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Production and characterization of monoclonal antibodies for aflatoxin B1

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K.T. DEVI, M.A. MAYO, K.L.N. REDDY, P. DELFOSSE, G. REDDY, S.V. REDDY AND D.V.R. REDDY. 1999. Hybridomas that secreted antibodies for aflatoxin B1 were selected using two immunization protocols referred to as A and B. Protocol A is a standard immunization method and resulted in the selection of only two clones that produced monoclonal antibodies against aflatoxin B1. In protocol B a unique immunization schedule which resulted in the generation of 10 hybridomas is described. Of the 10, one antibody was highly specific to B1, four antibodies reacted equally strongly with B1, G1 and weakly with B2. Another four reacted strongly with B1 and weakly with B2 and G1. One clone reacted equally strongly with B1, G1 and B2. Interestingly all the 10 antibodies showed little or no cross-reaction with G2.

INTRODUCTION

Agricultural products are often contaminated with fungi that can produce toxic metabolites referred to as mycotoxins. Among these, aflatoxins have assumed economic importance because of their influence on the health of human beings and livestock, and on the marketability of agricultural products. Aflatoxins are potent carcinogenic and immunosuppressive agents. In most developing countries limited or no facilities exist for monitoring these toxins in foods or feeds. They are based on physicochemical methods such as TLC and to a limited extent high performance liquid chromatography (HPLC). These methods are laborious and require expensive instrumentation and clean-up of the samples. Immunological methods which are cost effective and adaptable to the situation in developing countries have been reported for the estimation of aflatoxins using polyclonal antibodies (Chu and Uneo 1977; Anjaiah *et al*. 1989; Zhang and Chu 1989). However, commercial kits using immunological methods are expensive, and in many countries can be problematic to import. Therefore, the main aim of this investigation was to generate highly sensitive antibodies for the precise analysis of samples using hybridoma technology. They are preferred over polyclonal antibodies because their affinity and specificity do not vary from bleed to bleed and additionally, unlike polyclonal antibodies, do not react with other aflatoxin analogues. Most of the reports that have appeared so far on the production of monoclonal antibodies for aflatoxins have involved standard immunization protocols which include four intraperitoneal injections at weekly intervals (Candlish, Stimson and Smith 1985) or monthly intervals (Ward *et al*. 1990) with a final immunization 3 days prior to fusion. The numbers of clones obtained in each fusion have varied from one to a maximum of seven. This paper describes the production and characterization of monoclonal antibodies with high sensitivity and varying specificities for aflatoxins using a novel immunization schedule.

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MATERIALS AND METHODS

Production of monoclonal antibodies

Immunization. The immunogen used was AFB1–BSA conjugate (250 μg) dissolved in 250 μl of 0·01 mol/L PBS and emulsified with an equal volume of Freund's adjuvant (Sigma). This was injected intraperitoneally into each of several 6-week-old female Balb/c mice. The first injection was given using Freund's complete adjuvant and the rest of the injections, except for the booster, were given with Freund's incomplete adjuvant. No adjuvant was used for the booster injections. After completing the immunization schedule,

approximately 5 μl of blood was drawn from each mouse after cutting the tip of the tail.

Initially four injections were given to each of four mice at 1-week intervals, followed after 3 weeks by a booster. The mouse that gave the highest titre was used for preparing monoclonal antibodies. This procedure is referred to as protocol 'A'. The remaining three mice were kept for 8 months without any immunization and a booster injection was given at the end of the eighth month. Sera from the three mice were tested two weeks after the booster injection. Again the mouse that gave the maximum titre was used for antibody preparation (referred to as protocol 'B').

Fusion and cloning. The spleen was removed aseptically and splenocytes were fused with cells of the Sp2/0-Ag14 murine myeloma cell line at a ratio of 1 : 10 in the presence of polyethylene glycol, molecular weight 1500 (Boehringer Mannheim cat.no.783641, Mannheim, Germany). After fusion, cells were suspended in Iscove's Modified Dulbecco's medium (IMDM) (Gibco cat. no. 12200–036) containing 20% foetal bovine serum (FBS) (Gibco cat. no. 263000–061), hypoxanthine, aminopterine and thymidine medium (Gibco cat. no. 31062–011) and added to 96-well microculture plates. After 12 days, culture supernatants from each well were assayed using indirect competitive ELISA (see below). Culture supernatants from the cells that gave an absorption value of over 3 as a difference between 0·4 and 400 ng ml^{-1} were transferred to 24-well microculture plates in IMDM containing 20% FBS, hypoxanthine and thymidine. Supernatants from 24-well culture plates were tested again and only those clones that maintained absorption values over 3 in ELISA tests were chosen for further selection. Cell suspensions from each well of the 24-well-cultured plate were diluted to give approximately one cell per well when distributed into a 96-well culture plate. The plates were examined for the presence of number of hybridomas. Those that contained a single hybridoma in each well were retained and were screened for neutralization titres.

