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Preparation of the Monoclonal Antibodies against the Zoospores of *Allomyces macrogynus*

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Abstract

Monoclonal antibodies against the zoospores of *Allomyces macrogynus* were prepared using standard hybridoma technique. Mice were immunized either with the fixed zoospores or the zoospore proteins, and the production of the antibodies from the resulting hybridomas were screened by enzyme-linked immunosorbent assay (ELISA). Thirty hybridomas were initially identified and six hybridomas were purified to the single cell clones. Culture supernatants from the hybridomas were tested for the effects on the growth of the germ tubes, and some of the hybridoma culture supernatants studied showed growth stimulatory effects.

Key words : *Allomyces macrogynus, zoospore, hybridoma, monoclonal antibody*

INTRODUCTION

Allomyces macrogynus, a member of the Class *Chytridiomycetes*, is an aquatic fungus living in fresh water. This fungus exhibits clear alternation of haploid gametothalli and diploid sporothalli[1], and produces four types of zoospores containing single posterior flagellum[2]. During asexual reproduction, diploid zoospores, lacking cell wall, are released[3,4]. When the zoospores meet suitable nutritional condition, they stop swimming and start to form cell wall, giving rise to round cysts. Following the rhizoid formation, hyphal growth of the fungus resumes.

A. macrogynus is the one of the best known of all fungi in terms of the morphology, cytology and life cycle[3]. Although the development of its zoospore has been extensively studied cytologically, previous studies failed to explain the fate of cell membrane and various cellular organelles during the zoospore development. One of the reasons for the failures might be from the lack of a suitable marker necessary for tracing of the fate of the various cellular organelles and for purification of the specific cellular organelles.

In order to deveplope markers suitable for the study of the fate of cell membrane during zoospore development and for the isolation of a specific component affecting zoospore development, we decided to raise the monoclonal antibodies against zoospores of *A. macrogynus*. Here, we report the production of monoclonal anti-

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bodies against zoospores of *A. macrogynus* and discuss the effects of the monoclonal antibodies on the zoospore development.

MATERIALS AND METHODS

Organisms, Mice and Cell Lines

Allomyces macrogynus (Emerson strain Burma 3−35) was kindly provided by Dr. M. S. Fuller of the University of Georgia, USA. Six week old female BALB/c mice were purchased from the Korean Chemical Research Institute, Taejun. The myeloma cell line P3X63Ag8.653 (ATCC CRL1580) was obtained from Korean Cell Line Bank, Seoul.

Chemicals

Most of the reagents used for immunization, hybridoma preparation, cell culture and antibody production were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A. Alkaline phosphatase conjugated goat anti-mouse IgG, TRITC conjugated goat anti-mouse IgG and Protein A-Sepharose were also obtained from Sigma Chemical Co. Fetal bovine serum used in the cell cultures was purchased from Gibco-BRL, Gaithersburg, MD, U.S.A. Most of the chemicals for the cultivation of *A. macrogynusus* were purchased from Difco Labs, Detroit, MI, USA.

Preparation of zoospore and its protein

Maintenance of culture and production of zoospores were performed as described by Fuller and Jaworski[5]. The sporothalli of *Allomyces macrogynus* were submerged in the dilute salts (DS) solution and the diploid zoospores were harvested two hours later. One of the zoospore preparations was fixed with 4% formaldehyde and subsequently used for the immunization. For the preparation of the soluble zoospore proteins, unfixed zoospores were lysed by the incubation with five volume of the spore lysis solution of 300mM NaCl, 50mM Tris (pH 7.0), 0.5% Triton X−100 at 4℃ for 30min. Following centrifugation of the spore lysates, the soluble proteins in the supernatants were collected. The amounts of the spore proteins were determined by Bradford method[6], and the quality of the protein preparations were examined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The soluble zoospore proteins were used for immunizations and screenings.

Preparation and screening of the hybridomas

Preparation of the hybridomas and the production of monoclonal antibodies were performed with a slight modifications of the method described by Harlow and Lane[7]. Six week-old female BALB/c mice were immunized intraperitoneally either with the formalin-fixed diploid zoospores or with the soluble zoospore proteins. The soluble protein solutions were mixed with suitable Freund's adjuvants prior to immunization, while fixed zoospores were immunized directly without adjuvant. After immunization of the mice twice with the same antigen, antibody productions in the mice were identified by mouse tail bleeding. Three weeks later, the antibody producing mice were immunized again with the same antigens, and sacrificed on three days after immunization to prepare spleen cell suspensions. After removal of the red blood cell, the spleen cells were fused with P3X 63Ag8.653 myeloma cells using polyethylene glycol of molecular weight 1500 (PEG 1500). The fused cells were selected in HY medium (Sigma Chemical Co.) supplemented with hypoxanthin-aminopterine-thymidine (HAT) for 10 days and subsequently in the medium supplemented with hypoxanthin-thymidine (HT).

