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Production and Characterization of Aflatoxin B_{2a} Antiserum

P. K. GAUR,† H. P. LAU, J. J. PESTKA, AND F. S. CHU*

Food Research Institute and Department of Food Microbiology and Toxicology, University of Wisconsin, Madison, Wisconsin 53706

The specificity and sensitivity of antiserum elicited from rabbits against aflatoxin B_{2a}-bovine serum albumin conjugates were characterized with a radioimmunoassay (RIA) and an enzyme-linked immunosorbent assay (ELISA). Aflatoxin B_1 was first converted to aflatoxin B_{2a} and then conjugated to bovine serum albumin and horseradish peroxidase by a reductive alkylation method. The antiserum was developed in New Zealand white rabbits by multiple-site injection with the aflatoxin B_{2a}-bovine serum albumin conjugate. Antibody titers were determined by both RIA and ELISA. Competitive RIAs with various aflatoxin analogs indicated that the antiserum was most reactive with aflatoxin B_1 and slightly cross-reactive with aflatoxins B_{2a}, B_2, and M_1. Competitive ELISAs showed the antiserum to be equally specific for aflatoxins B_{2a} and B_1 and less reactive with aflatoxins B_2 and M_1. The relative sensitivities of RIA and ELISA for aflatoxin B_1 quantitation were 100 and 10 pg per assay, respectively.

Aflatoxin B_1 (afla B_1), one of the most potent naturally occurring carcinogens known, is produced by toxic strains of *Aspergillus flavus* and *Aspergillus parasiticus* (2, 4). The ubiquitous nature of this toxin and its related metabolites requires a rapid and sensitive means for monitoring their presence in the food supply and in animal tissues. During the past 5 years there has been considerable interest in the development of quantitative immunoassays for afla B_1 (8, 13, 14, 15, 21). Two basic approaches for eliciting antiserum specific for afla B_1 have been devised. In the first method, afla B_1 is converted to afla B_1-*O*-carboxymethyloxime (7) and then conjugated to bovine serum albumin (BSA) to yield an effective immunogen. Antibody generated in this manner (afla B_1 oxime antiserum) has been used in the radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) of afla B_1, and its specificity has been well characterized (8, 13, 15, 21). In the second approach, afla B_1 is chemically converted to afla B_{2a} and then coupled to a protein for immunization (14). The resultant antibody (afla B_{2a} antiserum) has been used in an ELISA of afla B_1 (14) but its specificity has not been fully characterized with respect to afla B_1 structural analogs. Furthermore, the afla B_{2a} ELISA was subject to a high degree of nonspecific binding, had low maximal absorbance, and required overnight incubation. Subsequent investigations on the effectiveness of the afla B_{2a} antiserum in the quantitation of afla B_1 have not been reported.

† Present address: Division of Dermatology, University of Maryland School of Medicine, Baltimore, MD 21201.

In this paper, new procedures for the preparation of the afla B_{2a}-BSA (or enzyme) and for the production and assay of afla B_{2a} antiserum are described. Details on specificity determinations of afla B_{2a} antiserum by both RIA and ELISA as well as the relative sensitivities of the two methods in quantitating afla B_1 are also presented.

MATERIALS AND METHODS

Chemicals. BSA (RIA grade), Tween 20, horseradish peroxidase (type VI), 2,2'-azino-di-3-ethyl-benzthiazoline-6-sulfonate, water-soluble 1-ethyl-3,3-dimethylaminopropyl carbodiimide, and hydrogen peroxide were purchased from Sigma Chemical Co., St. Louis, Mo. Sephadex G-15 fine was a product of Pharmacia Fine Chemicals, Uppsala, Sweden. Sodium borohydride was obtained from Fisher Scientific, Pittsburgh, Pa. Complete (Difco 3110-60-5) and incomplete (Difco 0639-60) Freund adjuvants were purchased from Difco Laboratories, Detroit, Mich. ^{3}H-afla B_1 (15 Ci/mmol) was obtained from Moravek Biochemicals, City of Industry, Calif. Albino rabbits were supplied by Klubertanz's Rabbit Farm, Edgerton, Wis., and tested to be *Pasteurella* spp. negative before use. Afla B_1, B_2, G_1, G_2, and M_1 and aflatoxicol were either prepared from *Aspergillus* cultures directly or by chemical conversions previously described (3, 12, 19, 20). Afla B_{2a} was prepared from afla B_1 according to the method of Pohland et al. (16).

