Production of Antibody Against Aflatoxin B_1

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Antibody against aflatoxin B_1 was obtained after one multiple-site injection of bovine serum albumin-aflatoxin B_1 conjugate into rabbits. The antibody has greatest binding efficiency for aflatoxin B_1, less efficiency for B_2, G_1, and Q_1, and least for aflatoxicol, G_2, and M_1. Sterigmatocystin, coumarin, and 4-hydroxycoumarin did not give a cross-reaction with the antibody. The sensitivity of the binding assay for detection of aflatoxin B_1 is in the range of 0.2 to 2.0 ng per 0.5-ml sample. Detailed methods for the preparation of the conjugate, production of immune serum, and methods for antibody titer determination are described.

Aflatoxins are a group of toxic metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Among this group of toxins, aflatoxin (Afla) B_1 was found to be one of the most potent environmental carcinogens. The biological and toxicological properties of aflatoxin and the impact of this series of mycotoxins on human and animal health have been well documented (6, 7, 12). Because of the widespread occurrence of toxin-producing fungi, and of the occurrence of the toxin in a number of agricultural commodities, efforts were made by various investigators to develop new methods for toxin detection. Methods for aflatoxin analysis used currently involve chemical analysis, biological assay, or a combination of both (6, 7, 11). These methods, however, are either lacking in specificity or limited in sensitivity. More recently, Langone and Van Vunakis (8) described radioimmunoassays for aflatoxins and suggested that these assays were sufficiently sensitive and specific to determine the toxin in contaminated foodstuffs and biological samples. Along with our immunochemical studies on ochratoxins (3), we have also investigated the possibility of production of an antiserum specifically against aflatoxins using an approach different from that of Langone and Van Vunakis (8). In this paper, details of the preparation of aflatoxin-protein conjugates and the method for antiserum production are described. The properties of the antibody obtained in our laboratory are compared with those of the antibody obtained by Langone and Van Vunakis (8).

MATERIALS AND METHODS

Materials. Aflatoxins B_1, B_2, G_1, and G_2 were prepared by the method of Chu (2) and by preparative thin-layer chromatography (TLC). Aflatoxin M_1 and sterigmatocystin were kindly supplied by R. D. Stubblefield, E. B. Lillehoj, and A. Ciegler of the Northern Regional Research Laboratory, U. S. Department of Agriculture, Peoria, Ill. Tritiated Afla B_1 (^{3}H-labeled Afla B_1, 13 Ci/mmol) was obtained from Moravek Biochemicals (City of Industry, Calif.). Water-soluble carbodiimide 1-ethyl-3,3-dimethylaminopropyl-carbodiimide (EDPC) was obtained from Story Chemical Corp., Muskegon, Mich. Bovine serum albumin (BSA) and polylysine (PL, molecular weight 30,000) were products of Sigma Chemical Co., St. Louis, Mo. 4-Hydroxycoumarin (4-OH coumarin) was kindly provided by C. Schroeder of WARF Institute, Madison, Wis. Coumarin was obtained from ICN-K and K Laboratories, Inc., Cleveland, Ohio. Complete Freund adjuvant and *Mycobacterium tuberculosis* (H37Ra) were purchased from Difco Laboratories, Detroit, Mich. Pertussis vaccine was an Eli Lilly product (Indianapolis, Ind.). Diethylaminoethylcellulose was obtained from Schleicher and Schuell, Inc., Keene, N.H. Albino rabbits were obtained from Klubertanz's Rabbit Farm, Edgerton, Wis., and tested to be *Pasteurella* negative before use. All other chemicals were either reagent grade or chemically pure.

Preparation of Afla-protein conjugates. Since Afla B_1 has no reactive group for coupling reactions, Afla B_1 was first converted to Afla B_1-O-carboxymethyloxime (Afla B_1-oxime) by the method described by Dean et al. (5) in their steroid work. The preparation gives a single spot in TLC and has an R_f of 0.20 in a 10% acid-benzene solvent system. The maximum absorption of Afla B_1-oxime was found to be at 362 nm in methanol with a molar absorptivity of 20,950. Details for the preparation of Afla B_1-oxime and the chemical and physical properties are described elsewhere (4).

