran moiety of afla B_{2a}, using the tetrabenzidene method of DeCarvalho et al (7). Hence, the antibody generated also cross-reacted with afla M_1 and B_{2a}. Conjugates prepared for the microplate method were bound at the reactive carboxyl of afla B_1-oxime and did not cross-react with these aflatoxins. Two other problems associated with the tube ELISA were high background levels and low maximal absorbance. The high background can probably be attributed to the use of a two-step assay similar to the one attempted in this report. The background was virtually eliminated in the microplate competitive ELISA by incubating free aflatoxin together with the afla B_1-peroxidase conjugate. Maximal absorbance levels obtained with the microplate method were nearly 10 times higher than those reported by Lawellin et al. (15). This was due to the use of 2,2'-azino-di-3-ethyl-benzthiazoline-6-sulfonate which has been reported to have a higher sensitivity and greater stability than conventional peroxidase substrates (17). A modified tube afla B_1 ELISA, recently reported by Bierman and Terplan (1), also yielded significantly less color than the method described here.

This paper has described an enzyme immunoassay which readily detected afla B_1 in the range of 25 to 2,500 pg per assay. The sensitivity was equivalent to or better than those of the double-antibody RIA (14), solid-phase RIA (21), high-performance liquid chromatography (19), and thin-layer chromatography (12). Whereas sensitivities of less than 1 pg per assay were reported for the tube ELISAs (1, 15), the problems encountered with low absorbance levels, the logistics of using plastic culture tubes, and the cross-reactivity with most aflatoxins suggest the microplate ELISA reported herein to be a safer and more reliable procedure for the routine assay of afla B_1.

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