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Research Article

Monoclonal Antibody Production against *Aspergillus Fumigatus* in Ascetic Fluid of Balb/C Mice

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Abstract: Monoclonal antibodies (mAbs) due to their binding specificity, their homogeneity and great ability to be produced in unlimited quantities are important reagent in diagnosis and treatment of diseases like infections and cancers. Hybridoma cell lines grown as ascites tumours in Pristane primed mice will frequently yield milligram quantities of monoclonal antibody per milli liter of ascites fluid. For large scale production of monoclonal antibodies, hybridoma cells that produce monoclonal antibody against *Aspergillus fumugatus* were injected into the peritoneum of the Balb/c mice which have previously been primed with 0.5 mL Pristane. After 10 days, approximately 4-5 mL ascitic fluid was harvested from the peritoneum of each mouse. Ascitic fluid was assayed for the titer of monoclonal antibody in reaction with crude extract of Aspegillus fumigatus and its cross reactivity in reaction with other pathogenic species of *Aspergillus* and *Candida*. The titer of mAb was 100,000 and didn't show cross reactivity with other pathogenic species of *Aspergillus* and Candida. Immunoblotting was used to study the specificity and nature of epitopes recognized by the antibodies and also, for confirmation of the result of cross reactivity with other species of *Aspergillus* and *Candida* by ELISA method. Periodate oxidation of crude extract of *Aspergillus fumigatus* on a PVDF membrane resulted in reduction of carbohydrate residue of the glycoproteins, and appearance of sharp bands. The proteins binding had a molecular mass of 58 kDa. The subclass of antibody was IgG1 and its light chain was kappa. Ascitic fluid was purified by ion exchange chromatography.
Keywords: Monoclonal antibodies, *Aspergillus fumugatus*, *Candida*, Hybridoma cell

INTRODUCTION

Antibodies are important tools used by many investigators in their research and have led to many medical advances. Monoclonal antibodies have been used as essential research tools for multiplicity of research purposes, including Western blotting, immuno histo chemistry, immuno cyto chemistry, enzyme-linked immuno sorbent assay (ELISA), immuno precipitation and flow cytometric analysis. In addition, antibodies are now being designed for therapeutic applications, including suppression of the immune system after organ transplantation [1, 2] treatment of cancers such as leukemia and inhibition of angiogenesis [3]. *Aspergillus fumigatus* is an opportunistic and one of the most ubiquitous of the airborne saprophytic fungi which causes a number of life threatening diseases in humans and animals. Invasive aspergillosis which occurs in a wide range of clinical scenarios, including allergic aspergillosis, colonization of cavities with or without the formation of a fungus ball (mainly in the lungs, paranasal sinuses, bronchiectasis), acute to chronic necrotizing invasive forms, ocular infections (keratitis), otomycosis, endocarditis, osteomyelitis and skin infections, is protean in its manifestations, and is still associated with an unacceptably high mortality rate. People most at risk for aspergillosis include patients with advanced AIDS, prolonged neutropenia, allogeneic hematopoietic stem cell transplant recipients, solid organ transplant recipients, and chronic granulomatous disease [4, 5].

Injection of requested clone into the peritoneum of the mouse that has previously been primed with Pristane and culture of the hybridoma cells

in tissue culture flasks are two common methods for large scale production of the monoclonal antibodies [6, 7]. Large-scale production of mAbs, usually takes place by propagating hybridomas as ascites tumours in pristanated mice [8]. The success rate in tumour development and the probability of ascites formation are increased by intraperitoneal injection of 0.5 ml Pristane (2, 6, 10, 14-tetramethyl pentadecane) or incomplete Freund's adjuvant a few days prior to injecting the cells [8, 9]. When hybridoma cells are injected into the peritoneum of mouse, the cells grow and produce ascitic fluid. This fluid has high concentration of antibody. The method of ascitic fluid production in peritoneum of mouse is simple and economic [10]. But reproduction of cells in cell culture media needs special skills, special medium and FCS that is expensive and an idling technique [11]. Furthermore, the loss of antibody's original glycosilation by in vitro culture method makes it unsuitable for long time functions. So, antibody production in ascitic fluid can be a suitable and economical method.