Preparation of afla B_{2a}-protein conjugates. The afla B_{2a} was conjugated to BSA and peroxidase by a modification of the reductive alkylation method reported earlier (1). A 20-mg amount of afla B_{2a} was dissolved in 3.0 ml of methanol and added to 200 mg of BSA in 40 ml of 0.05 M sodium phosphate buffer (pH 7.2). The reaction mixture was then incubated at 37°C for 30 min. This was followed by the addition of

2.0 ml of sodium borohydride ($NaBH_4$) solution (0.013 M) and then incubation for another 30 min at 4°C. One milliliter of 0.1 N HCl was added to the reaction mixture with gentle stirring to destroy excess $NaBH_4$. The protein solution was passed through a Sephadex G-15 column (2 by 40 cm) by using 0.05 M phosphate buffer (pH 7.2) as the eluant. The protein fractions were pooled together and dialyzed for 24 h against 1 liter of 0.05 M phosphate buffer (pH 7.2) and 24 h against distilled water. The product was lyophilized in 30-mg batches, yielding a bright yellow powder.

The same procedure was used for afla B_{2a}-peroxidase conjugation, except that 5 mg of horseradish peroxidase (Sigma; 370 U/mg) was conjugated to 0.1 mg of afla B_{2a}, and that product was dialyzed for 48 h against several changes of 0.05 M phosphate buffer (pH 7.2) instead of being passed through a Sephadex column. By using this method, approximately 12 and 2.5 mol of afla B_{2a} were covalently bound to 1 mol of BSA and peroxidase, respectively.

Production of afla B_{2a} antisera. Five New Zealand white female rabbits were immunized against afla B_{2a}-BSA by a modification of the method of Chu and Ueno (8). For initial injections, each rabbit received 2 ml of emulsion containing 1 volume of afla B_{2a}-BSA (400 μg) in saline and 3 volumes of Freund complete adjuvant. Booster injections were given in the thigh muscles by using 2 ml of an emulsion prepared by mixing 1 volume of the antigen (400 μg) with 2 volumes of incomplete adjuvant. The rabbits were bled via the marginal ear vein at weekly intervals, and the immunoglobulin G (IgG) from blood was purified by the ammonium sulfate precipitation method of Herbert et al. (10).

Antibody titer determination by RIA. Antibody titers were determined by a binding RIA method (9). The titer was defined as the reciprocal of the antibody dilution required for 50% binding of 10,000 cpm of ^{3}H-afla B_1 with a specific activity of 15 Ci/mmol. The specificity of antibody against afla B_1 was determined by a competitive binding assay (9).

Antibody titer determination by ELISA. The enzyme immunoassay used was a modification of the method developed by Saunders and co-workers (17, 18) for the detection of staphylococcal enterotoxin A. A report has been published describing the use of this technique in the assay of antiserum raised against afla B_1-*O*-carboxymethyloxime-BSA conjugates (15). Polystyrene microtest plates (Falcon 3040) were prepared by air drying 50 μl of 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 normal saline for 30 min, washed exhaustively in distilled water, and then allowed to air dry overnight. Purified antiserum was diluted in 0.1 M phosphate buffer (pH 7.5), and 50-μl samples were added to each well. The plates were dried under a current of forced air at room temperature. Microtest plates prepared in this manner could be stored for as long as 2 weeks. Before use, plates were washed three times in 0.1 M phosphate buffer (pH 7.5) containing normal saline and 0.05% (vol/vol) Tween 20.

For determination of the antibody titer, afla B_{2a}-peroxidase conjugate (1 mg/ml) was diluted (1:100) in 0.1 M phosphate buffer (pH 7.5) containing 1.0% BSA. Samples (50 μl) were then added to microtest plate wells containing serial dilutions of antiserum. Plates were incubated for 2 h at 37°C, washed three times in phosphate-buffered saline–Tween 20 (0.2 ml per well), and reacted for 5 min with 0.1 ml of substrate consisting of 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. The reaction was terminated by the addition of 0.1 ml of hydrofluoric acid-edetic acid stopping reagent (17). 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. The ELISA titer was defined as the serum dilution which gave a higher absorbance than the pooled preimmune control serum in the first dilution.

