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Preparation and characterization of a monoclonal antibody against aflatoxins B_1, B_2, G_1, and G_2

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Abstract. By using an indirect enzyme-linked immunosorbent assay (Indirect ELISA), a monoclonal antibody (McAb) designed as 1E3 was generated after fusion of mouse (P3-NS1-Ag4-1) myeloma cells with spleen cells isolated from BALB/c mice, which were immunized with aflatoxin B1-BSA conjugated. The McAb has a high affinity for aflatoxins B_1, B_2, G_1, and G_2. Specially, it has the greatest binding efficiency for aflatoxin B_1 based on an indirect competitive ELISA (indirect cELISA). The cross reactivities of the antibody with a family of aflatoxin group were 0.44 ng/ml (22 pg/assay), 2.1 ng/ml (105 pg/assay), 3.0 ng/ml (150 pg/assay), 7.3 ng/ml (365 pg/assay), and 1.4 ng/ml (70 pg/assay) for aflatoxins B_1, B_2, G_1, G_2 and total aflatoxins (B_1, B_2, G_1, and G_2), respectively. Aflatoxins M_1, M_2, B_{2a}, G_{2a}, R_0, P_1, Q_1, and sterigmatocystin showed almost no cross-reaction with the antibody. The antibody producing cell line (A25-2A3-3E6-1E3), which has been maintained for more than three years, secreted murine IgG class immunoglobulin and was characterized as IgG_1 with Lambda light chains. The potential to use this antibody to detect the four natural occurence of aflatoxins (B_1, B_2, G_1, and G_2) in foods or agricultural products was stressed.

Key words: Aflatoxins B_1, B_2, G_1, and G_2; Anti-aflatoxin monoclonal antibody; Competitive indirect enzyme-linked immunosorbent assay.

Introduction

The four natural occurring aflatoxins (B_1, B_2, G_1, and G_2), which are carcinogenic and teratogenic secondary metabolites of *Aspergillus flavus* and *A. parasiticus*, contaminate some agricultural commodities including peanuts, maizes, and feeds (Smith and Hacking, 1983; Stoloff, 1976; Stoloff, 1977). Epidemiological surveys have revealed a close association between liver cancer and a high level of aflatoxin B_1 (AFB_1) in foods in the areas such as South Africa (van Rensberg *et al.*, 1985), Philippines (Bulatao-Jayme *et al.*, 1982), and China (Sun *et al.*, 1983). The potent hepatocarcinogenicity of AFB_1 has been demonstrated in many animals including rats, rabbits, and monkeys (Sinnhuber *et al.*, 1977; Stoloff, 1977; Wogan, 1977).

In the past, analysis of aflatoxins in natural substrates is based on chemical methods including solvent extraction and clean-up. The final detection is achieved by thin-layer chromotography, gas chromotography, high performance liquid chromotography and gas chromotography mass spectroscopy. These methods are time-consuming, labour intensive and expensive instruments are also needed. Accordingly, it is necessary to develope a simple, rapid and sensitive method for detection of aflatoxins in foods or agricultural products. Immuonoassay is so far the best way to solve the problems. The advantages of the detection of aflatoxins by immunoassay are free from pretreatment of the samples, easy to operate and high specificity. Recently, several laboratories have used rabbit polyclonal antibodies to detect aflatoxins with radioimmunoassay and enzyme-linked immunosorbent assay

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(ELISA) (Biermann and Terplan, 1980; Chu *et al.*, 1985; Gaur *et al.*, 1981; Groopman *et al.*, 1984; Pestka *et al.*, 1980; Sizaret *et al.*, 1980). Monoclonal antibodies are also available for aflatoxins (Candlish *et al.*, 1985; Woychik *et al.*, 1984; Labet *et al.*, 1983; Sun *et al.*, 1983; Groopman *et al.*, 1984; Kawamura *et al.*, 1988; Tseng and Chung, 1990; Ramakrishna *et al.*, 1990). However, there are little reports related to the detection of total amount of aflatoxins in foods or agricultural products by using monoclonal antibody.

In this report, we describe the production and characterization of a monoclonal antibody with high affinity to aflatoxins B_1, B_2, G_1, and G_2. This antibody can be used to detect the total amount of aflatoxins in foods or agricultural products by using ELISA.