Antigens were prepared by conjugation of Afla B_1-oxime with BSA or PL in the presence of EDPC. In a typical experiment, 10 mg of BSA in 1 ml of distilled water was added to 20 ml of Afla B_1-oxime solution (6 mg of Afla B_1-oxime in 25% ethanol-water) containing 376 mg of EDPC. The reaction mixture was stirred at room temperature, in complete darkness, for 48 h. Two additional 376-mg portions of EDPC

were made to the reaction mixture during this period. After reaction, the mixture was dialyzed against 3 liters of distilled water and changed daily for 5 days. The final solution was passed through a membrane filter (Millipore Corp.) and kept frozen before being used for immunization. The amount of Afla B_1-oxime conjugated to the BSA was determined by a spectrophotometric method using the molar absorptivity of 20,950 at 362 nm. Data obtained from both the unreacted toxin and the toxin conjugated to protein were used for final calculation.

Immunization schedule and antibody titer determination. For immunization, the multiple-site intradermal method of Nieschlag et al. (10) was followed, except that pertussis vaccine was omitted as an additional adjuvant in the initial immunization. In summary, a water-in-oil emulsion was prepared by using a sterilized Potter-Elvehjem type homogenizer and complete Freund adjuvant (1:2 to 1:3 [vol/vol]) but incorporating additional dried *M. tuberculosis* (20 mg per rabbit, or 10 mg per ml of emulsion). Sufficient antigen was dissolved in 0.9% NaCl solution, prior to making the emulsion, so that each milliliter of final emulsion would contain between 25 and 105 μg of protein (1.5 to 6 μg of Afla B_1). The back of each rabbit was shaved and 2 ml of the emulsion was injected intradermally into each rabbit at 20 to 40 sites along the shaved area. Trial bleedings via the marginal ear vein were made once every week, starting 3 weeks after immunization. Except when otherwise stated, no booster injections were made after initial injection.

For antibody titer determinations, a binding assay method was used (3). Antisera obtained from immunized rabbits were precipitated two times with $(NH_4)_2SO_4$ at a final saturation of 33.3%. The precipitate was redissolved in sufficient 0.02 M sodium phosphate buffer (NaPB), at pH 7.4, to give a volume equal to that of the original serum sample. Appropriate dilutions (at least four serial dilutions) of the preparation were made for binding studies. In general, 0.5 ml of each diluted antibody solution was dialyzed against 0.5 ml of ^{3}H-labeled Afla B_1 in 0.02 M NaPB, pH 7.4 (approximately 8,400 counts/min per 0.5 ml), in a cold room in complete darkness overnight. The radioactivities of 0.4-ml portions from both sides were counted after dialysis. The degree of binding was then determined. Antibody titer was defined as the reciprocal of the dilution which was required for 50% binding of ^{3}H-labeled Afla B_1 under the stated conditions.

Determination of specificity of antibody against aflatoxins. The method for determining specificity of antibody against aflatoxin was essentially the same as those we have previously described for the immunochemical study for ochratoxins (3). Immunoglobulin G (IgG) was purified from the antisera and was used in the equilibrium dialysis studies (3). Three-tenths or five-tenths milliliter of IgG-^{3}H-labeled Afla B_1 complex (60% binding) was dialyzed against either equal volume of 0.02 M buffer (pH 7.4) or unlabeled aflatoxins of different concentrations. The degree of displacement of radioactivity of the IgG-^{3}H-labeled Afla B_1 complex was then determined.

Determination of radioactivity and protein concentration. For radioactivity determination, an appropriate amount (in general less than 0.5 ml) of the test solution was mixed with 10 ml of Bray solution (1), and the radioactivity was counted in a Beckman model LS-330 liquid scintillation spectrometer. The protein concentration was determined by the Lowry method (9) using BSA as the protein standard.

RESULTS AND DISCUSSION

Aflatoxins are low-molecular-weight, toxic fungal metabolites and thus are devoid of antigenicity. The toxins also lack a reactive group for the coupling of the toxin to a macromolecule carrier for antibody production. However, through derivation, a free carboxylic group is introduced to the toxin molecule, thus permitting the molecule to react covalently with proteins (Fig. 1). Our results showed that 10 and 13 mol of Afla B_1-oxime were successfully coupled to 1 mol of PL and BSA, respectively.

