Production and characterization of antibodies cross-reactive with major aflatoxins

G. Zhang and F. S. Chu

Food Research Institute and Department of Food Microbiology and Toxicology, University of Wisconsin-Madison, Madison (Wisconsin 53706, USA)

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Summary. Antibodies cross-reactive with 4 major aflatoxins were demonstrated three weeks after immunization of rabbits with an immunogen which was prepared by conjugating aflatoxin B_3 to bovine serum albumin. Aflatoxin B_3 was first converted to its hemisuccinate before conjugation to the protein. Tritiated aflatoxin B_1 (AFB_1) was used as the marker ligand both for antibody titer determination as well as for analysis of antibody specificity. Competitive RIA revealed that the antibodies have good cross-reactivity with aflatoxins B_1, B_2, G_1, and G_2 when tritiated AFB_1 was used as the marker ligand. The concentrations causing 50% inhibition of binding of ^{3}H-AFB_1 to the antibodies by unlabeled aflatoxins B_1, B_2, G_1, G_2 and B_3 were found to be 0.25, 3.34, 0.32, 4.0 and 0.53 ng/assay, respectively. The antibodies could be used for simultaneous analysis of aflatoxins B_1 and G_1, two of the most important toxic metabolites produced by *Aspergillus flavus* and *A. parasiticus*.

Key words. Aflatoxins; immunoassay; antibody; rabbit; cross-reactivity.

Aflatoxins (AFs) are a group of toxic secondary metabolites produced by *Aspergillus flavus* and *A. parasiticus*. Aflatoxin B_1 (AFB_1), the most toxic compound in this series, has been found to be one of the most potent carcinogens occurring naturally[1]. Because of frequent contamination of AFB_1 in agricultural commodities such as peanuts, corn, and animal feedstuffs, aflatoxin problems become a potential hazard to human and animal health[1]. Recent investigations have led to the production of specific antibodies against AFB_1 and several simple, sensitive and specific immunoassay methods for the analysis of aflatoxin in different commodities have been developed[2–4]. However, depending on the approaches that have been used for raising antibodies, the degree of cross-reactivity of these antibodies with their respective structural analogs varied considerably[2–4]. The accuracy of immunoassay of aflatoxins for the naturally-contaminated samples generally is affected by the cross-reactivity of antibodies with different types of aflatoxins present in the sample. For example, if the antibodies used in the immunoassay are 100% cross-reactive with AFB_1, but only 33% with AFG_1[5], the presence of a large amount of AFG_1 in the sample would result in a lower 'apparent' total aflatoxin level by the immunoassay method. Thus, interpretation of immunoassay data should be exercised carefully if the samples contain structurally-related mycotoxins. This problem was illustrated in a recent collaborative study in which equal amounts of AFB_1 and AFG_1 were present in the test samples[6].

To overcome the cross-reactivity problem of antibodies with structural analogs in the immunoassays, we have undertaken the task of developing a generic antibody against mycotoxins within a group of mycotoxins. Our objective is to develop antibodies which have equal cross-reactivity with the most important mycotoxins within a group of mycotoxins. Such antibodies could then be used for monitoring all the toxins within a group of compounds. For example, a method for the production of generic type of antibodies against group A type trichothecenes was recently developed in our laboratory[7]. These antibodies have good cross-reactivity with most mycotoxins in the group A trichothecenes[7]. In the present study, a method to produce polyclonal antibodies that have almost equal cross-reactivity with AFB_1 and AFG_1 has been developed. Details for the production and characterization of these new antibodies are presented in this paper.

