HYBRIDOMA
Volume 27, Number 4, 2008

DOI: 10.1089/hyb.2008.0011

A Novel Approach to Development of Monoclonal Antibodies Using Native Antigen for Immunization and Recombinant Antigen for Screening

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Abstract

The production of monoclonal antibodies (MAb) specific to microbes is rapidly growing. Finding an appropriate antigen to screen hybridoma clones has become increasingly important. However, the conventional method, in which the purified antigen from the microbe is routinely used for screening, cannot avoid selection of false positive hybridoma clones, since even highly purified antigen is found to be contaminated with some other proteins from the microbe. In this study, MAbs against anthrax protective antigen (PA), the central component of the three-part toxin secreted by *Bacillus anthracis* were developed using a pair of the roughly purified native PA as an immunogen and the recombinant PA as a screening antigen without any possibility of false selection, since the recombinant PA was produced by a gene engineering approach and impossible to be contaminated with any other proteins from *B. anthracis*. In total, nine stable hybridoma clones secreting anti-PA MAbs were developed. All of them had the same type of heavy and light chains, IgG1/κ. The binding profiles for these anti-PA MAbs were investigated by ELISA. This novel approach to the development of MAbs should be applicable to the production of MAbs to other microbes, especially to those from which antigens can hardly be purified to a high degree.

Introduction

THE PRODUCTION OF MONOCLONAL ANTIBODIES (MAb) specific to microbes is rapidly growing. Finding an appropriate antigen to screen hybridoma clones has become increasingly important. However, the conventional method, in which the purified antigen from the microbe is routinely used for screening[1,2] cannot avoid selection of false positive hybridoma clones, since even highly purified antigen is found to be contaminated with some other proteins from the microbe. In order to eliminate any possibility of selection of false positive hybridoma clones, the recombinant antigen is a good alternative to the native antigen as a screening antigen, since it is produced by a gene engineering approach and impossible to be contaminated with any other proteins from the same microbe as the native antigen.

Bacillus anthracis, the cause of anthrax, is classified as one of category A (most dangerous and likely to be used) biological warfare agents due to its high lethality and the ease of production and dissemination.[3,4] The virulence of *B. anthracis* is attributed to the anthrax toxin composed of three protein components: protective antigen (PA), lethal factor (LF), and edema factor (EF).[5,6] The toxicity is expressed only when PA is combined with LF or EF to form the lethal toxin or the edema toxin.[7,8] Thus, PA plays a central role in anthrax pathogenesis.

Currently, prevention and treatment of anthrax rely mainly on post-exposure antibiotics to eliminate the bacteria and pre- and post-exposure vaccination against PA. Since the initial symptoms are non-specific, when the specific symptomatic stage is reached due to massive amounts of the toxin produced by the bacteria, the disease is often beyond

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treatment.[9] Therefore, early diagnosis of anthrax is imperative for efficient treatment of anthrax. It was reported that the appearance and concentration of PA in the sera of the infected animals directly correlated with the development and progression of anthrax.[10] Since the time for immunodetection of PA can be very short, PA can be a marker for fast immunodiagnosis of anthrax in a relatively early stage.

The currently licensed human anthrax vaccine, designated "anthrax vaccine adsorbed (AVA)" is PA-based and highly effective,[11] but the use of the PA-based vaccine after exposure to *B. anthracis* may be limited. Since PA is a natural component of the anthrax toxin and may contribute to toxin formation, it may not be safe to administer the PA-based vaccine to the persons who have been or are suspected of having been exposed to anthrax. In view of the fact that the efficacy of the PA-based vaccine correlates with the development of humoral responses to PA, anti-PA antibodies play a key role in protection against anthrax.[12] Therefore, anti-PA antibodies might be an alternative to the PA-based vaccine in post-exposure prophylaxis or treatment of anthrax.

Taken together, antibodies against PA have the potential to be developed as diagnostic and therapeutic agents for diagnosis, prevention, and treatment of anthrax. As a result, increasing attention has been focused on development of anti-PA MAbs.[2,13–20] In this study, in order to develop anti-PA MAbs, the gene encoding PA of *B. anthracis* was cloned into a bacterial expression vector, pET19b. The poly-His-tagged PA expressed in the form of inclusion bodies in *Escherichia coli* was solubilized, purified, and refolded. The recombinant PA was used as an antigen to detect anti-PA MAbs by an enzyme-linked immunosorbent assay (ELISA) for screening the hybridoma clones resulting from the fusion of Sp 2/0 myeloma cells and splenocytes from the mice immunized with AVA, a roughly purified native PA. Nine stable hybridoma clones secreting anti-PA MAbs were isolated. The binding profiles for these MAbs to the PA were investigated by ELISA.

