Original Report

Preparation and Purification of Monoclonal
Antibodies for *Aspergillus fumigatus*

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Monoclonal antibodies were raised against *Aspergillus fumigatus*, a fungus which is an etiological agent of aspergillosis. Male SPF BALB/c mice were immunized subcutaneously with *A. fumigatus* culture-filtrate or mycelial antigen. Their splenocytes were hybridized with mouse myeloma cells, P3X63 Ag 8.653. The hybridoma supernatants were screened by enzyme-linked immunosorbent assay (ELISA), using *A. fumigatus* culture-filtrate antigen. Of the 123 hybridoma clones, 27 cell lines secreting antibodies positive for *A. fumigatus* were obtained. Two of these cell lines were further grown to larger volumes. The monoclonal antibodies obtained were immunoglobulin-M (IgM) antibodies, which were then purified by ammonium sulphate precipitation and Mono Q anion exchange chromatography. The purified monoclonal antibodies showed reactivity against the protein constituent of the culture-filtrate antigen.

Key words : *A. fumigatus*, culture-filtrate antigen, monoclonal antibodies, ELISA

Introduction

A. fumigatus, a fungus which can cause systemic, pulmonary and allergic aspergillosis, is still difficult to diagnose clinically. There have been many reports on the different antigens of this fungus¹⁻¹⁰⁾. In our study, the culture-filtrate antigen was chosen because it was easily prepared and was soluble enough to be used in the enzyme-linked immunosorbent assay. Identification of the exoantigens, which are present in the culture-filtrate of a fungus, results in its specific identification¹¹⁾. In the determination of the antigenicity of *A. fumigatus* culture-filtrate, its reactivity against

rabbit polyclonal antibody would not provide sufficient comprehensive information on the nature of the antigenic reactivity of the constituents. This is because an antigen is recognized by a polyclonal antibody via a number of different epitopes. In contrast, monoclonal antibodies represent a homologous population of immunoglobulins directed against a single antigenic determinant¹¹⁾. This prompted us to attempt the preparation of murine monoclonal antibodies. Monoclonal antibodies for other fungi have already been raised: those against fungi of medical importance¹²⁻¹⁴⁾ and fungal plant pathogens¹⁵⁾.

Materials and Methods

Culture-filtrate antigen: *A. fumigatus* strain NCPF 2109 was obtained from the Mycology

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Reference Laboratory, Central Public Health Laboratory, London, U.K. The preparation of the antigen was as described by Sakaguchi et al.¹⁾ with some modifications. The fungus was grown for 2 weeks, without shaking, in Sabouraud's dextrose broth at 28 °C. At the end of the cultivation, formalin was added to the culture to a final concentration of 0.5% and it was left overnight. A sterility test was carried out on Sabouraud's dextrose agar plates which were incubated at 28 °C. The mycelial mat was removed and the broth filtered through No.2 Toyo filter paper (Toyo Roshi Co., Ltd., Tokyo). The culture-filtrate was then concentrated by means of a rotary evaporator, dialyzed in distilled water overnight at 4 °C, reconcentrated and precipitated by 4 times v/v ethanol. The precipitate was then dehydrated in vacuo. The dried precipitate was ground to powder.

Mycelial antigen : Two 1,000ml flasks with 500ml of Sabouraud's dextrose broth were inoculated with a small amount of *A. fumigatus* conidia and incubated, without shaking, at 28 °C for 9 days. At the end of the incubation period, 2.5ml of formalin was added to each of the flasks. The flasks were shaken manually and left to stand in the incubator for 24 hours. A sterility test was then carried out on Sabouraud's dextrose agar plates, which were incubated at 28 °C. The mycelial mats were collected from both flasks and placed on a sterile piece of No.2 Toyo filter paper, diameter 18.5cm, then cut into small pieces with a pair of sterile scissors. The pieces of mycelial mat were further homogenized in 0.85% sterile saline to mycelial fragments with a homogenizer (Physcotron, Nichion Irikakiki, Tokyo).

Mice : Five-week-old male SPF, BALB/c mice (SLC, Shizuoka) were used.

Immunization protocol : The protocol used was based on that of Miyakawa et al.¹²⁾ with some modifications. The mice were immunized subcutaneously once a week for 3 weeks, each with 0.2ml of either 200mg of culture-filtrate antigen in 2ml sterile 0.85% saline and 2ml

Freund's complete/incomplete adjuvant (complete adjuvant for the first immunization) or 1ml of the homogenized mycelium in sterile 0.85% saline mixed with 1ml of Freund's adjuvant. Antibody response was monitored by ELISA.

Murine myeloma cell line : Cells of the myeloma line, P3X63Ag8.653, obtained from Tohoku University, Sendai, were used. These were cultured in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo), supplemented with 10% foetal bovine serum (Cosmo Bio, Tokyo).