Materials and methods. Aflatoxins B_1, B_2, G_1, G_2 were produced by *Aspergillus parasiticus* NRRL 2999 and were purified according to the method of Chu[8]. Aflatoxin B_3 was either prepared from *A. parasiticus* culture according to the method of Heathcote and Dutton[9] or prepared from AFG_1 according to the method of Cole and Kirksey[10]. An AFB_3 standard was kindly supplied by Dr Cole of the National Peanut Laboratory, USDA. Tritiated AFB_1 (14 Ci/mmol) was obtained from Moravek Biochemicals, City of Industry, CA. Bovine serum albumin (BSA, RIA grade) was purchased from Sigma Chemical Co. (St. Louis, MO). Water soluble carbodiimide, i.e. 1-ethyl-3,3-dimethylaminopropyl-carbodiimide (EDPC) and succinic anhydride (SA) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Complete Freund's adjuvant containing *Mycobacterium tuberculosis* (H 37 Ra) and incomplete Freund's adjuvant were obtained from Difco Laboratories (Detroit, MI). Albino rabbits (female) of approx. 2-kg size were purchased from Smith's Rabbitry (Seymour, WI). The health status of the rabbits were analyzed by a swab test; rabbits demonstrated to be *Pasteurella* negative were selected for immunization. All chemicals and organic solvents were reagent grade or better.

Preparation of immunogen: The protocol for the preparation of immunogen is summarized in figure 1. Aflatoxin B_3 was first converted to the AFB_3-hemisuccinate (AFB_3-HS) under conditions similar to those for the preparation of T-2 toxin to its hemisuccinate in the presence of dimethylaminopyridine (DAMP)[11]. Conjugation of AFB_3-HS to BSA was then carried out in the presence of a water soluble carbodiimide (EDPC) by the procedures previously described for T-2 toxin[11]. The molar ratio of hapten to carrier protein in the reaction mixture was 26. After reaction and dialysis to remove the free AFB_3-HS, the molar ratio was found to be around 10 as determined according to the method of Habeeb[12].

Production of antibody: The immunization schedule and methods of immunization were essentially the same as those described for T-2 toxin[12] by the multiple injection technique. Three rabbits were used. Each rabbit was injected intradermally with 500 µg of the immunogen in 1.0 ml of 0.1 M sodium phosphate buffer (pH 7.4) containing 0.85% NaCl (PBS), (emulsified with 2.0 ml of complete Freund's adjuvant) on the back of each rabbit (40 sites) using the multiple-injection technique[5]. For booster injections, 500 µg of antigen in 1.0 ml of PBS and 2.0 ml incomplete Freund's adjuvant was used and the injection was made in the thighs (4 sites). The collected antisera were precipitated with $(NH_4)_2SO_4$ to a final saturation of 33.3% using a 100% saturated $(NH_4)_2SO_4$ solution. The precipitates were redissolved in water and reprecipitated twice with $(NH_4)_2SO_4$ solution. Finally, the precipitates were reconstituted to half of the original volume with distilled water, dialyzed against distilled water for 0.5–1.0 h (membrane cut-off was 10,000 daltons), against 0.01 M PBS overnight at 6 °C, and then lyophilized.

Radioimmunoassay (RIA): Protocols for RIA were essentially the same as those described for AFB in which an ammonium sulfate precipitation method was used to separate

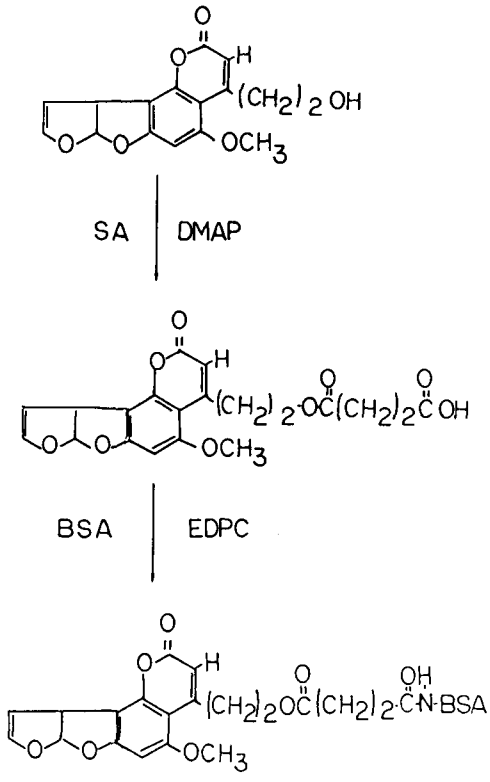

Figure 1. Preparation of AFB_3-HS-BSA conjugate. Aflatoxin B_3 was first converted to AFB_3-hemisuccinate (HS) in the presence of succinic acid anhydride (SA) and diemthylaminopyridine (DMAP) and then conjugated to BSA using EDPC as the coupling reagent to form AFB_3-HS-BSA.

