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A monoclonal antibody recognizing the specific epitope of aflatoxin analogs

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Abstract. A monoclonal antibody designated as 4A5 was obtained after fusion of mouse P3-NSl-Ag4-1 myeloma cells with spleen cells isolated from BALB/C mice that had been immunized with aflatoxin B_1 conjugated to bovine serum albumin. Using a competitive indirect enzyme-linked immunosorbent assay, the antibody has greatest binding efficiency for aflatoxin B_1 (AFB_1) and G_1. The sensitivity to AFB_1 was 250 pg/ml (12.5 pg/assay). Cross-reactivity of the antibody with each toxin, as determined by the concentration of aflatoxin necessary to cause 50% decrease in maximum absorbance at 405 nm, was 6.2, 5.5, 13, 54 and 170 ng per mililiter for aflatoxin B_1, G_1, Q_1, R_0 and P_1, respectively. Aflatoxin M_1, M_2, B_2, B_{2a}, G_2, G_{2a} and sterigmatocystin showed almost no cross-reaction with the antibody. These results strongly suggest that the dihydrodifurocumarinic moiety (7aα, 10aα-dihyro-furo[3′, 2′ 4, 5]-furo[2, 3-h]-1-benzopyran-2-one) of aflatoxin analogs is the determinant of specificity for the antibody.

Key words: Aflatoxin monoclonal antibody; Dihydrodifurocumrinic moiety; Epitope.

Introduction

The aflatoxins are highly toxic and carcinogenic compounds, which are frequent contaminates of human food supplies in many areas of the world, and are epidemiologically associated with human liver cancer (Busby and Wogan, 1979; Stoloff, 1976; Wogan, 1973). The potent hepatocarcinogenicity of aflatoxin B_1 (AFB_1) has been demonstrated in many animal species including rats, rainbow trouts, and monkeys (Sinnhuber *et al.*, 1977; Stoloff, 1977; Wogan, 1977). This apparent health hazard has prompted the search for a fast, reliable, and sensitive screening system for AFB_1 in foods and feeds. Most established analytical methods for AFB_1 detection involve thin-layer chromatography and high-pressure liquid chromatgraphy which are sensitive but time consuming. In the past few years several laboratories have used rabbit polyclonal antibodies (Biermann and Terplan, 1980; Chu *et al.*, 1985; Chu *et al.*, 1987; Gaur *et al.*, 1981; Groopman *et al.*, 1984; Pestka *et al.*, 1980; Pestka *et al.*, 1981; Sizaret *et al.*, 1980; Stoloff, 1977) and monoclonal antibodies (Candlish *et al.*, 1985; Woychik *et al.*, 1984) to detect aflatoxins with radioimmunoassay and enzyme linked immunosorbent assay. Monoclonal antibody against AFB_1 was prepared using spleen cells obtained from BALB/c which had been immunized with AFB_{2a}-BSA conjugate (Lubet *et al.*, 1983; Sun *et al.*, 1983). Groopman *et al.* (1984) have used AFB_1-γ globulin as an antigen to generate monoclonal antibody against AFB_1. Recently Kawamura *et al.* (1988) used AFB_1-BSA conjugate as an immunogen to prepare anti-AFB_1 monoclonal antibodies.

We report here the production of a high affinity IgG monoclonal antibody that recognizes AFB_1, AFG_1 and AFB_1 metabolites (AFQ_1, AFR_0, and AFP_1). This antibody has a paratope specific for the structure of dihydrodifurocumarinic moeity of aflatoxin analogs.

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Materials and Methods

Materials

Aflatoxin standards including AFB_1, AFB_2, AFB_{2a}, AFG_1, AFG_2, AFG_{2a}, AFM_1, AFM_2, AFR_0, and AFP_1, sterigmatocystin, AFB_1-BSA conguate, and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, Mo., U. S. A. Freund's complete adjuvant, RPMI 1640 medium, fetal bovine serum, goat anti-mouse IgG alkaline phosphatase conjugate, and p-nitrophenyl phosphate were purchased from Bio-Rad Laboratories, Richmond, California. Polyethylene glycol m. w. 1,500 was obtained from E. Merck Co., Darmstadt, W. Germany. All inorganic and organic chemicals were of reagent grade quality.