Materials and Methods

Chemicals

Aflatoxin standards including aflatoxins B_1, B_2, B_{2a}, G_1, G_2, G_{2a}, M_1, M_2, R_0, and P_1, sterigmatocystin, bovine serum albumin (BSA), and AFB_1-BSA conjugate were purchased from Sigma Chemical Co., St. Louis, MO; Freund's complete adjuvant, RPMI 1640 medium, fetal bovine serum, goat anti-mouse IgG (H+L) alkaline phosphatase conjugate, and p-nitrophenyl phosphate were purchased from Bio-Rad Laboratories, Richmond, California. Polyethylene glycol (PEG, m.w. 1,500) was obtained from E. Merck Co., Darmstadt, Germany. All inorganic and organic chemicals were reagent grade.

Immunization

Animals used in this study were female, 8-week -old BALB/c mice obtained from Department of Plant Pathology, National Taiwan University. Aflatoxin B_1 -BSA conjugate (ratio of 23 moles of AFB_1 per mole of BSA) was served as immunogen. Fifty microliter (500 μg) immunogen was added to the mixture of 1 ml complete Freund's adjuvant and 1 ml sterilized water. This solution was mixed well until forming gel solution to serve as mixture of immunogen. A 0.4 ml immunogen mixture was injected into the peritoneum of the immunizing mice. After one week, 0.4 ml immunogen mixture was injected into the mice by the same way. After four weeks, the animals were boosted with 0.1 ml (1,000 μg) immunogen without complete Freund's adjuvant by an intravenous route and three days later the mice were sacrificed. The protocal for cell fusion was described in the previous report (Tseng and Chung, 1990).

Screening of the Hybridoma Cell Line

The hybridoma cells were cultured in selective medium gradually, i. e., first cultured for one day in HT medium (Hazoleton, USA), then cultured for 2 weeks in HAT medium (Hazleton, USA) and replaced with 50% HAT medium every 4-7 days. Again, the cells were cultured in HT medium one day and dispensed into CRPMI 1640 medium (Gibco, USA). The plates were screened for the desired antibodies about 18-21 days post-fusion by an indirect ELISA according to the modified method of Woychik *et al.* (1984), with the exception that plates were coated with antigen in this study by incubation of 50 μl AFB_1 or AFB_1-BSA (50 ng per well) in 0.05 M sodium carbonate buffer (pH 9.6) at 4°C overnight. The antibody-producing hybridomas were identified by the wells which had an absorbance at 405 nm significantly above backgrounds.

Cloning

Positive hybridomas were subsequently expanded into flasks and cloned by limiting method (Oi, 1980) into 96-well plates in CRPMI 1640 medium, which containing 30% fetal bovine serum, 0.1 M 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 a week to 10 days later. Clones active in producing anti-AFB_1 and AFB_1-BSA antibodies were cultured in CRPMI medium, maintained at a cell density between 10^5 to 10^6 per ml or frozen in 1 ml amples by storage at -70°C or liquid nitrogen.

Hybrid cells producing anti-AFB_1-BSA antibodies were cultured to obtain antibodies for further characterization. Cultures were grown to late-stationary phase, and the media were precipitated with ammonium sulfate at a 50% final saturation. The precipitates were suspened in phosphate-buffered saline (PBS). It was dialyzed for 24 hours at 4°C and then kept at -70°C for further experiments.

Isotypes of anti-AFB_1 and anti-AFB_1-BSA monoclonal antibodies were determined by using the All Immunochemical Kit (SBA Clonotyping System III, Horseradish peroxidase labelled antibodies for the sub -type), a product of Southern Biotechnology Associates Inc., USA.

Table 1. *Specificity of 1E3 monoclonal antibody against aflatoxin analogs in indirect competitive enzyme-linked immunosorbent assay*

Toxin	Minimal inhibition[a] ng/ml (pg/assay)	50% inhibition[b]
B_1	0.02	0.44
	(1.0)	(22)
B_2	0.05	2.1
	(2.5)	(105)
G_1	0.05	3.0
	(2.5)	(150)
G_2	0.40	7.3
	(20)	(365)
$B_1+B_2+G_1+G_2$	0.03	1.4
	(1.5)	(70)
B_{2a}	>1,000.00	>1,000.0
G_{2a}	>1,000.00	>1,000.0

[a]ng/ml or (pg)/assay of aflatoxin required for first significant inhibition of binding of antibody to the AFB_1-SBA solid plate.
[b]ng/ml or (pg)/assay of aflatoxin required to inhibit binding of antibody by 50% to the AFB_1-BSA solid plate. Calculated by regression analysis.