the free and bound toxin[11]. In general, 50 µl of tritiated AFB_1 (10,000–12,000 dpm) was incubated with 0.15 ml of antiserum solution of various dilutions in PBS at room temperature for 30 min and then in a cold room (6 °C) for at least 1 h, followed by separation of the bound and free ligand with an ammonium sulfate precipitation method as described earlier[11]. Antibody titer was defined as the reciprocal of the antiserum volume (ml) required to give 50% binding of tritiated toxin under the conditions described.

Analysis of antibody specificity: Protocols for determination of antibody specificity were essentially the same as for the titer determination, except that various unlabelled aflatoxins at concentrations between 0.1 and 1000 ng/ml, were present in the reaction mixture. Different derivatives were first dissolved in methanol and then diluted with 0.1 M PBS (pH 7.4). The final volume of the reaction mixture was 0.2 ml, and the final methanol concentration was 5%.

Determination of radioactivity: Radioactivity was determined in a Beckman Instrument, Inc. (Fullerton, CA) model LS-5801 liquid scintillation spectrometer using 4.5 ml of Aquasol (New England Nuclear Corp., Boston, MA).

Results and discussion. Approaches used by various laboratories for the preparation of immunogens for production of antibodies against aflatoxins generally can be classified into two groups[2–4]; in the first group, aflatoxin is conjugated to a protein carrier through the carbonyl group at the cyclopentane ring of the molecules by making a carboxymethyl oxime derivative of AFB[5,13]. In the second group, conjugation is made through the dihydrofuran portion of molecule by using the hemiacetal-type derivatives[14,15]. Immunization of these immunogens in animals elicit antibodies with different specificities. The antibodies generally recognized the dihydrofuran portion of the molecule when the animals were immunized with the first group of immunogens, whereas the antibodies had a specificity directed toward the cyclopentane ring when the second group of immunogens were used. Consequently, the antibodies are highly specific to each group of aflatoxins and are useful for the determination of individual major aflatoxins. In recognizing that there is a need for generic type of antibody for the major aflatoxins, the present study was carried out. Since AFB_3 has structural features for both B and G types of aflatoxins[9,10], it was selected as the starting material for the preparation of immunogen. Results obtained from the present study indicate that the antibodies produced by this method, indeed, have cross-reaction with both aflatoxins B_1 and G_1.

Results for the responses of three rabbits during the first 14 weeks after immunization with AFB_3-HS-BSA are shown in figure 2. The rabbits responded to the immunogen very rapidly and started to elicit antibodies which were capable of binding with tritiated AFB_1 as early as three weeks after immunization. Variation in antibody production between rabbits, however, was observed. The antibody titer of the rabbit giving the best response reached a peak, with a titer around 11,000, at 8 weeks, after one booster injection.

The specificity of the antibodies obtained from rabbits that had been immunized with the new immunogen was analyzed by a competitive RIA and the results are presented in figure 3. In general, the antisera shows good cross-reactivity with

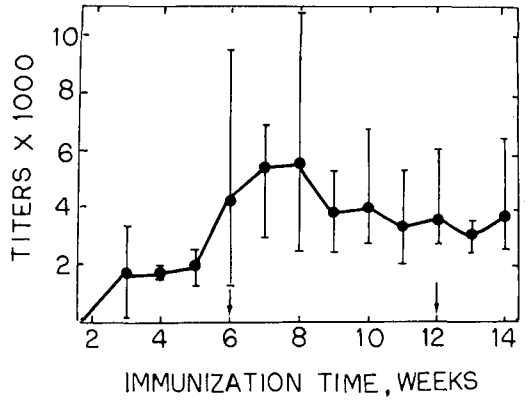

Figure 2. Antibody titers of three rabbits after immunization with AFB_3-HS-BSA. Tritiated AFB_1 was used as the marker ligand in the binding assay, and an ammonium sulfate precipitation method was used to separate the free and bound ligand. Arrows indicate booster injections. Data are the average of three rabbits with high and low range indicated as bars.