For displacement, 25 μl of afla in phosphate-buffered saline was added to microtest plate wells previously treated with a 1:100 dilution of antibody. This was followed by the addition of 25 μl of a 1:100 dilution of the afla B_{2a}-peroxidase conjugate containing 1.0% (vol/vol) BSA in phosphate-buffered saline. Plates were incubated, and bound enzyme was determined as described above.

RESULTS

Production of afla B_{2a} antibody. Antibody levels in rabbits immunized with afla B_{2a}-BSA conjugate were monitored by a binding RIA in which ^{3}H-afla B_1 was used as the marker ligand. The results obtained for three rabbits (Fig. 1) indicate that antibody titers were demonstrable as early as 3 weeks after immunization. The highest titers were obtained 13 weeks after the

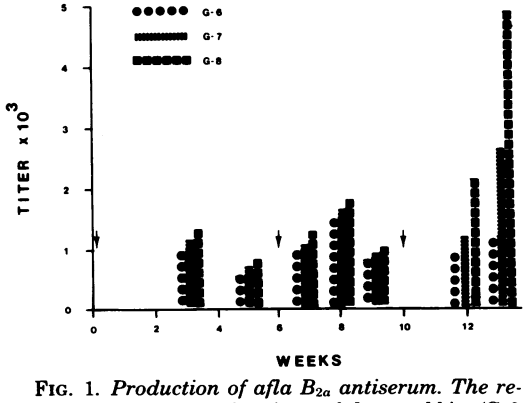

FIG. 1. *Production of afla B_{2a} antiserum. The results show representative titers of three rabbits (G-6, G-7, and G-8) as determined by RIA. Arrows indicate initial immunization (week 0) and subsequent booster injections (weeks 6 and 10).*

initial immunization, during the course of which two booster injections were given.

Determination of antibody titers by ELISA. Afla B_{2a} antiserum preparations were also titrated by an ELISA technique in which an afla B_{2a}-peroxidase conjugate was incubated with serial dilutions of the antiserum, and total bound enzyme was then determined. Typical results obtained from the ELISA titration of three rabbit antisera are shown in Fig. 2. Dilutions of the preimmune control serum showed negligible binding of the afla B_{2a}-peroxidase conjugate. Only a low degree of nonspecific binding was detected when serial dilutions of the pooled rabbit antisera were tested against control peroxidase (unconjugated). The three antiserum preparations exhibited visually distinct titration endpoints as well as maximal absorbance plateaus at the low dilutions of antibody. These results indicate that the peroxidase conjugate bound in a highly specific manner.

Titers determined for afla B_{2a} antiserum by ELISA and RIA showed close correlation. For example, the titers obtained for three rabbit antisera by the ELISA method (Fig. 2) were 1,280, 2,560, and 5,120, respectively. The titers measured by the RIA method for the same rabbits were found to be 1,140, 2,630, and 4,880, respectively. These results suggest that the ELISA could be used as a means for routinely titrating afla B_{2a} antibody. Similar results were found when afla B_1 oxime antibody titers determined by RIA and ELISA were compared (15).

Specificity and sensitivity of afla B_{2a} antibody. Figures 3 and 4 show the sensitivity and specificity of the afla B_{2a} antibody as determined by competitive RIA and ELISA, respectively. The sensitivity for afla B_1 detection with this antibody in the RIA fell in the range of 0.1 to 10 ng per assay and was comparable to previously published results for afla B_1 oxime antibody (15). The limits for afla B_1 detection by ELISA were between 0.01 and 10 ng per assay. These data show that ELISA was nearly 10 times more sensitive than RIA and allowed for a greater range of detectability for afla B_1. The RIA competition curve determined for the various afla analogs indicated that the assay was most specific for afla B_1 and showed limited cross-reactivity with the other forms as follows: afla B_1 > afla B_{2a} > afla M_1 > afla G_1 > afla G_2 > aflatoxicol (Fig. 3). Although a similar range of specificities was observed for ELISA, the assay showed greater reactivity with afla B_{2a} than with afla B_1 (Fig. 4). The effective displacement by afla B_{2a} in ELISA was most likely due to the use of afla B_{2a}-peroxidase as the marker ligand rather than free afla B_1 as was used in RIA.