Indirect competitive ELISA procedure for screening hybridomas. Microtitre plates (Maxi-sorp-Nunc) were used and at each step the plates were incubated for 1 h at 37 °C. Initially plates were coated with 150 ng ml^{-1} of AFB1-BSA in 0·2 mol/L carbonate coating buffer (150 μl/well, Hobbs *et al.* 1987). In the second step plates were filled with a suspension of 4% dried milk prepared in phosphate-buffered saline containing 0·05% Tween 20 (PBS-T). Aflatoxin B_1 (0·4 or 400 ng ml^{-1}) in 100 μl, were added to each well and mixed with 50 μl of tissue culture supernatant (TCS). Goat antimouse IgG conjugated to alkaline phosphatase (1 : 1000 dilution) was used to detect mouse antibodies. Substrate, *p*-nitrophenyl phosphate at 1 mg ml^{-1}, was allowed to develop for 1 h at room temperature and absorbance at 405 nm was read in an ELISA plate reader.

Characterization of antibodies

Determination of isotype and cross-reactivity. Commercially available ISO-2 kits from Sigma were used to determine the isotypes of the monoclonal antibodies produced by various hybridoma cell lines.

To evaluate the cross-reactivity of each of the monoclonal antibodies, it was essential to determine the optimum conditions for neutralization. These included coating antigen (aflatoxin B1-BSA) concentration and the dilution of the antibody required for neutralization. IgGs were extracted from tissue culture supernatants using 18% sodium sulphate (Hobbs *et al.* 1987). IgG concentration was determined spectrophotometrically and antibody titres were determined by the indirect competitive ELISA procedure as described. The optimum concentration required at each step to obtain the maximum sensitivity was determined by 50% displacement values of B/B_0, where B is the extinction of the well containing AFB1 and B_0 is the extinction of the well without toxin, derived from the slope of calibration curves. Using these parameters various concentrations of the IgG (50 μl/well) from different monoclonal antibodies were added to 100 μl of AFB1 at concentrations ranging from 100 ng to 100 pg ml^{-1}. The protocols used for the characterization of antibody specificity were similar to those used to determine antibody titres except that in addition to B1, aflatoxin B2, G1 and G2 were also included for determining neutralization titres.

RESULTS

Immunization

The sera of mice immunized using protocol A showed poor antibody responses. Antibody was used at a dilution of 1 : 500. In contrast, high antibody titres, exceeding 1 : 20 000, were recorded from the sera of mice immunized by protocol B.

Fusion and cloning

The fusion efficiency (number of wells showing cell multiplication in each well of the 96-well plates) was 100%. In the first fusion, supernatants from cells derived from eight wells showed specific binding to AFB1. However, after two successive transfers only two retained the antibody activity. In the second fusion, 41 clones that secreted AFB1-specific antibodies were selected. After two successive transfers, 10 clones continued to give high neutralization titres. These clones were transferred to 25 cm^2 flasks and IgGs were

extracted from culture supernatants. IgG concentrations varied from 150 to 185 μg ml^{-1} of the culture supernatant.

Optimum aflatoxin and antibody activity for ELISA

An aflatoxin B1-BSA conjugate concentration of 125 ng ml^{-1} was found to be optimum for coating the plates. The optimum concentration of antibody for neutralization depended on the titre of antibody and it varied from 5 to 50 μg ml^{-1}.