Table 2. *Specificity of 1E3 monoclonal antibody against aflatoxin analogs in indirect competitive enzyme-linked immunosorbent assay*

Toxin	Minimal inhibition[a]	50% inhibition[b]
M_1	10.0	>1,000.0
	(500)	
M_2	1.0	>1,000.0
	(50)	
P_1	>1,000.0	>1,000.0
Q_1	>1,000.0	>1,000.0
R_0	>1,000.0	>1,000.0
ST	>1,000.0	>1,000.0

Footnotes, a and b, same as described in Table 1.

Indirect Competitive Enzyme-linked Immunosorbent Assay

The procedures are the same as described for indirect ELISA except the different aflatoxin analogs in various concentrations were used. In general, the plates were coated with 50 ng of AFB_1-BSA conjugate per well. Competitive inhibition of antibody reacting with the antigen bound to the solid phase was carried out by incubation 50 μl of antibody (1:20 dilution of 50% ammonium sulfate fractionation) and 50 μl of an aflatoxin analog per well at 37°C for 1 hour. The plates were washed and incubated with 100 μl of goat antimouse IgG alkaline phosphatase conjugate (1:2,000 dilution) per well. Finally, 0.1 ml of p-nitrophenyl phosphate solution (1 mg/ml) was added to each well and the formation of p-nitrophenyl product was measured at 405 nm after 1 hour incubation. The assay was done in quadruplicate and repeated twice.

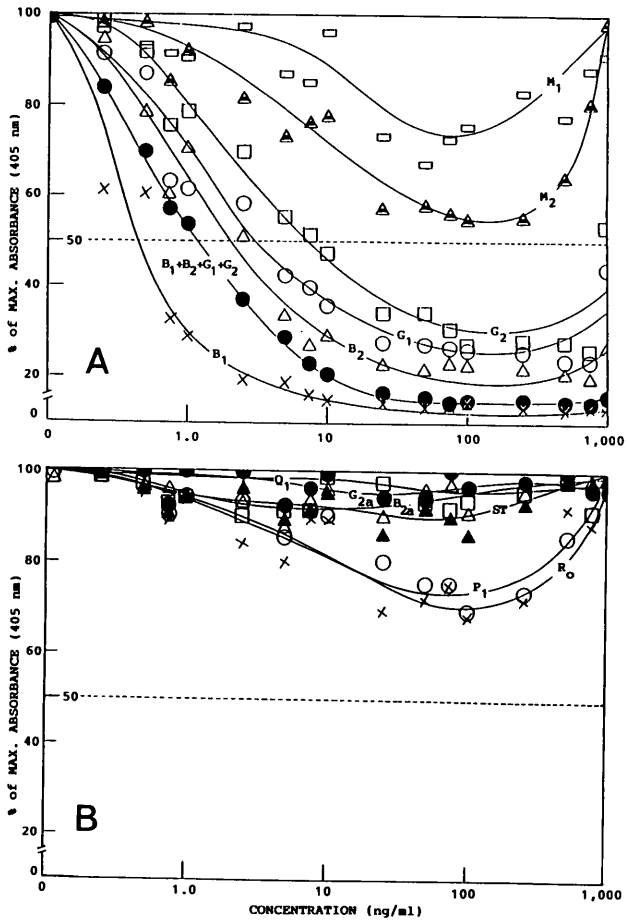

Fig. 1. Sensitivity and cross reactivity of aflatoxin analogs with 1E3 monoclonal antibody determined by indirect cELISA. The range for 100% maximal absorbance were 1.5 to 1.7 absorbance units. This figure is in a semi-log scale. In the indirect cELISA, 50 μl of sample was used in each analysis. Thus, the amount in each analysis was 20 times less on the ng/ml basis. The letters indicate B_1 (×), B_2(△), G_1(○), G_2(□), $B_1+B_2+G_1+G_2$(●), M_1(□), and M_2 (△) for Fig. 1-A; R_0(×), P_1(○), B_{2a}(△), G_{2a}(●), ST(▲), sterigmatocystin for Fig. 1-B.

