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Synthesis and Identification of Aflatoxin B_{2a} Artificial Antigens: The First Critical Step in Preparing Monoclonal Antibodies Against Aflatoxin B_1

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Abstract: Aflatoxins, a group of mycotoxins are both natural contaminants of food and feedstuff causing a serious health risk of human and animals. In the present study, Aflatoxin B_1 (AFB_1) was converted into Aflatoxin B_{2a} (AFB_{2a}) using citric acid and then conjugated to two carrier proteins, Bovine Serum Albumin (BSA) and Human Serum Albumin (HSA). The artificial antigens were analyzed by polyacrylamide gel electrophoresis and ultraviolet spectrophotometry. Five Balb/c mice were immunized with AFB_{2a}-HSA before serum titers were detected. Gel electrophoresis results showed that the migration patterns of the conjugated proteins were different from those of the carrier proteins alone. Ultraviolet spectrophotometry results revealed that the maximum absorption peak of the conjugates barely shifted. The immunized mice developed a titer up to 128,000. These results indicated that the artificial antigens were successfully synthesized and could be employed in the preparation of monoclonal antibodies against AFB_{2a}.

Key words: Aflatoxin B_1, aflatoxin B_{2a}, artificial antigen, AFB_{2a}-carrier protein conjugates, China

INTRODUCTION

Aflatoxins, a group of toxins structurally related to secondary metabolites are produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus* (Hussain, 1985). They are found in various agricultural commodities such as peanuts and corn (Roebuck, 2004). Among all the aflatoxins identified, AFB_1 is the predominant one and presents the highest toxic potential. It was classified as a Group 1 carcinogen for humans by the International Agency for Research on Cancer. Aflatoxicosis also represents one of the serious diseases of poultry, livestock and other animals causing heavy losses in the breeding industry. Broilers fed with AFB_1 was reported a significantly decreased body weight-gain, feed intake and impaired feed conversion rate (Madden *et al*., 1999). Aflatoxin residues were detected in eggs and breast muscles of AFB_1-fed laying hens (Pandey and Chauhan, 2007). Reduced feed intake, lowered daily gains and impaired liver function have been observed for swine fed contaminated feed (Rustemeyer *et al*., 2010). In dairy, AFB_1-contaminated feed could lead to liver cell injury, a fatty liver syndrome, poor feed conversion and a significant reduction in milk yield (Colvin *et al*., 1984; Cook *et al*., 1986; Cockcroft, 1995). Besides, AFB_1 was reported to show an adverse influence on immune system in poultry and swine increasing their susceptibility to infectious diseases (Bondy and Pestka, 2000; Cabassi, 2007; Meissonnier *et al*., 2008).

When oxidized in weak acid, AFB_1 will be converted into its hemiacetal form AFB_{2a} through the addition of a water molecule onto the vinyl ether double bond of the B_1 terminal ring. Because of the hydroxyl on the 2-C of the terminal furan ring (Fig. 1), characteristics of AFB_{2a} are distinct from those of AFB_1, AFB_2, AFM_1 and AFM_2. The toxicity of AFB_{2a} is much lower than that of AFB_1. A comparative evaluation of the capacity to initiate bile duct hyperplasia showed that AFB_{2a} was <200 times as toxic as AFB_1 in ducklings (Lillehoj and Ciegler, 1969). When bonded to DNA, the hypochromic effect evoked by AFB_{2a} was less remarkable than that of AFB_1 (Lillehoj and Ciegler, 1969). However, antisera against AFB_{2a} were capable of cross-reacting with AFB_1, AFB_2, AFM_1 but reacted primarily with AFB_1 (Gaur *et al*., 1981). There was

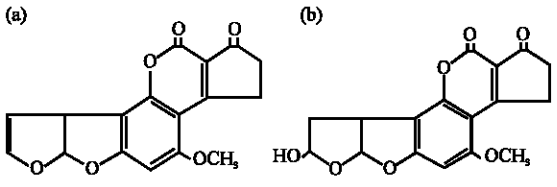

Fig. 1: a) Structures of AFB_1 and b) AFB_{2a}

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significant cross reaction between metabolites of AFB_1 *in vivo* such as AFB_1-N7-Gua, AFB_1-FAPyr and AFB_1-diol and antibodies against AFB_{2a} (Pestka *et al*., 1982). The AFB_{2a} antiserum demonstrated reactivity against the compounds indicating that it had a high degree of specificity for both the cyclopentenone and methoxy group of the parent aflatoxin molecule (Pestka *et al*., 1982). Antibodies against AFB_{2a}, especially monoclonal antibodies are significant for diagnosis and treatment of aflatoxicosis, studying AFB_1 metabolism and outcomes *in vivo* as well as tumor prevention and monitoring and food hygiene inspection. It was previously reported that AFB_{2a} antibody has been used as a histochemical probe in the indirect immunoperoxidase localization of AFB_1 bound to rat liver (Pestka *et al*., 1983). However, there is little literature describing the preparation of a monoclonal antibody against AFB_{2a}.