Characterization of antibodies

The designation, data on isotypes, cross-reactivity and the minimal inhibition values for 10 monoclonal antibodies are presented in Table 1. The antibodies showed a range of cross-reactivity (Fig. 1) and could be classified broadly into three distinct groups. Group 1 comprised one monoclonal antibody, 10D5–1A11, that was highly specific for B1 and showed a weak cross-reaction to G1. Group 2 (13D1–1D9) contained antibodies that recognized B1, G1 and B2, with a weak cross-reaction with G2. The remaining clones that recognized B1 and G1 with equal efficiency were grouped in the category 3. Cell lines produced in the first fusion (protocol 'A') had a detection range from 10 to 100 ng ml^{-1} in contrast with that of the clones produced in the second fusion (protocol 'B') which gave values ranging from 0·001 to 1 ng ml^{-1}. One clone from the second fusion (10D5–1A11) is highly sensitive to aflatoxin B1 with a 50% inhibition at 0·006 ng ml^{-1} (Fig. 2).

DISCUSSION

The main aim of generating monoclonal antibodies was to obtain antibodies with appropriate antigen affinity, cross-reactivity and sensitivity. The generation of monoclonal antibodies is largely dependent on the immunization schemes, fusion procedures, and stabilities of the hybrid clones. The first step is to obtain as many antigen-specific monoclonal antibodies as possible. When immunizations were done by conventional methods (Candlish, Smith and Stimson 1990; Wang *et al*. 1995), very few monoclonal antibodies were generated. Interestingly the same group of mice gave better immune response following a long resting period. This presumably resulted in the accumulation of B memory lymphocytes with high affinity to Ig receptors in the spleen, thereby enhancing the chance of selecting hybridomas. Booster injections have been given normally 3 days prior to removal of spleen for fusion (Candlish *et al*. 1990; Ward *et al*. 1990). In contrast, we gave boosters 18 days before fusion. This was attempted because booster injections given 3 days before fusion (protocol 1) resulted in the selection of only a few monoclonal antibodies. To our knowledge, this is the first time that a rest after initial immunization has been shown to contribute to enhanced generation of monoclonal antibodies. It is yet to be tested if this protocol will consistently lead to generation of antibodies, with wide variation in affinity, when different antigens are used for immunization. Results are not presented in this communication because the affinity of these antibodies to the four aflatoxins are yet to be evaluated.

Table 1 Isotypes, cross-reactions and minimal inhibition observed with 10 monoclonal antibodies

		Cross-reaction† (%)				Minimal inhibition‡ (ng ml^{-1})		
Designation	Isotype*	B1	B2	G1	G2	B1	B2	G1
10D5–1A11	IgG_1	100	2	12	< 1	0·001	–	–
5D8–2B1	IgG_1	100	–	110	–	1	–	1
13D1–1D9	IgG_1	100	2	100	< 1	0·01	–	0·01
5F2–1E8	IgG_1	100	12	100	3	0·1	–	0·1
3G7–1B8	IgG_1	100	22	100	< 1	0·1	1	0·1
11C8–1A8	IgG_{2a}	100	20	66	1	0·01	–	0·01
3F7–1B9	IgG_1	100	15	60	< 1	< 0·01	–	< 0·01
5H4–1B1	IgG_1	100	13	72	1	< 0·01	–	< 0·01
6G12–2B3	IgG_{2a}	100	7	50	< 1	< 0·01	–	< 0·01
6E12–1E5	IgG_1	100	60	75	5	0·1	0·1	0·1

*Determined using a commercial kit.
†Expressed as 50% displacement value of B/B_0 for aflatoxin B1 divided by the 50% displacement value for each of the aflatoxin under testing (see text).
‡Concentration of aflatoxin (ng ml^{-1}) required for first significant inhibition of binding of antibody to AFB1-BSA solid phase.