Immunization

Fifty microliter of AFB_1-BSA conjugate (500 μg) was dissolved in 1 ml of sterile distilled water and emulsified with an equal volume of complete Freund's adjuvant. Initially, 8-week-old female BALB/C mice were each given 98 μg (0.4 ml antigen gel solution) of the AFB_1-BSA conjugate (ratio of 23 mol of AFB_1 per mol of BSA) by intraperitoneal injection. The mice were given the same amount of AFB_1-BSA conjugate 1 week after the initial exposure. Four weeks after the initial exposure, the mice were given a final booster immunization of 1 mg of AFB_1-BSA in 0.1 ml sterile distilled water by tail vein injection.

Cell Fusion and Cloning

Four days after the final booster immunization, the mice were sacrified. Their spleens were aseptically removed, rinsed with RPMI medium, and teased apart with bent 25-gauge syringe needles to produce a single-cell suspension. The spleen cells (ca. 10^8/ml) were washed and combined with 10^7/ml P3-NS1-Ag4-1 myeloma cells. The cell mixture was pelleted at 1,500 rpm for 10 min, re-suspended in 0.5 ml of RPMI medium, and allowed to fuse. Cell fusion took place in 1.5 ml of prewarmed (37°C) 50% PEG 1,500 (dissolved in RPMI 1640) with gentle agitation for 45 sec in 37°C followed by incubation at the same temperature for 75 sec. Over the next 6 min 2 ml of RPMI was slowly added to the fused cells in the first min and the culture was maintained at 37°C for 1 min. This step was repeated and finally another 20 ml of RPMI was added over the last 2 min. The fused cells were centrifuged at 1,500 rpm for 10 min and the pellet was resuspended with an appropriate volume of HT medium, counted and then diluted to 10^6 cells per ml and distributed at 0.1 ml per well into two to three 96-well microwell plates (Nunc DK 4000 Roskilde, Denmark). Twenty four hours later, 0.1 ml of fresh HAT medium was added to each of the wells. The fused cells were fed by replacing 50% of the HAT medium every 4-7 days for 2 weeks followed by feeding with HT and CRPMI media. The plates were screened for the desired antibodies ca. 18-21 days post-fusion by an indirect enzyme-linked immunnsorbent assay (ELISA) as the modified method (Woychik *et al.*, 1984), with the exception of Nunc microwell plates (cat. No. 269620) which were coated with antigen by incubation of 50 μl of AFB_1 or AFB_1-BSA (50 ng per well) in 0.05 M sodium carbonate buffer (pH 9.6) at 4°C overnight. Normal mouse serum was used as control. The reaction plates were read at 405 nm in a SLT-Labinstruments Austria Easy Reader. The antibody-producing hybridomas were identified by the wells which had an absorbance significantly above backgrounds.

Hybridomas from positive wells were propagated in 24-well microtiter plates. Cultures that continued to show the antibody titre after 5-8 days of further growth and incubation were cloned by a limiting dilution technique (Oi, 1980) into 96-well plates in RPMI 1640 medium which containing 30% fetal bovine serum, 0.1 mM hypoxanthine, 16 μM thymidine, 0.03% L-glutamine and CR-Endothelial Cell Growth Supplement (CR-ECGS) as a feeder layer. Wells were examined for single clone and re-screened for specific antibody activity 1 week to 10 days later. Clones active in producing AFB_1 and AFB_1-BSA antibodies were grown in CRPMI medium and maintained at a cell density between 10^5 and 10^6 per ml or frozen in 1 ml ampules by storage at -70°C then transferred to liquid nitrogen.

Hybrid cells producing anti-AFB_1 and AFB_1-BSA antibodies were growing in culture to obtain antibody for further characterization. Cultures were grown to late-stationary phase, and the media were precipiated with ammonium sulfate at a final saturation of 50% $(NH_4)_2SO_4$. The precipitates were dissolved in an appropriate amount of phosphate-buffered saline. It was dialyzed for 24 h at 4°C and then kept at -70°C for further use.