As a hapten, AFB_{2a} has to be conjugated to a carrier protein so that the artificial antigen synthesized can efficiently stimulate activation, proliferation and differentiation of lymphocytes and induce the development of antibody. In the current study, AFB_1 was converted into AFB_{2a} in the presence of an acid and was then conjugated to two carrier proteins: Bovine Serum Albumin (BSA) and Human Serum Albumin (HSA). The artificial antigens were identified and employed in the preparation of monoclonal antibodies for AFB_{2a}.

MATERIALS AND METHODS

Reagents: Unless otherwise indicated, all chemicals were products of Beijing chemical works (analytical grade). AFB_1, BSA, HSA, Freund's complete adjuvant, Freund's incomplete adjuvant and goat anti-mouse IgG conjugated to horseradish peroxidase were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of artificial antigens for AFB_{2a}

Preparation of AFB_{2a}: AFB_{2a} was prepared from AFB_1 according to the method of Pohland *et al*. (1968). Briefly, 10 mg AFB_1 was added into 0.2 M citric acid at 28°C with stirring and allowed to react for 24 h. Thin-Layer Chromatography (TLC) was employed to monitor the reaction process. Any AFB_{2a} generated would emit strong blue fluorescence in chloroform and exhibit an Rf value of 0.18 in silica gel TLC. AFB_1 would show an Rf value of 0.88 in a chloroform:acetone system (97:3, v/v). Samples at an Rf value of about 0.2 were collected, filtered through a sintered glass filter and volatilized to dryness.

Preparation of AFB_{2a}-BSA and AFB_{2a}-HSA conjugates: The AFB_{2a} was conjugated to BSA and HSA through adaption of the reductive alkylation method reported previously (Ashoor and Chu, 1975a, b). An amount of AFB_{2a} (5 mg) was dissolved in 0.75 mL methanol and added to 50 mg BSA in 10 mL of 0.05 M phosphate-buffered saline (PBS; pH 7.2). The reaction mixture was then incubated at 37°C for 30 min. This was followed by the addition of 0.5 mL sodium borohydride ($NaBH_4$; 0.013 M) and then incubation for another 30 min at 4°C. A 0.25 mL volume of 0.1 M HCl was added to the reaction mixture with gentle stirring to neutralize any excess $NaBH_4$. The protein solution was applied to a Sephadex G-15 column (2×40 cm) with 0.05 M Phosphate Buffer (PB; pH 7.2) used as an eluant. The protein fractions were pooled together and dialyzed for 24 h against 1 L of 0.05 MPB and ten 24 h against distilled water. The product was lyophilized in 1 mg batches, yielding a bright yellow powder. The same procedure was used for AFB_{2a}-HSA conjugation.

Identification of AFB_{2a}-carrier protein conjugates

Analysis of conjugates by polyacrylamide gel electrophoresis: The AFB_{2a}-protein conjugates were analyzed by polyacrylamide gel electrophoresis using a modification of the method of Sambrook and Russell (2001). A 20 μL (0.1 g L^{-1}) volume of the sample was mixed with an equal volume of 2×SDS protein loading buffer [1 M Tris-HCl pH 6.8, 0.02% (w/v) bromophenol blue, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol] and boiled for 5 min. The stacking gel contained 5% (w/v) acrylamide and the separating gel contained 15% (w/v) acrylamide. Tris-glycine buffer with 0.1% SDS (w/v) was used as the electrophoresis buffer and electrophoresis was carried out at 90 V in the stacking gel and 120 V in the separating gel. When the dye front reached the end of the gel, the gel was soaked in Coomassie brilliant blue R-250 solution [0.1% (w/v) in 25% (v/v) isopropyl alcohol and 10% (v/v) acetic acid] for >4 h at room temperature with gentle shaking. Gels were then washed with a solution containing 50% (v/v) methanol and 10% (v/v) acetic acid 3-4 times until it was satisfactorily destained. Pictures of the gels were taken with a UVI gel auto imaging system.