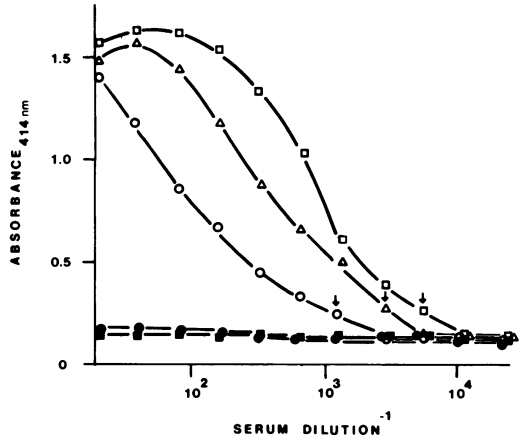

FIG. 2. *ELISA titration of afla B_{2a} antiserum. Titrations were performed as described in the text. Serum dilutions were plotted on a logarithmic scale. Symbols:* (○) *G-6 antiserum;* (△) *G-7 antiserum;* (□) *G-8 antiserum;* (●) *pooled G-6, G-7, and G-8 preimmune sera;* (■) *pooled G-6, G-7, and G-8 antisera assayed with control horseradish peroxidase (unconjugated). Arrows indicate wells used for determining titers.*

DISCUSSION

Immunoassays provide simple alternatives for

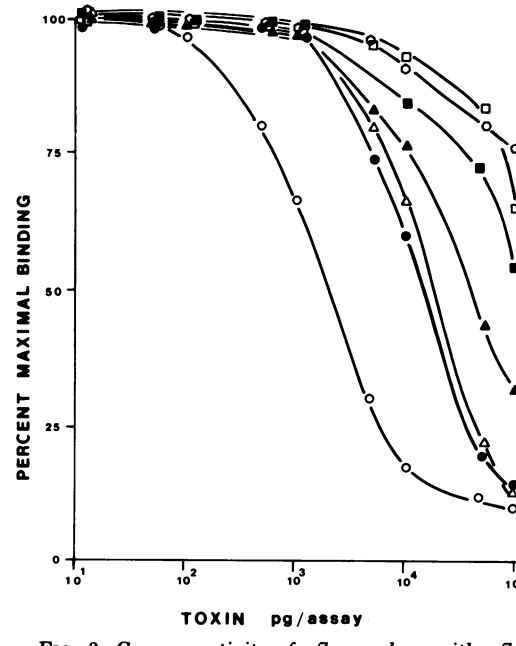

FIG. 3. *Cross-reactivity of afla analogs with afla B_1 antibody in competitive RIA. A dilution of G-8 antiserum to give 50% maximal binding was used. Symbols:* (○) *afla B_1;* (△) *afla B_2;* (□) *aflatoxicol;* (■) *afla G_1;* (○) *afla G_2;* (▲) *afla M_1;* (●) *afla B_{2a}.*

the analysis of mycotoxins when compared with high-performance liquid chromatography (19) and thin-layer chromatography (11). Methods for the production of antiserum specific for several mycotoxins, including afla B_1 (8, 13, 14), afla M_1 (9), ochratoxin A (5), and T-2 toxin (6), have been developed in our laboratory and others. A number of quantitative immunoassays for afla B_1 have also been reported (8, 13). For quantitation of afla B_1 in peanut buffer, corn, wheat, urine, and serum (13, 19), afla B_1 oxime antiserum is generally used. Little attention has been paid to an alternate approach in which antiserum is produced against an afla B_{2a}-BSA conjugate (14). Since two completely different approaches are used to produce the afla immunogens for the preparation of afla B_{2a} antiserum and afla B_1 oxime antiserum, obvious differences must exist in the quality and specificity of the two types of hyperimmune sera. In this report, we have described a new method for the preparation of afla B_{2a} antiserum and have determined both its specificity and sensitivity in RIA and ELISA.