Isotypes of anti AFB_1 and AFB_1-BSA monoclonal

antibodies were determined by using all immunochemicals (SBA Clonotyping System III, Horseradish peroxidase labelled antibodies for subtype identification kit from Southern Biotechnology Associates Inc., Birmingham A. L., U. S. A.) which were reconstituted and used according to the isotype kit specification.

Determination of Antibody Specificity

The procedures as described in the previous section for indirect ELISA was used to determine the specificity of the antibody except that different aflatoxin analogs in various concentrations were used. In general, the plates were coated with 50 ng of AFB_1-BSA conjuate per well. Competitive inhibition of antibody reacting with the antigen bound to the solid phase was carried out by incubating 50 μl of antibody (50% ammonium sulfate fractionation 1 : 20 dilution) and 50 μl of an amount of an aflatoxin analog per well at 37°C for 1 hr. The plates were then washed and incubated with 100 μl of anti-mouse IgG alkaline phosphatase conjugate (1 : 1,000 dilution) per well. Finally, 0.1 ml of p-nitrophenyl phosphate solution (1 mg/ml) was added to each well and the amount of p-nitrophenol product was estimated by optic density at 405 nm after 1 hr incubation. The assays were done in quadruplicate and repeated twice.

Results and Discussion

Female BALB/c mice were immunized with AFB_1-BSA conjugate in complete Freund's adjuvant. Six of the 10 mice injected were found to produce significant anti-AFB_1-BSA serum titers as measured by non-competitive ELISA. Spleen cells from four of these mice were fused with NS-1 myeloma cells. The fusion efficiency (number of wells with growing colonies per number of well seeds) was greater than 65%. A total of 985 hybridoma clones were produced in 4 independent fusions. In the ELISA screening tests, 54 hybridoma clones produced antibodies agaisnt AFB_1 and AFB_1-BSA. Two of these clones (A-11-5H3 and A-11-8E10) were used for monocloning. A total of 205 monoclones were obtained by the limiting dilution method. Only one of these possessing high-affinity to AFB_1 was designated as 4A5, which was produced by a A-11-5H3 (4A5) monoclonal hybridoma cell line. This 4A5 monoclonal antibody is an IgG_1 with Kappa light chains. In the present work, since the indirect ELISA used the IgG-specific antibody for screeing hybridoma, no IgA or IgM antibodies were expected.

The cross-reactivity of aflatoxin analogs with 4A5 monoclonal antibody was carried out by competitive indirect ELISA. The specificity of the antibody was shown in Fig. 1 and Table 1. The concentrations of AFB_1, AFG_1, AFQ_1, AFR_0 and AFP_1 necessary to cause 50% decrease in maximum absorbance at 405 nm were 6.25, 5.5, 13, 54 and 170 ng per ml, respectively. Aflatoxin M_1, M_2, B_2, B_{2a}, G_{2a} and sterigmatocystin