MATERIALS AND METHODS

Two inbreed, female, 6 to 8 weeks old Balb/c mice were obtained from the Institute of Medical Research (IMR, Kuala Lumpur, Malaysia). 0.5 ml Pristane (2, 6, 10, 14 tetra methyl penta decane, Sigma) was injected intra peritoneally into each mouse. Ten days after priming with Pristane, the cells of a suitable mono clone in density of 10^6 cells/0.5 mL PBS were injected intra peritoneally into each mouse [12, 13]. Five days after the injection of hybridoma cells, the mice were observed daily for production of ascitic fluid. About ten days after the injection of cells, abdomen of the mice were completely enlarged and their skins were extended. Ascitic fluids were harvested using 19 gage needles. After 4 days, ascitic fluid of the mice were harvested again and centrifuged. Determining the antibody titer and its cross reactivity with other medically important species of *Aspergillus* and *Candid*a. The titer of monoclonal antibody was assessed by ELISA method. In this assay, first crude extract of *Aspergillus fumigatus* (5 µg/mL) was coated for 45 minutes in 96 wells plate. After twice washing with PBS-Tween 20 (0.05%), non specific sites were blocked with 2% BSA and incubated at 37°C for 45 min. The washing was repeated and then 100 µL of the continuous dilution of ascitic fluid was added to each well and incubated for 1 hr at 37°C. Then the plate was washed 5 times. 100 µL of Rabbit Anti-mouse IgG conjugate with 1/4000 dilution (Sigma) was added to each well and incubated for 45 min at 37°C. After five times of washing, 100 µL of TMB substrate solution (Sigma) was added into each well and incubated for 15 min in dark place at 37°C. The reaction was stopped by 100 µL of 5% solution of Sulphuric Acid. The absorbance of each well was read by ELISA Reader at 450 nm and therefore the titer of monoclonal antibody in ascitic fluid was determined [10]. In order to determine the cross reactivity with IgM and IgA, the micro titer plates were coated with each class of the purified immunoglobulins (5µg/mL in coating buffer, 100 µL/well) and incubated for 1 hr at 37°C. The plates were blocked with 2% BSA as described above. Following a soak and wash cycle, the ascitic fluid with distinct dilution was added to the wells and incubated as described above. The rest of the assay was preceded as described for the ELISA procedure. In the end of the reaction and by reading their absorbance, cross reactivity of the monoclonal antibodies with other species of *Aspergillus* and *Candida* was determined.

Immunoblotting

This technique was used for confirming the result of cross reactivity with other species of *Aspergillus* and *Candida* by ELISA method. First, the nitrocellulose membrane and several thicknesses of Whattman chromatography paper were soaked in the transfer buffer (25 mm Tris, 192 mm glycine, 20% V/V methanol, pH 8.3). The wet nitrocellulose membrane was overlaid on the wet Whattman sheets by taking precaution to avoid bubbles. Then, the gel of SDSPAGE was placed on the wet nitrocellulose membrane and then several wet Whattman papers were placed on it. Transfer of the proteins from gel to nitrocellulose membrane was done in 100 V for 3 h. Then, non specific sites were blocked with 2% BSA solution. After three times of washing, the membrane was cut into strips and incubated for 2 h at 37°C using the ascitic fluid. Again, after five times of washing, the strips were incubated for 2 h at 37°C with Rabbit Anti-Mouse IgG conjugate (1/2000 dilution). The strips were washed and then developed in DAB substrate (Sigma). The sharp band was appeared in its molecular weight position [11].

Isotyping

The screening and determination of the classes and subclasses of mouse monoclonal antibodies was done using the Immuno Pure Monoclonal Antibody Isotyping Kits from Pierce. Fifty µL of the crude antigen of A. Fumigatus was used to coat each of 9 separate wells and incubated at room temperature for 2 hr. After washing, 125 µL of 1X 2% BSA was added as a blocking solution and incubated at 37°C for 1 h. Wells were washed 4 times with 125 µL of washing buffer (0.05% tween 20 in PB). Then, 50 µL of ascitic fluid with distinct dilution was added to each antigen-coated well on the plate and incubated at 37°C for 1 hr. The solution was then removed and washed 4 times with 125 µL of washing buffer. One drop (50 µL) of subclass-specific anti-mouse immunoglobulins was added to each antigen-coated well on the plate. The plates were incubated at 37°C for 1 h followed by 4 times washing with 125 µL of washing buffer. Fifty µL of horseradish peroxidase conjugated goat anti-mouse IgG (Sigma, USA) which had been diluted 1:5000 in blocking solution was added to each antigen-coated well and incubated at 37°C for 1 h. The solutions were

removed and wells were washed 4 times with 125 µL of washing buffer. One hundred µL of ABTS substrate solution (Pierce) was added to each antigen-coated well on the plate, incubated at room temperature and colour development was monitored for approximately 30 min. Colour changes were read both qualitatively by visual inspection and quantitatively with a spectrophotometer at 405 nm.