Preparation of hybridoma cells : After the final immunization, the mice were sacrificed and their spleens removed aseptically. Hybridoma cells were then prepared according to the method of Galfre et al.¹⁶⁾ In our preparation, untreated splenocytes were also added to each well to serve as feeder cells. After 7 to 10 days, wells showing vigorous growth indicated successful hybridoma clones. The supernatants from these wells were assayed for antibody production by ELISA. Positive clones were given nonselective medium and when the growth became confluent, they were transferred to 24- and 6-well plates successively. The most actively growing cell lines were recloned and grown to larger volumes.

Polyclonal Antibodies : The immunization protocol was adapted from that of Hearn and Mackenzie³⁾. White rabbits, weighing about 2kg, were immunized subcutaneously once a week for 4 weeks, each with 1ml of 100mg of culture-filtrate antigen of *A. fumigatus* in 1ml sterile saline and 1ml of Freund's complete /incomplete adjuvant (complete adjuvant for the first immunization). Antibody response was monitored by ELISA. The rabbits' sera were then used as the source of polyclonal antibodies.

ELISA method : The method followed was that of Voller et al.¹⁷⁾. The assays were carried out in flexible, flat-bottomed, 96-well assay plates (Becton Dickinson and Co., Oxnard, CA, U.S.A.). Wells were coated with 100 μ l of antigen solution (30 μ g ml⁻¹ of culture-filtrate antigen in 0.6 M carbonate buffer pH

9.6). Primary antibodies were the supernatants of hybridoma cell culture, polyclonal antibody or monoclonal antibodies. Secondary antibody conjugates were goat anti-rabbit immunoglobulin-G (No.F23385, Seikagaku Kogyo Co., Ltd., Tokyo), rabbit anti-mouse immunoglobulin-G (No.F44989, Seikagaku Kogyo Co., Ltd., Tokyo) or goat anti-mouse immunoglobulin-M (No. 31419, Organon Teknika Corporation, West Chester, PA, U.S.A.) horseradish peroxidase conjugates diluted 1,000-fold into phosphate-buffered saline with 0.5% Tween 20. The substrate was o-phenylene diamine. The presence of the antibody which reacted with the coated *A. fumigatus* culture-filtrate antigen was determined by measuring the absorbance at 492 nm with an Immuno Reader NJ-2000 (Inter Med Co., Tokyo).

Determination of immunoglobulin class : A modified ELISA method was used. After the primary antibodies had been washed off, the "Mouse Typer Kit" (Bio-Rad Labs., Richmond, CA, U.S.A.) was used according to the instructions provided by the manufacturer.

Purification of monoclonal antibodies : The monoclonal antibodies were purified from supernatants of the two selected hybridoma clones. The antibodies were precipitated from the culture supernatants with ammonium sulphate overnight at 4°C. The precipitate was centrifuged at 3,000 rpm for 30 minutes. The pellet was washed with 0.85% saline and dialyzed overnight at 4°C in 0.85% saline. The amount of NH_4^+ ions was monitored using "Ammonium-Test" strips (E. Merck, Postfach 41 19, D-6100 Darmstadt, Federal Republic of Germany). The solution was then lyophilized. The reconstituted monoclonal antibodies were purified according to Sampson et al.¹⁸⁾ with some modifications. The antibodies were purified by a fast protein liquid chromatography system, through a Mono Q HR 5/5 (Pharmacia LKB Biotechnology, Tokyo) anion exchange column, equilibrated in potassium phosphate buffer pH 6.5. The fractions were collected and their reactivity against the culture-filtrate antigen was determined by ELISA (data not

shown).

Protein assays : The method of Lowry et al.¹⁹⁾ was employed in the determination of protein contents of the culture-filtrate antigen and the antibodies. Bovine serum albumin (No.A-4378, Sigma Chemical Co., Tokyo), serially diluted from $100 \mu\text{g ml}^{-1}$ in purified water, was used as a standard.

Carbohydrate assays : Total carbohydrate of the culture-filtrate antigen was measured by a modified Molisch method²⁰⁾. Glucose, serially diluted from $100 \mu\text{g ml}^{-1}$ in purified water, was used as a standard.

Treatment of culture-filtrate antigen with protease : Two-fold dilutions of Pronase E (Kaken Chemical Co., Ltd., Tokyo), from the starting concentration of 10% in phosphate-buffered saline, was carried out in flexible, flat-bottomed, 96-well assay plates. The diluted enzyme was then added to the culture-filtrate antigen ($30 \mu\text{g ml}^{-1}$) and incubated at 37°C for 24 hours and 48 hours. The enzyme-treated antigen was subsequently used to coat the 96-well plates, which were first incubated overnight at 37°C and the normal ELISA was performed on the following day. The controls were held under the same conditions in the absence of enzyme.