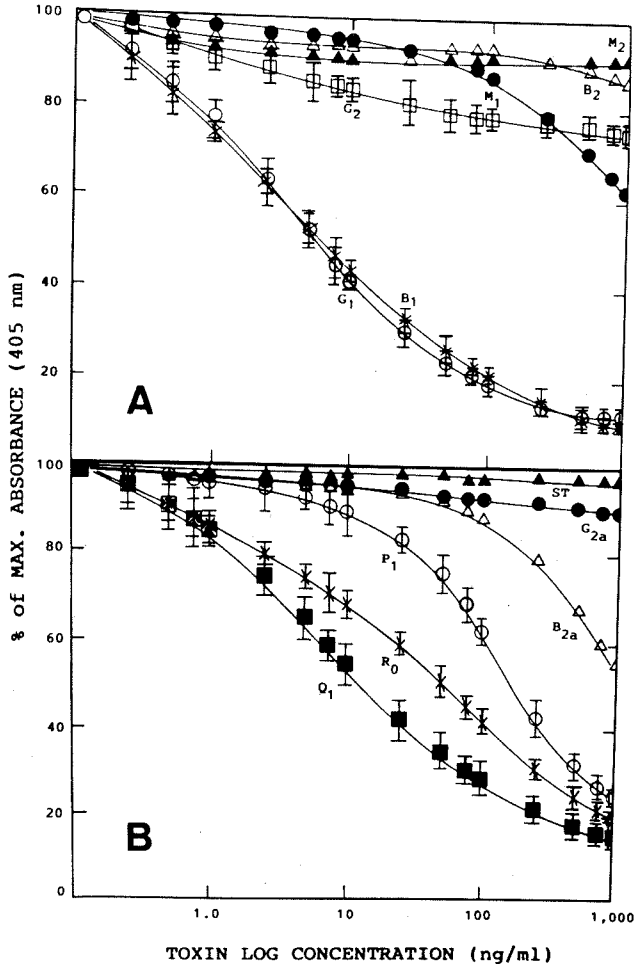
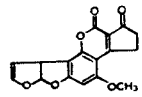
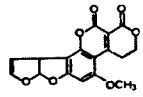
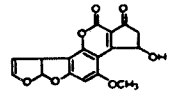
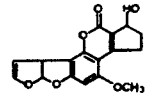
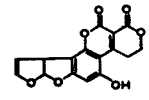
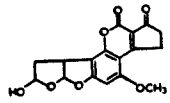
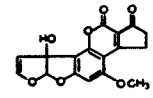
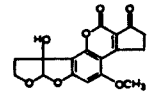
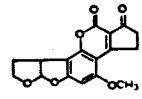
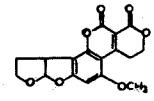
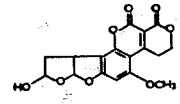
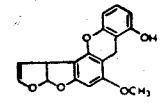


Fig. 1. Cross-reactivity of aflatoxin analogs wiht 4A5 monoclonal antibody determined by competitive indirect ELISA. The range of values for 100% maximal absorbance were 1.2 to 1.4 absorbance units. The results showed in the AFB_1, G_1, G_2, Q_1, R_0, P_1 assaies are expressed as mean±S. D. The letters B_1(X), G_1(○), B_2(Δ), G_2(□), M_1(●), and M_2(▲) for Fig. 1-A; Q_1(■), R_0(X), P_1(○), B_{2a}(Δ), G_{2a}(●); ST(▲), sterigmatocystin for Fig. 1-B.

Table 1. *Specificity of 4A5 monoclonal antibody against aflatoxin analogs in competitive indirect enzyme-linked immunsorbent assay*[a]

Toxin	Structure	Minimal inhibition[b]	50% inhibition[c]
B_1		0.25	6.2
G_1		0.25	5.5
Q_1		0.50	13.0
R_0		0.50	54.0
P_1		7.50	170.0
B_{2a}		100.00	>1,000.0
M_1		100.00	>1,000.0
M_2		>1,000.0	>1,000.0
B_2		>1,000.0	>1,000.0
G_2		>1,000.0	>1,000.0
G_{2a}		>1,000.0	>1,000.0
ST		>1,000.0	>1,000.0

[a] All values in ng/ml of aflatoxin analogs in 10% methanol/PBS. The assays were done in quadruplicate and repeated twice.
[b] ng/ml of aflatoxin required for detectable inhibition of binding of antibody to the immobilized AFB_1-BSA.
[c] ng/ml aflatoxin required to inhibit binding of antibody by 50% to the AFB_1-BSA solid plate. Calculated by regression analysis.