The surviving hybridoma cells were examined for the production of antibodies specific to zoospore proteins using ELISA as described by Hornbeck[8]. Briefly, 20μg of spore protein was allowed to bind to a 96 well ELISA plate at room temperature for 2 hours. Following the blocking with 0.25% BSA in 0.05% Tween 20, the culture supernatant from each hybridoma culture was added. After incubation for two hours, the wells were washed and then incubated for two hours with alkaline-phosphatase conjugated goat anti-mouse IgG. Enzyme activities were determined by the reaction with p-nitro-

phenyl phosphate (pNPP) and the measurement of absorbance at 420nm using Molecular Device UVmax ELISA Reader (Menlo Park, CA, USA). The resulting positive hybridoma cultures were subcloned once or twice by limiting dilution technique and subsequently screened by ELISA to isolate purified single cell clones.

Preparation of the monoclonal antibodies

Monoclonal antibodies were purified by the protein A-Sepharose affinity column chromatography using prepared mouse ascites[9]. Briefly, 5×10^6 cells from the single hybridoma clone were inoculated to the peritoneum of female BALB/c mouse pretreated with pristane, and the resulting ascitic fluids were collected from the mice. The ascites were diluted with phosphate buffered saline (PBS) and loaded to the protein A-Sepharose column at the flow rate of 0.2ml/min. The columns were then washed with 5 volumes each of 3N NaCl with 50mM sodium borate buffer (pH 8.0) and of 3N NaCl with 10mM sodium borate buffer (pH 8.9). Monoclonal antibodies bound to the column were then eluted using 100mM glycin buffer (pH 3.0) and the eluates were neutralized quickly by 1M Tris buffer, pH 8.0. Purified monoclonal antibodies were examined by SDS-PAGE under both reducing and non-reducing conditions[9].

Immunofluorescence analysis of the monoclonal antibodies

Bindings of the monoclonal antibodies to the zoospores were examined by indirect immunofluorescene assay as described by Park and Cho[10]. Briefly, 1×10^3 zoospores were fixed with formaldehyde on the glass slide, and reacted with the monoclonal antibodies for 30 min. After washing unbound antibodies, the spores were incubated with the TRITC-conjugated goat anti-mouse IgG antibody and were examined using the Olympus BH2-RFL fluorescence microscope (Olympus, Japan).

RESULTS AND DISCUSSION

Mice were immunized either with the formaldehyde fixed diploid zoospores or with the diploid zoospore proteins, and significant antibody production were confirmed by tail bleeding from both immunizations. In case of the fixed spore immunization, we had plated fused cells in about 100 wells, and approximately 20 wells showed the growth of the hybridomas after selection in the medium supplemented with HAT. For zoospore protein immunization, however, we had increased the number of the plating wells to approximately 200 and half of the wells, approximately 100 wells, showed hybridoma growth after selection in the medium supplemented with HAT, suggesting the fusions from both immunizations were successful. We have identified total of 30 positive primary hybridomas by ELISA, six from fixed spore immunization and 24 from the spore protein immunization.

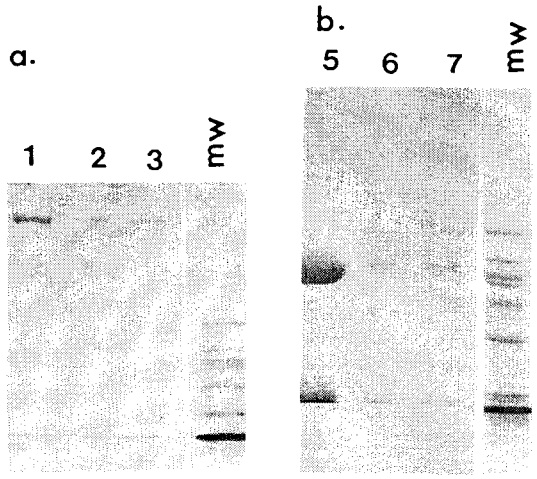

Fig. 1. SDS-polyacrylamide gel electrophoresis analysis of the purified monoclonal antibodies in the nonreducing condition (a) and the reducing condition (b). The lanes 2 and 6 represent monoclonal antibodies purified from the AM9D8A ascite, and the lanes 3 and 7 represent ones purified from the YF12D 12F ascite. The lanes 1 and 5 represent standard mouse immunoglobulin G. The "mw" represents molecular weight standard of 66K, 45K, 36K, 29 K, 24K, 20K and 14K daltons.