Results and Discussion

Aflatoxin B_1-BSA conjugate was used as im-

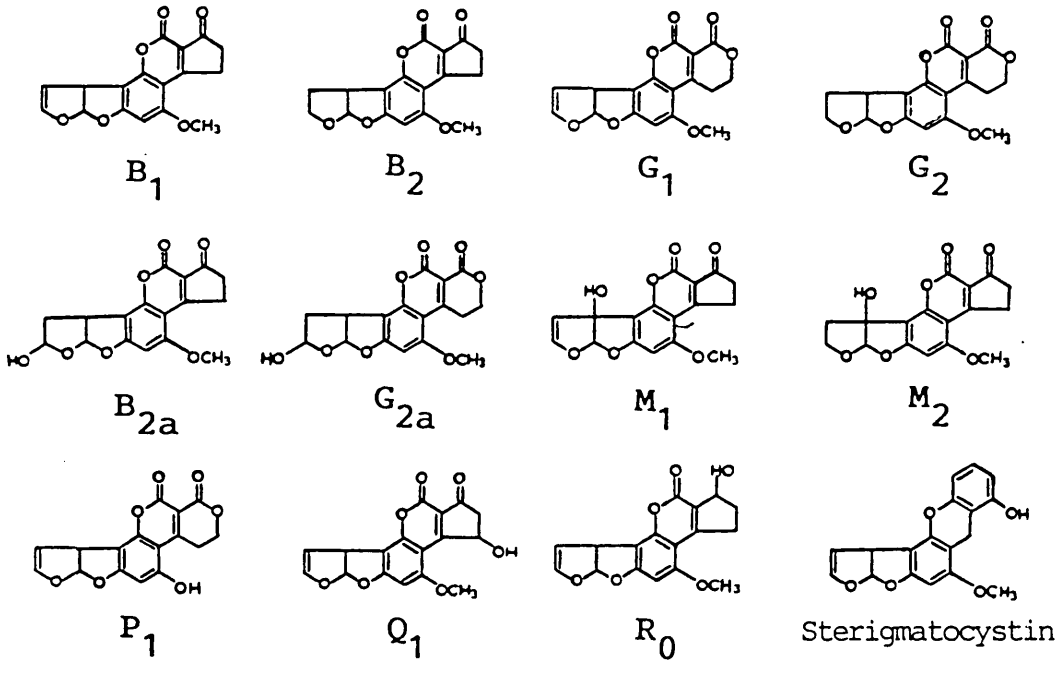

Fig. 2. Structure of aflatoxin B_1 and related aflatoxins.

munogen to immunize the BALB/c mice. After several challenges with immunogen, the mice were sacrificed, and their spleen cells were taken out and fused with mice myeloma cells by PEG. A total of 820 hybridoma clones was obtained in 3 indepentent fusions. By using ELISA screening tests, 58 hybridoma clones produced antibodies against AFB_1 and AFB_1-BSA. Three of the clones were used for monocloning. A total of 180 monoclones was obtained by the limiting dilution method. One of the clones producing McAb with high afinity to aflatoxins B_1, B_2, G_1, and G_2 was designed as IE3, which was generated by a A25-2A3-3E6-1E3 monoclonal hybridoma cell line.