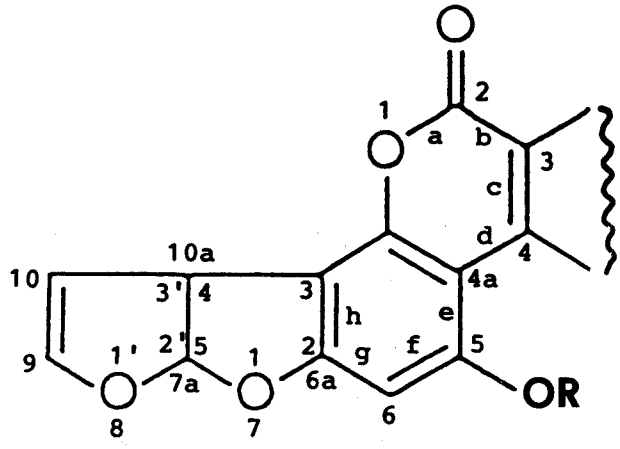


Fig. 2. The structure of dihydrodifurocumrinic moiety (7aα; 10aα-dihyro-furo[3', 2': 4,5]-furo[2, 3-h]-1-enzopyran-2-one moiety[DFFBP moeity]) of aflatoxin molecule an antigenic determinant.

displayed negligible cross-reactivity for the antibody. These data indicate that a specific epitope for 4A5 monoclonal antibody recognition of aflatoxin analogs lies in the dihydrodifurocumarinic moeity (7aα, 10aα -dihyro-furo [3', 2' : 4,5]-furo[2, 3-h]-1-benzopyran-2 -one moeity) of the aflatoxin molecule (Fig. 2). However, Lubet *et al.* (1983) has reported that the relative cross-reactivity of a monoclonal antibody for AFB_1 with different aflatoxins was 1.0, 1.2, 1.6 and >100 for AFB_1 B_2, M_1 and aflatoxicol, respectively, and that there was no cross-reactivity with AFG_1 and G_2. It is evident that the AFB_1 monoclonal antibody of Lubet *et al.* (1983) has a specificity toward the cyclopentenone ring portion of the aflatoxin molecule. These results are expected because these investigators used AFB_{2a} conjugate to protein for immunization, whereas AFB_1 which was first converted to its O-carboxymethyloxime at carbonyl group of the cyclopentenone ring of the AFB_1 molecule and then conjugated with BSA was used for the current study. The 4A5 antibody had a detection limt of 12.5 pg/assay for AFB_1 as compared to 250 pg/assay repoted by Lubet *et al.* (1983). The antibody producing hybridoma cell line (A-11-5H3-4A5) is very stable and has been maintained for more than two years, secreting IgG immunoglobulin.

In summary, we report here the production of an IgG monoconal antibody having high affinity for AFB_1, AFG_1 and some AFB_1 metabolites (AFQ_1, AFR_0 and AFP_1). This antibody should be useful in assaying for these carcinogenic mycotoxins occurring in agricultural commodities. In addition, the strong cross-reactivities with AFB_1 metabolites makes this antibody potentially useful in the diagnosis for aflatoxicosis, because these metabolites may be present in significant ammounts in tissues of animals with aflatoxicosis (Rodricks and Stoloff, 1977).

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一種可辨認黃麴毒素分子構造上，特異性抗原決定基之單源抗體

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我們研究室找到了一株命名爲 4A5 之單源抗體。該抗體係以與牛血清蛋白結合之 AFB_1，經免疫 BALB/c 老鼠之後，分離出其脾臟細胞，再以此細胞與 P_3-NSI-Ag_4-1 腫瘤細胞融合後篩選而得。當使用間接競爭性酵素免疫測驗抗體對各種毒素之反應時，發現其與 AFB_1 和 AFG_1 之親和性很高，對 AFB_1 之敏感度爲 250 pg/ml (12.5 pg/assay)。進一步從事交叉反應試驗結果發現其對 AFB_1, G_1, Q_1, R_0 和 P_1 之專一性，分別爲 6.2, 5.5, 13, 54 和 170 ng/ml (以抑制 50% 之酵素活性所需要之各種毒素重量爲計)；但它對於其他毒素 AFM_1, M_2, B_2, B_{2a}, G_2, G_{2a}, 和 sterigmatocystin，則幾乎沒有反應。本研究有一重要發現即證明該單源抗體所確認之抗源決定基 (Antigenic determinant)，爲黃麴毒素分子構造上之 dihydrodifurocumarinic moiety.