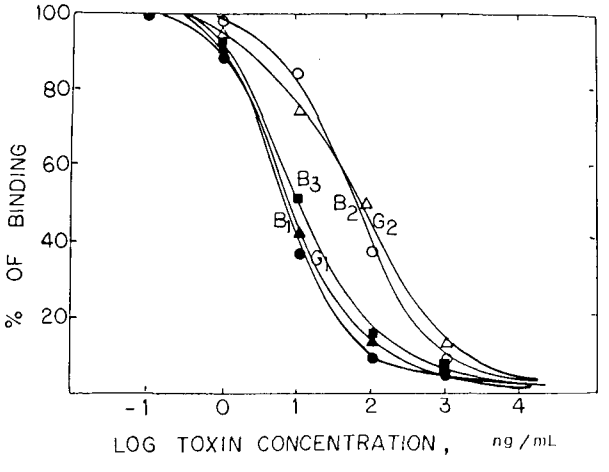

Figure 3. Effect of different aflatoxins on the binding of ^3H-AFB_1 with rabbit anti-AFB_3-HS-BSA.

most aflatoxins tested. The concentrations causing 50% inhibition of binding of ^{3}H-AFB_1 (values in the parentheses indicate relative cross-reactivity), to the anti-AFB_3-HS-BSA antibody by unlabeled AFB_1, AFB_2, AFG_1, AFG_2, and AFB_3 are found to be 0.25 (*100*), 3.34 (*7.5*), 0.32 (*78.1*), 4.0 (*6*), and 0.53 (*47.2*) ng/assay, respectively. From the displacement curve, it is apparent that the range for the detection AFB_1 and AFG_1 of the present RIA system falls between 1 and 100 ng/ml (or 0.05–5 ng/assay). Such sensitivity is within the range of most other RIA systems (0.5–5.0 ng/assay) for aflatoxins [5, 6]. Since the antibodies elicited have almost equal cross-reactivity with both B_1 and G_1, such antibodies would be very useful for simultaneous detection of both toxins. The low cross-reactivity of the antibodies with AFB_2 and AFG_2 would not limit the wide use of these antibodies for aflatoxin assay because these two aflatoxins rarely occur in agricultural commodities. Also, the toxicity and carcinogenicity of these two aflatoxins are relatively low. Since tritiated AFB_1 was used as the marker ligand in the RIA, the apparent cross-reactivity of the antibodies with AFB_3 was slightly lower than for AFB_1 and AFG_1. In view of the importance of use of this antibody for immunoassay of aflatoxins, studies such as the development of an ELISA method for simultaneous detection of both aflatoxins as well as using this new immunogen for generating monoclonal antibody for aflatoxins are currently underway in our laboratory.