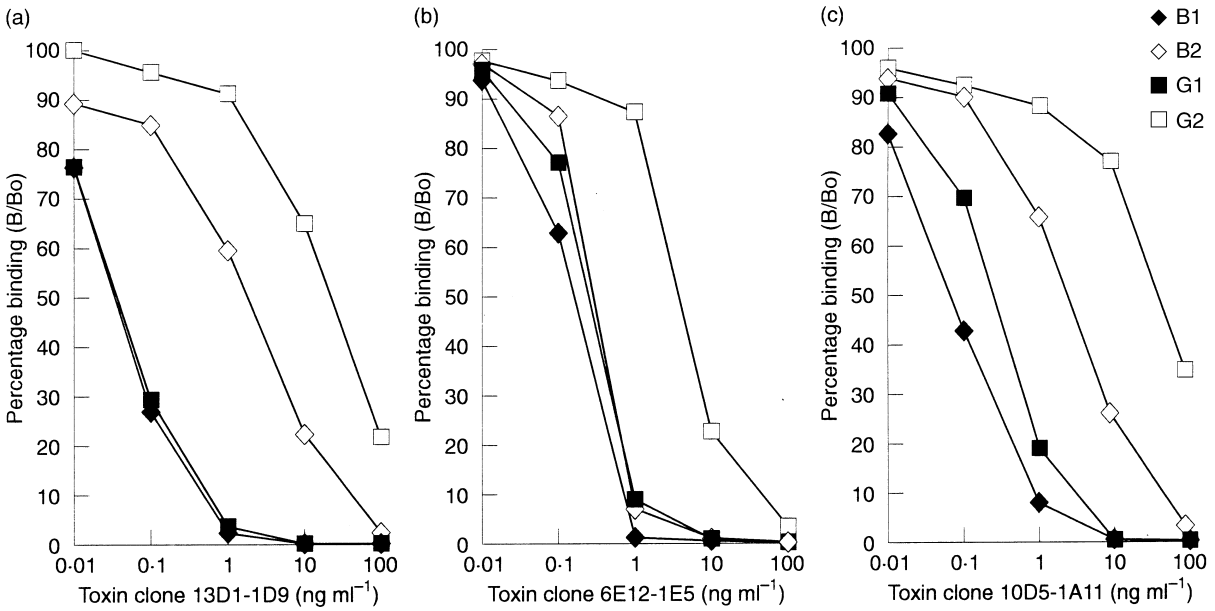

Fig. 1 Cross-reactivity of three groups of monoclonal antibodies to four major aflatoxins. Graphs plotted as percentage binding (B/Bo) against mass of toxin per millilitre (ng ml^{-1}). (a) Antibodies that recognize B1 and G1 with equal efficiency. (b) Antibodies that recognize B1, G1 and B2, with a weak cross-reaction to G2. (c) Antibody specific for B1 with varying degrees of cross-reaction to G1, B2 and G2

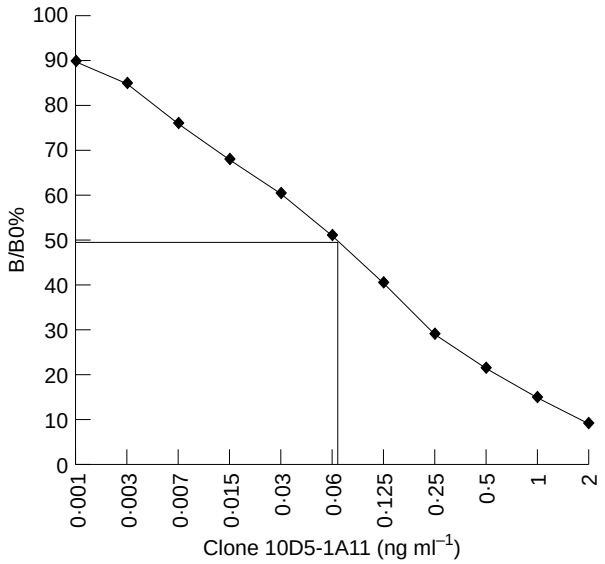

Fig. 2 Dose response curve of aflatoxin B1. Fifty percent inhibition value of AFB1 was 0·06 ng ml^{-1}

Monoclonal antibodies produced by Candlish *et al.* (1990) were largely specific to B1 with poor cross-reaction to B2, G1 and G2 aflatoxins. However, antibodies reported by Kawamura *et al.* (1988) were highly specific to B1 and showed partial reaction to G1. On the other hand Hefle and Chu (1990) reported equal cross-reactivities with all four aflatoxins. The wide variation in the specificities of monoclonal antibodies obtained in this study for four aflatoxins has so far not been reported to occur in the products of a single fusion. The antibodies produced can be used either to estimate B1 alone or B1, B2 and G1. Since G2 is the least toxic of all the four aflatoxins, and is not known to occur widely (FAO 1990) in foods and feeds, this deficiency is unlikely to contribute to inaccurate estimation of total aflatoxin content. The clone 10D5–1A11 (Fig. 2) could detect as little as 1 pg ml^{-1} of AFB1. This is in contrast with the limits of 200 pg ml^{-1} reported by Candlish *et al.* (1985) and 10 pg ml^{-1} reported by Ward *et al.* (1990).

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