The sensitivity and cross reactivity of aflatoxin analogs with 1E3 McAb were examined by indirect cELISA. The minimum detectable limits of aflatoxins B_1, B_2, G_1, G_2, and total aflatoxins were 0.02, 0.05, 0.05, 0.40, and 0.03 ng/ml, respectively (Table 1). The detection limits were expressed as concentrations which gave significant (over 10%) inhibition. The specificity of the antibody was shown in Fig. 1, Table 1 and 2. The concentrations of aflatoxins B_1, B_2, G_1, and G_2, which caused a 50% maximum absorbance at 405 nm were 0.44 ng/ml (22 pg/assay), 2.1 ng/ml (105 pg/assay), 3.0 ng/ml (150 pg/assay), 7.3 ng/ml (365 pg/assay), respectively; when the four aflatoxins were mixed, the cross reactivity was 1.4 ng/ml (70 pg/assay). Aflatoxins M_1, M_2, B_{2a}, G_{2a}, Q_1, P_1, R_0, and sterigmatocystin displayed negligible cross reactivity for the antibody (Fig. 2). From the above data, it demonstrated that 1E3 McAb had high affinity toward the four aflatoxins (B_1, B_2, G_1, and G_2). The antibody is most cross-reative with AFB_1 than AFB_2, AFG_1, and AFG_2 indicating that the dihydrofuran ring portion of the molecule is more important for recognization. However, the cyclopentenone moiety is still somewhat important for recognization. The antibody was an IgG with Lambda light chains and the approximate molecular weight was 15.4 Kd. The cloned hybridoma cell line A25-2A3-3E6-1E3 showed high stability. It was frozen in liquid nitrogen, or at -70°C and -150°C for more than three years, and the titer of the antibody has not been changed. Using monoclonal antibody to analyze aflatoxins has been reported in several instances. Lubet *et al.* (1983) disclosed an enzyme immunoassay to detect AFB_1 and M_1 by monoclonal antibody and the sensitivity of each analysis was 250 pg. Also in 1983, Sut *et al.* reported that a hybridoma cell line with B_{2a}-BSA complex as immunogen produced a specific McAb against AFB_1. The sensitivity toward AFB_1 was 50 pg. Groopman *et al.*

(1984) revealed the McAb to aflatoxins B_1, B_2, M_1, and aflatoxin-DNA complex, as well as the usage in solid phase immunoassay. Kawamura *et al.* (1988) disclosed a McAb-based enzyme-linked assay for AFB_1 in peanut products. Recently, several laboratories reported that McAb produced by hybridoma cell lines showing cross reactivity with four major aflatoxins (B_1, B_2, G_1, and G_2) (Hefle and Chu, 1990; Ward *et al.*, 1990). A monoclonal antibody recognizing the specific epitope of aflatoxin analogs in terms of aflatoxins B_1, G_1, Q_1, Q_0 and P_1 has been reported by Tseng and Chung (1990). In the world, aflatoxins in foods and agricultural products are most commonly measured by total amount, however, report on the detection of total aflatoxins by McAb is still meager.

We report a monoclonal antibody designated as 1E3, which has a high affinity for aflatoxin B_1, B_2, G_1, and G_2, but without any cross reaction to other aflatoxin family. The sensitivities of the antibody to AFB_1 and total aflatoxins were 1 pg and 1.5 pg per assay. This antibody should be useful for detecting or qualifying the total aflatoxins in foods or agricultural products. In addition, the antibody can be combined with an immunoassay system such as radioimmunoassay, immunofluorescence or ELISA to set up a method for detection of aflatoxin(s).

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對抗黃麴毒素 B_1, B_2, G_1 和 G_2 單源抗體之製備及特性

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利用間接性酵素免疫法，篩選到一株產生命名爲 IE3 之單源抗體。該抗體係以牛血清蛋白與 AFB_1 結合爲抗源，以老鼠 (BALB/c) 爲試驗動物。經免疫後，分離其脾臟細胞，再以此細胞與 P_3-NS1-Ag_4-1 腫瘤細胞融合後，篩選而得。進一步使用間接競爭性酵素免疫法，測定抗體對各種毒素之反應結果，發現其與四種黃麴毒素 (AFB_1, B_2, G_1 和 G_2) 具親和性，尤其對於 AFB_1 之親和性最高。根據交叉反應試驗結果，顯示抗體對於 AFB_1, B_2, G_1, G_2 以及四種黃麴毒素之專一性，分別爲 0.44 ng/ml (22 pg/assay), 2.1 ng/ml (105 pg/assay), 3.0 pg/ml (150 pg/assay), 7.3 ng/ml (365 pg/assay) 和 1.4 ng/ml (70 pg/assay)；而對於其他毒素，AFM_1, B_{2a}, G_{2a}, R_0, P_1 及 ST (sterigmatocystin)，則幾乎沒有反應。產生抗體之細胞系，曾保存於液態氮，歷時三年，仍然具產生 IgG1λ 輕鍊抗體之能力。本抗體因對於上述四種黃麴毒素具有高親和性，將適用於偵測存在於食物及農業品中，四種黃麴毒量。