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1 Busby, W. F. Jr, and Wogan, G. N., in: Food-borne Infections and Intoxications, 2nd edn, p. 519. Eds H. Riemann and F. L. Bryan. Academic Press, New York/London 1979.
2 Chu, F. S., J. Fd Prot. *47* (1984) 562.
3 Chu, F. S., in: Mycotoxins and Phytotoxins, p. 277. Eds P. S. Steyn, and R. Vleggaar. Elsevier, Amsterdam 1986.
4 Chu, F. S., in: Modern Methods in the Analysis and Structural Elucidation of Mycotoxins, p. 207. Ed. R. J. Cole. Academic Press, New York 1986.
5 Chu, F. S., and Ueno, I., Appl. envir. Microbiol. *33* (1977) 1125.
6 Chu, F. S., Lee, R. C., Trucksess, M. W., and Park, D. L., J. Ass. off. analyt. Chem. *71* (1988).
7 Wei, R. D., and Chu, F. S., Analyt. Biochem. *160* (1987) 399.
8 Chu, F. S., J. Ass. off. analyt. Chem. *54* (1971) 1304.
9 Heathcote, J. G., and Dutton, M. F., Tetrahedron *25* (1969) 1497.
10 Cole, R. J., and Kirksey, J. W., Agric. Fd Chem. *19* (1971) 222.
11 Chu, F. S., Grossman, S., Ru-Dong Wei, and Mirocha, C. J., Appl. envir. Microbiol. *37* (1979) 104.
12 Habeeb, A. F. S. A., Analyt. Biochem. *14* (1966) 326.
13 Harder, W. O., and Chu, F. S., Experientia *35* (1979) 1104.
14 Gaur, P. K., Lau, H. P., Pestka, J. J., and Chu, F. S., Appl. envir. Microbiol. *41* (1981) 478.
15 Sizaret, P., Malaveille, C., Montesano, R., and Frayssinet, C., J. natl Cancer Inst. *69* (1980) 1375.

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Profile of sequential determinants in tissue polypeptide antigen BrCN:B fragment

A. Chersi, M. Camera, M. L. Trinca and M. Castelli

Regina Elena Institute for Cancer Research, Lab. of Biochemistry, Viale Regina Elena 291, I-00161 Roma (Italy)
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Summary. A synthetic approach has been applied to determine the profile of sequential determinants of one immunodominant region of *T*issue *P*olypeptide *A*ntigen (TPA). Five overlapping peptides, covering 30 of the 32 amino acid residues of this fragment, were chemically synthesized, and their antibody-binding activities for rabbit anti-TPA antibodies determined by enzyme-linked immunoadsorbant assays.
Anti-TPA reacted with two overlapping fragments at the COOH-terminal end of the fragment, but not with peptides that include Arg 15 considered as essential for the antigenicity of the whole fragment. This might suggest that this critical residue is involved in the formation of a complex conformational determinant.
Key words. TPA; synthetic peptides; sequential determinants.

The *T*issue *P*olypeptide *A*ntigen (TPA), discovered in 1957 by Bjorklund [1] has been demonstrated by several authors to be a potential marker in different neoplasias [2]; this protein is apparently produced and released by cancer cells, and its level in serum is correlated significantly with the progression of the neoplastic disease [3].
Because of the role that TPA plays in cancer detection and diagnosis, there has been much interest in isolating this protein in a pure form, and in defining its biochemical and biophysical properties. Also, since TPA is detected and quantitated in biological fluids by the aid of anti-TPA sera, much effort has been dedicated to the identification of the immunogenic regions of this antigen.
In aqueous solution, TPA has the tendency to form high molecular weight aggregates, that can be dissociated by SDS into subunits. The B1 subunit (mol.wt 43,000) has been partially characterized from the biochemical point of view, and several cyanogen bromide fragments have been isolated and sequenced [4]. TPA fragment BrCN:B, a 32-residue-long peptide that shares over 70% homology with sequence 56–87 of human epidermal 50 K keratin, seems to include at least one antigenic determinant, since anti-TPA sera react with synthetic peptides that closely resemble in sequence this cyanogen-bromide fragment [5]. The exact location and nature of the epitope(s) is unknown, although preliminary investigations indicate that Arg 15 is essential for the binding of anti-TPA antibodies, and that one antigenic site should be therefore located around this amino acid. In this investigation, we examined the profile of continuous antigenic determinants recognized by commercial anti-TPA sera in this immunogenic 32-residue fragment. Five synthetic peptides, that were from 9- to 15-residues-long and covered the full length of the BrCN:B fragment, were chemically synthesized by the solid-phase method [6] using a DuPont-Vega Coupler