Immunization of rabbits with these two Afla B_1-protein conjugates revealed that BSA-Afla B_1-oxime was a good antigen (Fig. 2). Antibody titers were demonstrated 3 weeks after immunization, and maximum production occurred in 5 to 8 weeks. Among five rabbits tested, three produced usable antisera (titers more than 1,000) in 5 to 8 weeks. Subsequent experiments using the highest antigen concentration (210
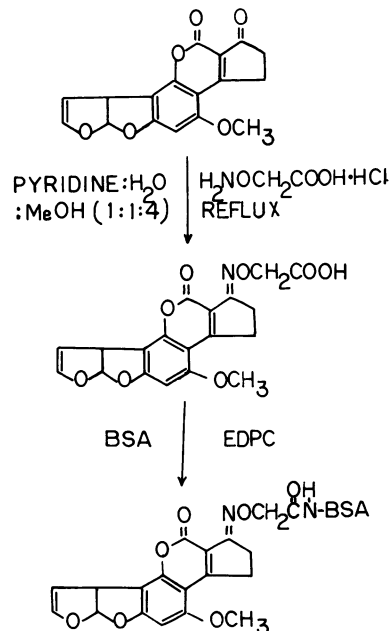

FIG. 1. *Schematic diagram for the preparation of BSA-Afla B_1-oxime conjugate. Abbreviations are as follows: BSA, bovine serum albumin; EDPC, 1-ethyl-3,3-dimethylaminopropyl-carbodiimide.*

μg per rabbit) showed that all of the 11 rabbits tested gave high antibody titers.

PL-Afla B$_1$-oxime was not a good antigen. The antisera obtained from the three rabbits, 4 weeks after being immunized with 250 μg each, bound less than 30% of ^{3}H-labeled Afla B$_1$ at a 1-to-5 dilution. A booster injection with 250 μg of PL-Afla B$_1$-oxime to each of the rabbits was performed on the fifth week, along with the simultaneous injection of 0.5 ml of pertussin vaccine. Again, no improvement in the antibody production was observed. This result is different from that of Langone and Van Vunakis (8). The discrepancy may be due to either the different injection method or the molecular size of polylysine. The molecular weight of polylysine used in the present study (30,000) was considerably smaller than that used by Langone and Van Vunakis (8) (90,000).

The specificity of antisera against Afla B$_1$, produced by rabbits after a multiple-site injection of BSA-Afla B$_1$ conjugate, was determined by a competitive binding assay by using purified IgG fraction as described by Chu et al. (3), and the results are shown in Fig. 3. The concen-

FIG. 3. Effect of different aflatoxins on the binding of ^{3}H-labeled Afla B$_1$ with IgG. Three-tenths to five-tenths milliliter of an Afla B$_1$ (15,000 to 20,000 counts per min)-IgG (30 μg/ml) mixture, in 0.02 M NaPB, pH 7.4, was dialyzed against equal volumes of different unlabeled aflatoxins, at different concentrations. The extent of binding of ^{3}H-labeled Afla B$_1$ with IgG in the absence of unlabeled toxin, i.e., by dialyzing against buffer alone, was considered as 100% of binding. The letters B$_1$, B$_2$, G$_1$, G$_2$, B$_1$-O, and M$_1$ represent different aflatoxins. Ster. represents sterigmatocystin.
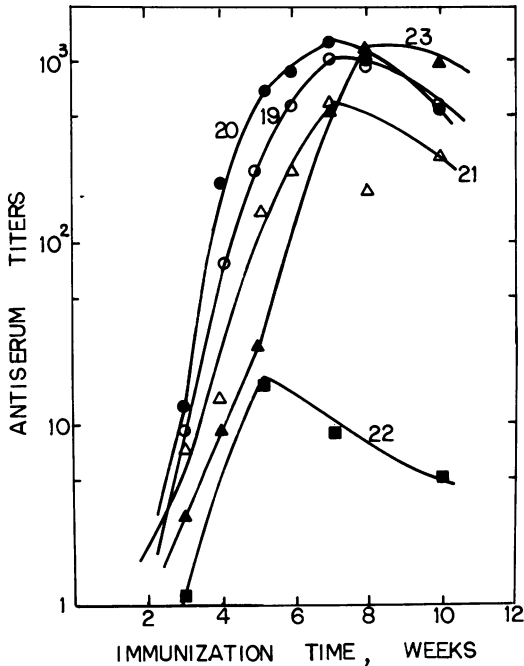