Analysis of conjugates by ultraviolet spectrophotometry: Different substances, owing to their different molecular structures have diverse absorption spectrums as well as maximum absorption wavelengths (λ_{max}) so it is possible to identify and analyze structures of substances according to their absorption spectra (Liu, 2001). The λ_{max} of the carrier protein will alter following conjugation with the hapten and the alteration indicates whether the conjugation is successful or not. The concentration of

protein samples was appropriately adjusted using PBS (0.01 M, pH 7.4) containing 20% methanol before analysis. The conjugates were scanned over a wavelength range of 190-400 nm on a Varian Cary 500 UV-Vis-NIR spectrophotometer (Varian; Palo Alto, CA, USA).

Determination of antibody titers in immunized mice: Five female Balb/c mice, at 8 weeks old and weighing 19±1 g were purchased from the Changchun Institute of Biological Products (Jilin province, China). The immunogen employed was AFB_{2a}-HSA (150 μg) dissolved in 150 μL of 0.01 M PBS and emulsified with an equal volume of Freund's adjuvant. The first injection was given intraperitoneally using Freund's complete adjuvant and the next two injections were administered with Freund's incomplete adjuvant at multiple sites by subcutaneous injection. Approximately 100 μg of AFB_{2a}-HSA conjugate without any adjuvant was used for the booster intraperitoneally. All four injections were given to the five mice at fortnightly intervals.

At 1st week following every injection, 5-10 μL of blood was drawn from each mouse by cutting the tip of the tail. An indirect and non-competitive Enzyme-Linked Immunosorbent Assay (ELISA) was employed for titer detection. AFB_{2a}-BSA (2 μg mL^{-1}) was used as the coating antigen with 10% rabbit serum as the blocking agent. Goat anti-mouse IgG conjugated to peroxidase (1:5000) was used to detect mouse antibodies. The substrate, O-Phenylenediamine Dihydrochloride (OPD) was allowed to develop for 1 h at 37°C and absorbance at 490 nm was recorded.

RESULTS AND DISCUSSION

Analysis of conjugates by polyacrylamide gel electrophoresis: The results of polyacrylamide gel electrophoresis on AFB_{2a}-protein conjugates are shown in Fig. 2. The patterns of migration for the AFB_{2a}-BSA and AFB_{2a}-HSA conjugates were different from those of BSA and HSA alone. The migration velocity of BSA and HSA was faster than that of AFB_{2a}-BSA and AFB_{2a}-HSA. It could be found that the molecular weight of the AFB_{2a}-BSA and AFB_{2a}-HSA conjugates were increased compared BSA and HSA.

Analysis of conjugates by ultraviolet spectrophotometry: The spectra of BSA, HSA, AFB_{2a}-BSA and AFB_{2a}-HSA were determined, respectively and recorded from 190-600 nm to identify artificial antigens (Fig. 3). The results showed that the absorption values for conjugates as well as the spectral shape were distinct from those of BSA and HSA. The maximum absorption wavelengths of

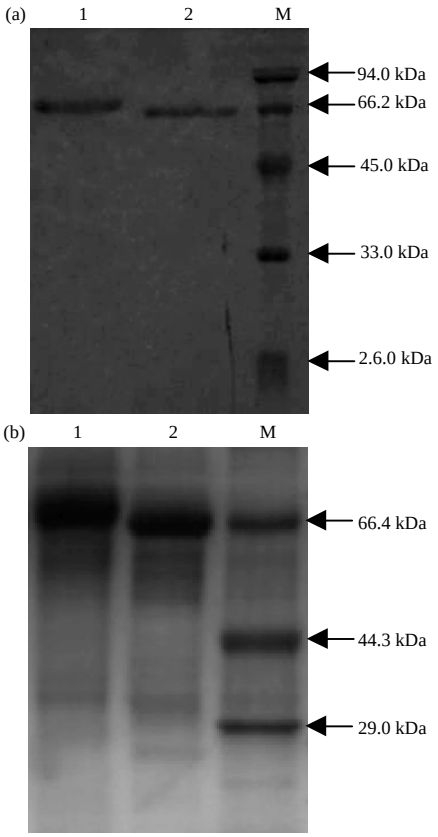

Fig. 2: a) Analysis of AFB_{2a}-BSA and BSA by polyacrylamide gel electrophoresis. Lane 1, AFB_{2a}-BSA; 2, BSA; 3, maker and b) Analysis of AFB_{2a}-HSA and HSA by polyacrylamide gel electrophoresis. Lane 1, AFB_{2a}-has; 2, HSA; 3, maker

BSA and HSA were 210 and 204 nm, respectively. The maximum absorption wavelengths of AFB_{2a}-BSA and AFB_{2a}-HSA were 196 and 198 nm, respectively. Compared to the carrier proteins, the maximum absorption peak of conjugates showed a little hypochromic shift (Fig. 2).