Purification

The ascitic fluids were diluted 1: 2 with PBS and fractionated with 40% saturated ammonium sulphate. After several times of washing with 40% ammonium sulphate, the fraction was centrifuged for 15 min in 5000 g. The precipitated fraction was dialyzed against 0.05 M phosphate buffer pH 7.4 containing 0.05 M Nacl. The final dialyze was exchanged against the column washing buffer (Tris 40 mm). Purification of ascitic fluid was done by Ion exchange chromatography (DEAE- Sepharose 6B) which is a simple and economical method [9]. At first, the column was eluted with washing buffer (Tris 40 mm, pH 8.1) in order that the pH of the external buffer to be the same as the PH of internal buffer. Then the dialyzed sample in 60 mg/3 mL concentration was run to the column with dimensions of 1.6×15 cm. Distinct antibody was eluted from the column through washing buffer containing 50 mm Nacl and the fractions were collected in 5 mL within 20min. Confirmation of the purified fractions was done by SDS-PAGE in reducing condition.

RESULTS

About 1×10^6 hybridomas cells that produce monoclonal antibody against *Aspergillus fumigatus* were suspended in 0.5 mL of sterile PBS and injected into the peritoneum of the Balb/c mice which have previously been primed with 0.5 mL Pristane. After 10 days, approximately 4-5 mL ascitic fluid was harvested from the peritoneum of each mouse. Again, after 4 days, about 3-4 mL ascitic fluid was harvested from their peritoneum. The titer of monoclonal antibody in ascitic fluid was assessed by ELISA method. Absorbance (OD) of 1/100,000 dilution was above 1.1. With the same titer, the suitable mAb didn't show any cross reactivity with other medically important species of *Aspergillus* and *Candida* (Figure 1). Immunoblotting was used to study the specificity and nature of epitopes recognized by the antibodies. Also, it was used to confirm the result of cross reactivity with other types of medically important species of *Aspergillus* and *Candida* by ELISA method. Periodate oxidation of crude extract of A. Fumigatus on a PVDF membrane resulted in reduction of carbohydrate residue of the glycoproteins, and appearance of sharp bands. The proteins binding with mAbs 7D7 and 4C6 had a molecular mass of 58 kDa.

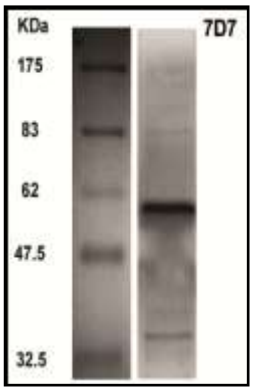

Fig-1: The immunobloting of ascitic fluid related to suitable mono clone.

The antibody isotype was determined according to the Isotyping kit (Immuno Pure, Pierce). The subclasses of mAbs were IgG_1 and Kappa (κ) light chains were found in that. To check the purity, mAb was applied to 12% SDS-PAGE. The staining of gel and analyzing the results by Quantity One analysis software resulted that the mAb 4C6 consists of two (51.34 kDa and 25.11) fragments.

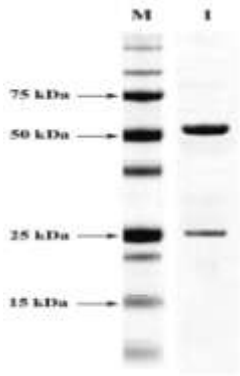

Fig-2: SDS-PAGE analysis of fraction from Ion exchange chromatography purification. Reducing SDS-PAGE (1) of produced monoclonal antibody.

The diluted ascitic fluids were precipitated with saturated ammonium sulphate followed by dialyzing against PBS (pH 7.4). Concentration of the dialyzed product in assay with UV at 280 nm was about 100 mg. Its purification with ion exchange chromatography yielded about 30 mg of purified monoclonal antibody. The purified monoclonal antibody was conjugated with peroxidase. The titer of conjugate was 100,000 and didn't show any cross reactivity with other species of *Aspergillus* and *Candida.*

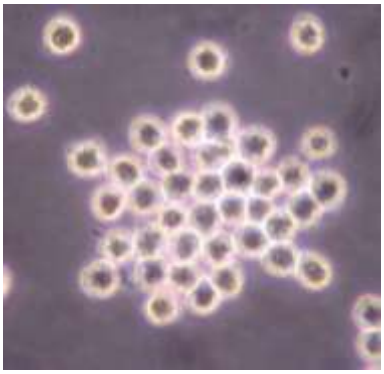

Fig-3: Proliferated suitable mono clone selected for injection into the peritoneum of mice.