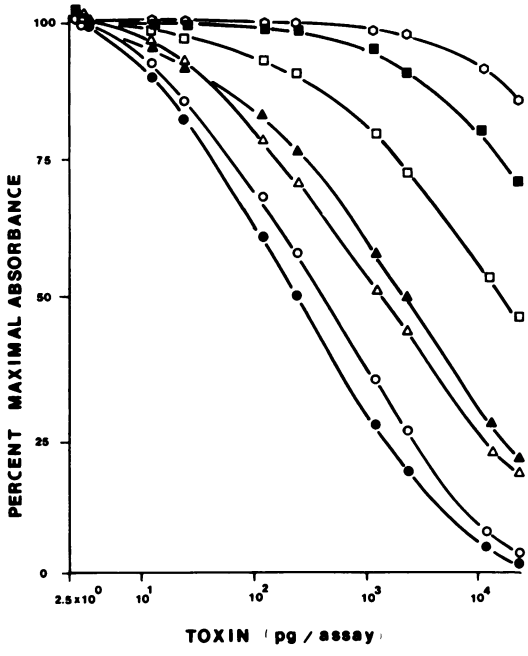

FIG. 4. *Cross-reactivity of afla analogs with afla B_1 antibody in competitive ELISA. A 1:400 dilution of G-8 antiserum was used. Symbols:* (○) *afla B_1,* (△) *afla B_2;* (□) *aflatoxicol;* (■) *afla G_1;* (○) *afla G_2;* (▲) *afla M_1;* (●) *afla B_{2a}.*

A comparison of RIA and ELISA specificity studies for afla B_{2a} and afla B_1 oxime antiserum which was complied from this paper and previous reports (8, 15) is shown in Table 1. Immune reactivity is represented by the total nanograms of toxin necessary to cause 50% inhibition of binding in the assay as well as by comparison with afla B_1. The two ELISA methods, although more sensitive than the RIA procedures, were also subject to more cross-reactivity with the afla analogs. In both the RIA and ELISA, afla B_{2a} antiserum and afla B_1 oxime antiserum showed different spectra of cross-reactivity for the various toxins. This effect was due to the different techniques used in preparing the BSA immunogen. For afla B_{2a} antibody, BSA was coupled to the difuran moiety of the afla molecule by reductive alkylation. The cyclopentenone ring of afla B_{2a} thus acted as the immunodominant portion of the afla B_{2a}-BSA conjugate and was critical in determining the specificity of the hyperimmune sera. Hence, the afla B_{2a} antibody showed negligible binding with afla G_1, afla G_2, and aflatoxicol. The opposite was true for the afla B_1 oxime antiserum (8, 15) in which the difuran ring of afla B_1 is dominant in determining antibody specificity. In this case, the

TABLE 1. *Cross-reactivity of afla B_1 oxime and afla B_{2a} antisera to afla analogs*

Afla analogs	Afla B_1 oxime antiserum[a]		Afla B_2 antiserum[a]	
	RIA[b]	ELISA[c]	RIA	ELISA
Afla B_1	2 (1)[d]	0.25 (1)	2.5 (1)	0.21 (1)
B_2	16 (8)	0.20 (1)	20 (8)	1.25 (4)
G_1	20 (10)	0.80 (3)	120 (32)	>25 (>81)
G_2	320 (160)	19 (76)	>1,000 (>400)	>25 (>81)
M_1	>1,000 (>500)	>25 (>100)	40 (16)	25 (81)
B_{2a}	ND[e]	>25 (>100)	12 (5)	0.25 (1)
Aflatoxicol	80 (40)	0.15 (1)	200 (80)	18.80 (61)

[a] Nanograms of toxin which gave 50% inhibition of binding. For details of experiments, see the text.
[b] Data from Chu and Ueno (8).
[c] Data from Pestka et al. (15).
[d] Number within parentheses indicates the ratio of cross-reactivity relative to afla B_1.
[e] ND, Not determined.

antiserum does not bind with afla M_1 or afla B_{2a} but shows significant reactivity with afla B_2, G_1, and aflatoxicol.

The results presented herein show that afla B_{2a} antiserum could be used for the analysis of afla B_1 by either RIA or the more sensitive ELISA. The RIA and ELISA presented here have detection limits similar to those previously reported by this laboratory (8, 15) for afla B_1 oxime antibody. The new afla B_{2a} ELISA was preferable to that reported by Lawellin et al. (14) on the basis of assay time, color development, and laboratory safety. Since afla B_{2a} antiserum was directed against the cyclopentenone ring of the aflatoxin molecule and showed negligible cross-reactivity with afla G_1 and afla G_2, it might be more effective for the analysis of afla B_1 than afla B_1 oxime antibody.

Reactivity of the afla B_{2a} antiserum with afla B_2 should not greatly affect assay precision in food products because afla B_2 is present at very low levels relative to afla B_1 in natural contamination. The presence of afla B_{2a} and afla M_1, could, however, interfere with an afla B_{2a} immunoassay. This problem could be overcome by a Sep-Pak silica gel treatment to separate afla B_1 from afla B_{2a} and afla M_1 or by employment of another immunoassay with afla B_1 oxime antibody (8, 15) or afla M_1 antibody (9).

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