Some of the primary hybridoma clones were subcloned to the single hybridoma clones using the limiting dilution technique. Hybridoma cells were diluted with the conditioned single cell cloning medium (Harlow and Lane, 1988), and the cells were plated at the average of 0.3 cells per well. Approximately 30 wells from the 96 wells plated showed up with the hybridoma cell growth, suggesting that the hybridomas are well separated into the single cell clones. Following identification of the antibody producing hybridoma clones by ELISA, at least one clone from each single cell cloning was chosen and allowed to grow further. The cells from a single cell hybridoma clone were injected into the pristane treated BALB/c mice to produce ascites. Six monoclonal antibodies were purified from the ascites using protein A-Sepharose column chromatography, and the monoclonal antibodies from the YF12D12F and the AM9D8A clones were studied further. They showed similar molecular weight as that of the standard mouse IgG in non-reducing SDS-PAGE (Fig. 1a), and also similar light and heavy chain subunits as those of the standard mouse IgG, indicating the monoclonal antibodies purified were the antibodies of the IgG class (Fig. 1b).

Binding of the purified monoclonal antibodies to *Allomyces macrogynus* zoospores was examined by indirect immunofluorescence (Fig 2). Immunofluorescence with the monoclonal antibodies from the YF12D12F and AM 9D8A clones showed bright areas along the periphery of the zoospore, suggesting that they might bind to the surface of the zoospore (Fig. 2a and c). We had studied whether these antibodies were bound to the intracellular components or cell surface by pretreating zoospores with triton X-100 to allow penetration of the antibodies into the cytoplasm of the zoospores. Both antibodies were still limited to the periphery of the zoospores upon treatment of the triton X-100, suggesting the binding of these antibodies to the zoospore surface. It is assumed that antibodies from other AM hybridomas may include the antibodies binding to the cytoplasmic proteins or cellular organelles, since they were produced from the soluble protein immunization.

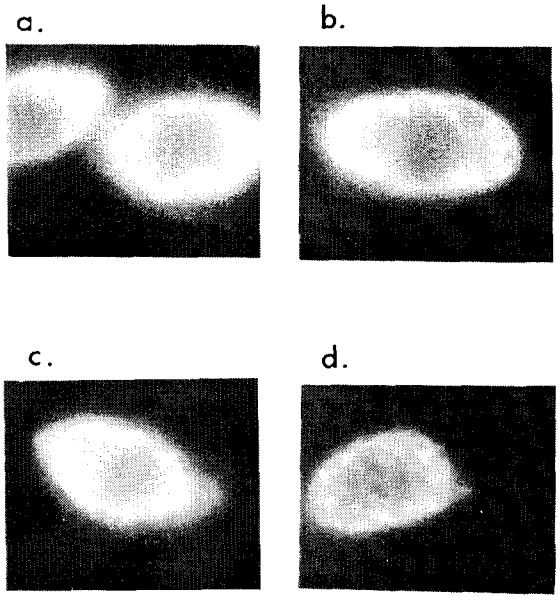

Fig. 2. Immunofluorescence analysis of the purified monoclonal antibodies. Pictures (a) and (b) represent the immunoflurescences of the triton-X100 untreated and treated zoospores, respectively, with the monoclonal antibodies from the YF12D 12F hybridoma. Pictures (c) and (d) represent the immunoflurescences of the triton-X100 untreated and treated zoospores, respectively, with the monoclonal antibodies from the and AM9D8A hybridoma.

As a preliminary study of the function of the monoclonal antibodies, we have incubated zoospores with the culture supernatants from the various hybridomas to select antibodies affecting zoospore growth. Among 30 hybridoma culture supernatants tested, the culture supernants from the AM9D8A and the YF12D12F hybridoma clones stimulated hyphal growth while the others including the control HY culture medium did not (Fig. 3). The zoospores incubated with the culture supernata-