Antibody titer determination in mice immunized with AFB_{2a}-HSA: The titers in all five mice were plotted in Fig. 4. The second mouse showed the highest titer (128,000) with the other mice having titers greater than or equal to 40,000 after completion of the immunization protocol. This indicated that the mice showed strong antibody responses against AFB_{2a}-HSA.

The successful preparation of monoclonal antibodies specific to AFB_{2a} is essential for establishing immunoassays in order to detect AFB1 as well as studying its metabolic intermediates and metabolic pathway *in vivo*. Synthesis of artificial antigens is the first

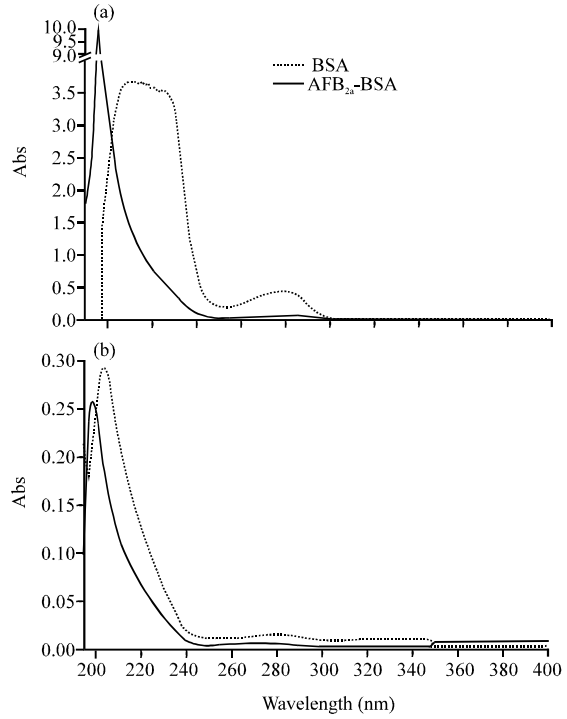

Fig. 3: a, b) Analysis of AFB_{2a}-BSA, AFB_{2a}-HSA, BSA and HSA by ultraviolet spectrophotometry

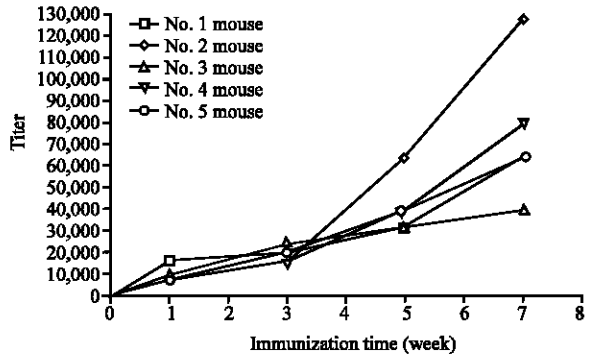

Fig. 4: Titers of artificial antigens in mice immunized with AFB_{2a}-HSA

critical step for production of monoclonal antibodies. In the present study, AFB_1 was first converted into AFB_{2a}, then BSA and HSA were chosen as carrier proteins and conjugated to AFB_{2a} by the Reductive Alkylation Method. Ashoor and Chu (1975a, b) had studied the interaction of AFB_{2a} with amino acids and proteins and proposed a mechanism for the interaction known as formation of a Schiff base. However, the ultimate aim of artificial antigens is to raise antibodies therefore, the best way to identify an artificial antigen is to verify whether it can elicit an antibody response. Five mice were immunized with AFB_{2a}-HSA following preliminary analysis of non-denaturing polyacrylamide gel electrophoresis and ultraviolet spectrophotometry of AFB_{2a}-carrier protein conjugates. The antigen titers in serum samples were also detected with AFB_{2a}-BSA as the coating antigen.

CONCLUSION

The results indicate that artificial antigens were successfully prepared with this study laying the foundation for AFB_{2a} monoclonal antibody production.

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