DISCUSSION

Monoclonal antibodies are proteins, possessing exquisite specificity and remarkable sensitivity in their reactions with specific sites on target molecules that they have become reagents of central importance in modern biological and microbiological research. Optimum interval between injecting Pristine and injecting hybridomas and also, optimum quantity of Pristine used are two main parameters that affect the ascites production [8, 12]. In this study, to obtain the maximum yield of ascitic fluid an interval of one week and 0.5 mL of Pristane were applied to each Balb/c mouse. Each of mice received 0.8 x 10^6 hybridoma cells intra-peritonealy. The mice were weighed and observed on a daily basis for physical changes. The physical changes were observed in abdomen of mice after 8 days and also on average, the weight of mice increased 14 gram. Approximately, 4-5 ml ascitic fluid were extracted and harvested from the peritoneum of each mouse. For the production of this amount of antibody by the in vitro method, several liters of DMEM medium and FBS as well as several months of continuous struggle and attention are needed. Therefore, it is obvious that ascitic fluid production is a very useful and economical method. The produced ascitic fluid with 1/100,000 dilution was very promising and had absorbance above 1. Based on cross-reactivity test, we found that mAb binds to a unique epitope which distinguish *Aspergillus fumigatus* from the other pathogenic species of *Aspergillus* and *Candida.* The produced monoclonal antibody may be used as reference antisera and/or for the purification of the antigen employed in the test and allowing for standardization of the immunoassay for detecting the *Aspergillus fumigates* in tissue and other specimens [12, 13, 14].

REFERENCES

1. Koch M, Niemeyer G, Patel I, Light S, Nashan B; Pharmacokinetics, pharmacodynamics, and immuno dynamics of daclizumab in a two-dose regimen in liver transplantation. Transplantation, 2002; 73(10): 1640-1646.
2. Bumgardner GL, Hardie I, Johnson RW, Lin A, Nashan B, Pescovitz MD; Results of 3-year phase III clinical trials with daclizumab prophylaxis for prevention of acute rejection after renal transplantation. Transplantation, 2001; 72(5): 839-845.
3. Stephan S, Datta K, Wang E Li J, Brekken RA, Parangi S; Effect of rapamycin alone and in combination with anti angiogenesis therapy in an orthotropic model of human pancreatic cancer. Clin Cancer Res, 2004; 10(20): 6993-7000.
4. Stevens DA, Kan VL, Judson MA, Morrison VA, Dummer S, Denning DW; Practice guidelines for diseases caused by *Aspergillus*. Infectious Diseases Society of America. Clin Infect Dis, 2000; 30(4): 696-709.
5. Fujimura M, Ishiura Y, Kasahara K., Amemiya T, Myou S, Hayashi Y; Necrotizing bronchial aspergillosis as a cause of hemoptysis in sarcoidosis. Am J Med Sci, 1998; 315(1): 56-58.
6. Jackson LR, Trudel LJ, Fox JG, Lipman NS; Monoclonal antibody production in murine ascites. II. Production characteristics. Lab. Anim. Sci., 1999, 49: 81-86.
7. Baradaran B, Majidi J,Hassan Z.M, Abdolalizadeh J; Large Scale Production and Characterization of Anti-Human IgG Monoclonal Antibody in Peritoneum of Balb/c MICE. American Journal of Biochemistry and Biotechnology, 2005; 1 (4): 189-192.
8. Hoogenraad N, Helman T, Hoogenraad J; The effect of pre-injection of mice with pristane on ascites tumour formation and monoclonal antibody production. J Immunol Methods, 1983; 61(3): 317-320.
9. Mueller UW, Hawes CS, Jones WR; Monoclonal antibody production by hybridoma growth in Freund's adjuvant primed mice. J Immunol Methods, 1986; 87(2): 193-196.
10. Mc Ardle J; Alternatives to ascites production of monoclonal antibodies. Anm. Welfare Inform. Center Newslett, 1998; 8: 3-4.
11. Lang AB, Schuerch U, Cryz SJ Jr.; Optimization of growth and secretion of human, 1991.
12. Koushan Sineh Sepehr, Behzad Baradaran, Jafar Majidi, Jalal Abdolalizadeh, Leili Aghebati, Fatemeh Zare Shahneh. Mass-Production and Characterization of Anti-CD20 Monoclonal Antibody in Peritoneum of Balb/c Mice, Advanced Pharmaceutical Bulletin, 2013, 3(1), 109-11.
13. Brodeur BR, Tsang P, Larose Y; Parameters affecting ascites tumour formation in mice and monoclonal antibody production. J Immunol Methods, 1984; 71(2): 265-272.
14. Monoclonal antibodies by hybridomas cultued in serum-free media. Hybridomas, 10: 401-409.