FIG. 2. Production of antibody against aflatoxins. The rabbits were immunized once with 50 (rabbit no. 23), 100 (no. 21 and 22) and 210 (no. 19 and 20) μg of BSA-Afla B$_1$-oxime. Antisera titers were expressed as the reciprocal of the serum dilution required for 50% binding of ^{3}H-labeled Afla B$_1$ (8,400 counts/min in 0.5 ml of solution).
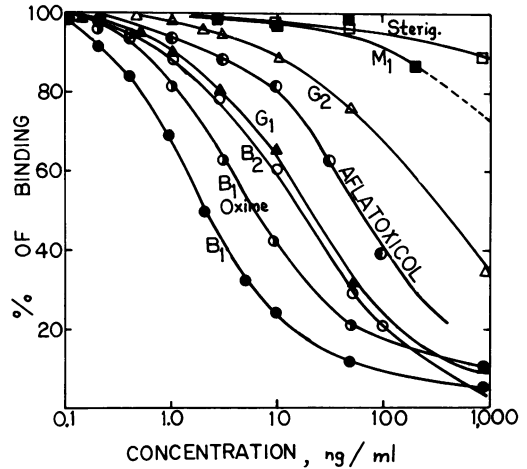
tration to give 50% inhibition of binding of ^{3}H-labeled Afla B$_1$ with IgG by unlabeled aflatoxins B$_1$, B$_1$-O-carboxymethyl-oxime, B$_2$, G$_1$, Q$_1$ (not shown in Fig. 3), aflatoxicol, G$_2$, and M$_1$ were 1.8, 6, 16, 20, 30, 80, 320, and >1,000 ng/ml, respectively. Sterigmatocystin, coumarin, and 4-OH-coumarin had little or no effect on the binding of ^{3}H-labeled Afla B$_1$ with IgG at the concentrations tested (up to 1,000 ng/ml). In the binding system described, the volume of the sample tested varied between 0.3 and 0.5 ml. A total of 5,000 to 10,000 counts/min of radioactive Afla B$_1$ was used. Therefore, the sensitivity for Afla B$_1$ detection in our system falls in the range of 0.2 to 2.0 ng. The sensitivity, however, can be improved by using higher-specific-activity ^{3}H-labeled Afla B$_1$.

In the present study, a small amount of antigen has been shown to be sufficient for production of antisera against Afla B$_1$. The rapid increase in antibody titers within a 2-month period is in agreement with the original work for the production of antibody against steroid hormones (10). However, antibody titers begin to decrease 6 weeks after initial injection, and the response for individual rabbits to the antigen also appears to vary considerably. Since Langone and Van Vunakis (8) did not report the details for antibody production such as the time

required to evoke useful antisera as well as the antisera titers, it is difficult to compare their method with the present procedures. Nevertheless, the present method was found to be better than the single injection technique described earlier (3), by which we were unable to obtain useful antibody titers against aflatoxins for radioimmunoassay purposes (S. H. Ashoor and F. S. Chu, unpublished observation).

Although the present study confirmed the investigation of Langone and Van Vunakis (8) that Afla B_1-protein (polypeptides) conjugates are capable of producing antibodies against Afla B_1, our results suggest that BSA-Afla B_1 is a better antigen than the polylysine-Afla B_1. Whereas the sensitivity of antibodies obtained in both laboratories using either BSA-Afla B_1 or polylysine-Afla B_1 falls in the same range, the antibody obtained in the present study using BSA-Afla B_1 as antigen appears to be more specific for Afla B_1. Langone and Van Vunakis (8) reported that aflatoxins B_2, G_1, and G_2 inhibited the binding of ^3H-labeled Afla B_1 (determined at the 50% inhibition level) 2.3, 6.6, and 21 times less effectively than B_1, but the present study, using the antibody produced from BSA-Afla B_1, shows that aflatoxins B_2, G_1, and G_2 are 9, 11, and 89 times less effective. In addition, we found that the binding of two other major Afla B_1 metabolites, namely aflatoxicol and aflatoxin M_1, with the antibody varied considerably. Aflatoxicol, a metabolite formed by reduction of the carbonyl group in the cyclopentenone of Afla B_1, binds about 40 times less than Afla B_1; but aflatoxin M_1, which is formed by hydroxylation at the 4 position in the dihydrofuran part of the Afla B_1 structure, lost practically all of the binding capability. These results indicate conformation of aflatoxins greatly affecting their interaction with the antibody against Afla B_1.

Whereas the antibody obtained in the present study is specific for Afla B_1, aflatoxins B_2 and G_1 also show some cross-reactivity at a concentration 10 times greater than that of B_1. since Afla B_2 is generally produced in much smaller quantities relative to B_1, B_2 may not present much of a problem in the application of radioimmunoassay for Afla B_1. Aflatoxin G_1, on the other hand, may affect the assay if the concentration is proportionally greater than that of B_1. Problems currently under investiga-

tion in our laboratory include the development of a solid-state radioimmunoassay for Afla B_1, the development of antibodies specifically against other aflatoxin derivatives and metabolites, and the in vivo neutralization of the Afla B_1 toxic effect in rabbits immunized with the Afla B_1-BSA conjugate.

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