Results

The hybridization of immunized mouse splenocytes with mouse myeloma cells resulted in 123 hybridoma clones. During growth, some of the clones lost activity, some retained activity while a few increased their activity. Twenty-seven of these cell lines secreted antibodies positive for *A. fumigatus* culture-filtrate antigen. The two most actively growing cell lines, though not the ones secreting antibodies with the highest titres, were further grown to larger volumes; these lines were 8-3C F5 G9 and 20-4C 3F4 E10, which were prepared from mice immunized with culture-filtrate and mycelial antigens, respectively. Their supernatants were then purified by ammonium sulphate precipitation, followed by Mono Q anion exchange chromatography. Immunoglobulin-M

Fig. 1. Mono Q anion exchange chromatography profile of the monoclonal antibody, 8-3C F5 G9, as monitored by the U.V. monitor.

Fig. 2. Mono Q anion exchange chromatography profile of the monoclonal antibody, 20-4C 3F4 E10, as monitored by the U.V. monitor.

Table 1. Protein contents of the polyclonal and monoclonal antibodies

	Protein ($\mu\text{g ml}^{-1}$)
Polyclonal antibody	94,000
Monoclonal antibodies	
8-3C F5 G9 before purification	4,960
20-4C 3F4 E10 before purification	5,100
8-3C F5 G9 after purification	30
20-4C 3F4 E10 after purification	110

(IgM) was separated from immunoglobulin-G (IgG) and immunoglobulin-A (IgA) by Mono Q chromatography (Figs. 1 and 2). The two monoclonal antibodies were IgM antibodies.

The protein contents of the polyclonal antibody and those of the two monoclonal

antibodies, before and after purification, as determined by Lowry's method are given in Table 1. After purification, the titres of the monoclonal antibodies had increased by about 24 times for 8-3C F5 G9 and about 41 times for 20-4C 3F4 E10 (Fig. 3). The titres of the purified monoclonal antibodies were also higher than that of the polyclonal antibody; that of 8-3C F5 G9 was about 7 times higher while that of 20-4C 3F4 E10 was about 5 times higher (Fig. 3).

The protein content of the culture-filtrate antigen, as determined by Lowry's method, was 61.7% while its total carbohydrate content, as determined by the modified Molisch method was 6.17%. To determine against which of the constituents our antibodies were reactive, we tested the effect of protease on the reactivity of the antigen against the antibodies. After the protease treatment, the reactivity

Fig. 3. Reactivity of the antibodies (○) 8-3C F5 G9 before purification, (●) 8-3C F5 G9 after purification, (△) 20-4C 3F4 E10 before purification, (▲) 20-4C 3F4 E10 after purification and (□) polyclonal antibody. The concentration of the antibodies in $\mu\text{g ml}^{-1}$ is plotted on a log scale versus the absorbance at 492 nm.

Fig. 4. Reactivity of polyclonal antibody (□), 8-3C F5 G9 (○) and 20-4C 3F4 E10 (△) against Pronase E-treated culture-filtrate antigen for 24 hours and 48 hours. The percentage of enzyme added is plotted versus the absorbance at 492 nm. The mean plus or minus one standard deviation of 3 assays is given.

of the culture-filtrate against polyclonal and the two monoclonal antibodies was reduced (Fig. 4).

Discussion

We were able to prepare hybridoma cells capable of producing antibodies reactive against *A. fumigatus* culture-filtrate antigen. These monoclonal antibodies were IgM antibodies. The other researchers^{12,14,15)} also obtained all or nearly all IgM monoclonal antibodies in their preparations of monoclonal antibodies. The two selected hybridomas, irrespective of their origins, showed reactivity against *A. fumigatus* culture-filtrate antigen. This finding is consistent with those of other immunological studies of fungi^{1,5,11,15)} in that fungi produce specific exoantigens which are readily detected in culture broths or surface washings.

By employing Mono Q anion exchange chromatography in the purification of the monoclonal antibodies, the fractionation of IgM from the IgG and IgA fractions was rapid and reproducible¹⁸⁾.

The polyclonal and monoclonal antibodies showed reactivity against the protein constituent of the culture-filtrate, as demonstrated by the reduction in reactivity after the protease treatment. This suggests that the active sites of the culture-filtrate antigen are protein moieties.

Kurup²¹⁾ produced an IgM κ monoclonal antibody against *A. fumigatus* antigen having IgG and IgE antibody-binding activities against patient sera. It reacted with 12 of 16 antigens extracted from 9 different *A. fumigatus* strains and with all 3 *Aspergillus flavus* antigens studied. The nature of species-specific antigens of *A. fumigatus* has to be determined and is the subject of our continuing study. It is hoped that these monoclonal antibodies will be valuable in the precise determination of the antigenicity of the *A. fumigatus* culture-filtrate. The derivation of hybridomas producing antibodies against *A. fumigatus* antigen may also prompt the production of standard supplies of monoclonal antibodies for clinical

diagnosis of aspergillosis, especially systemic aspergillosis in which the detection of circulating antigens by monoclonal antibodies will be useful.

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