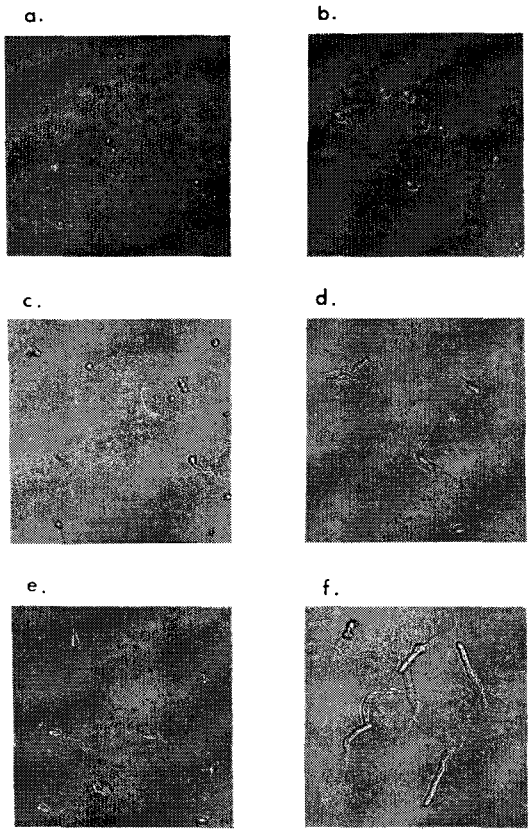

Fig. 3. Hyphal growth of the zoospores incubated with the culture supernatants from various hybridomas. Pictures (a), (c) and (e) represent the zoospores incubated with the culture medium used for hybridoma culture (HY with 10% FCS), and pictures (b), (d) and (f) represent the zoospores mixed with the culture supernatants from YF12D12F. Pictures (a) and (b) were taken in 210 min of incubation, pictures (c) and (d) in 340 min, and pictures (e) and (f) in 485 min of incubation. The open triangle and closed triangle in picture (b) represent a rhizoid and a germ tube, respectively. The double triangle in the picture (d) represents growth of hyphae. The growth patterns of the zoospores mixed with the other culture supernatants were similar to that of the zoospore treated with the control medium.

nts from YF12D12F culture soon became round cell as the other cultures. The zoospores incubated with the supernatant from YF12D12F started to form rhizoids in 90 min while the zoospores incubated with the control supernatant started to form rhizoids between 120min and 210min of incubation (Fig. 3a and b). By 210min of incubation, most of the zoospores in the supernatant from YF12D12F were evident to form multiple rhizoids and started to form germ tubes (Fig. 3b). By 340 min of incubation, the growth of the hyphae in the culture supplemented with the supernatant from YF12D12F was clearly different from those in the control cultrue (Fig. 3c, d). By that time, most of zoospores in the supernatant from YF12D12F started to form germ tubes while the zoospores in the control culture did not show any germ tube formation. By 485 min of incubation, the zoospores incubated with the supernatant from YF 12D12F formed much longer hypha than those incubated with the control supernatant (Fig. 3e and f).

Although we do not have any idea what was exactly happened at this moment, the following explanations could be possible for this phenomenon. First, the monoclonal antibodies from the AM9D8A and the YF12D12 F cultures may act as the ligands for the cellular receptors which regulate cell growth, and the bindings of the receptors with the antibodies may send the growth signals into the cells as if the receptors were occupied by the native growth factors as seen in the case of other monoclonal antibodies[11]. Second, the culture supernatants may possess growth inhibitory substances and the monoclonal antibodies may bind to the receptors of these inhibitory molecules to block the effects of inhibitory subatances. Third, some of the molecules in the culture supernatants may act directly onto the zoospores and regulate hyphal growth regardless of the antibodies in the culture supernatants. Considering that the culture supernatants from various hybridoma cultures were supposedly composed of similar constituents due to the si-

milar cellular characteristics of the hybridomas, the idiotypes of the monoclonal antibodies in the culture supernatants would be the major differences. Together with the findings that the antibodies from AM9D8A and YF12D12F clones may bind to the cell surface, the growth stimulatory effect of these hybridoma clones may be resulted from the binding of the antibodies to the cell surface. Currently, examinations of these possibilities were under progress.

ACKNOWLEDGEMENT

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초록 : *Allomyces macrogynus*의 유주자와 반응하는 단일클론항체의 준비

최소영, 황정숙, 김정섭, 박경희, 조정원, 윤현주(인제대학교 미생물학과)

*Allomyces macrogynus*의 유주자에 대한 단일클론항체를 만들었다. 고정된 유주자를 주사하거나 유주자 단백질 용액을 주사함으로서 생쥐를 면역화 하였으며, 하이브리도마 세포들은 효소면역흡착법을 이용하여 검색하였다. 약 30개의 하이브리도마 클론이 유주자에 대한 항체를 생산하는 것으로 확인 되었으며, 이들중 일부는 단일세포클론으로 분리되었다. 이들이 만들어내는 항체는 정제되어, 간접면역형광법에 의하여 유주자의 표면에 반응하는 항체로 확인 되었다. 또한, 하이브리도마 배양상등액을 이용하여 유주자의 성장에 영향을 미칠 수 있는 클론을 조사하여 보았다. 조사한 배양상등액중 두가지의 하이브리도마에서 얻은 배양상등액이 germ tube의 성장을 촉진하는 것으로 확인 되었다.