Materials and Methods

Gene cloning, expression, purification, and refolding of the recombinant PA

The gene encoding PA of *B. anthracis* strain Ames was amplified by PCR using a pair of gene-specific primers flanking the full-length sequence of the PA gene except for the portion encoding the signal sequence. *Nde I*/*BamH I* sites were designed in the primers to facilitate cloning of the PCR product into the *Nde I* and *BamH I* sites of the bacterial expression vector pET19b (Novagen, San Diego, CA). The resultant plasmid was designated as pET19b-PA. Expression, purification, and refolding of the recombinant PA were performed using a method described previously.[21] Briefly, after expression in *E. coli*, the cell pellet was sonicated and solubilized in an urea buffer. The solubilized recombinant PA was purified by Talon metal affinity resin (Clontech, Palo Alto, CA) and then refolded in the buffer containing glycerol and non-detergent sulfobetaines. The purified and refolded recombinant PA was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE and Western blot analysis

Proteins were separated by 10% SDS-PAGE gels by use of a Mini-PROTEAN II apparatus (Bio-Rad Laboratories, Mississauga, Canada). The bands were visualized by Coomassie blue staining. Gels were immunoblotted to Immunobilon-P membranes (Millipore, Bedford, MA) using a Western blot semi-dry transfer apparatus (Bio-Rad Laboratories). After blocking, blots were incubated with anti-poly-His MAb (Invitrogen, Burlington, Canada). Following washes, the blots were incubated with horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and finally the specific binding was detected by an enhanced chemiluminiscence kit (Amersham Pharmacia Biotech, Baie d'Urfe, Canada).

Immunization

Female BALB/c mice were obtained from the pathogen-free mouse breeding colony at Defence Research and Development Canada (DRDC)-Suffield with the original breeding pairs purchased from Charles River Canada (St Constant, Canada). The use of these mice was reviewed and approved by the Animal Care Committee at DRDC-Suffield. Care and handling of the mice followed the guidelines set out by the Canadian Council on Animal Care. Mice (6–8 weeks old) were intraperitoneally immunized with 100 μL of AVA (Bioport, Lansing, MI) emulsified in 100 μL of TiterMax Gold adjuvant (Sigma-Aldrich, Oakville, Canada). Booster immunizations were carried out every other week with the same dose of AVA emulsified in TiterMax Gold adjuvant. The mice were bled from the tail vein, and the anti-PA antibody titers were checked by an ELISA.

Cell fusion

Spleens were aseptically dissected from the immunized mice 3 days after the last booster and were ground gently with autoclaved frosted-glass slides in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) and then filtered through a wire mesh screen to prepare splenocytes. Hybridomas were produced by fusing the splenocytes with Sp

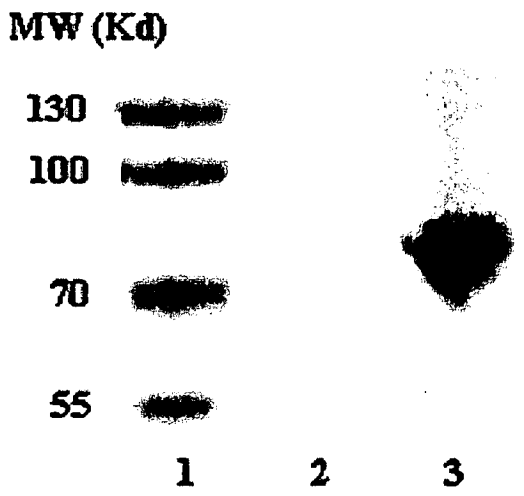

FIG. 1. Western blotting analysis of bacterial lysate probed with an anti-poly-His antibody. Lane 1, molecular weight marker; lane 2, bacterial lysate from mock *E. coli* (negative control); lane 3, bacterial lysate from pET19b-PA plasmid-transformed *E. coli*.

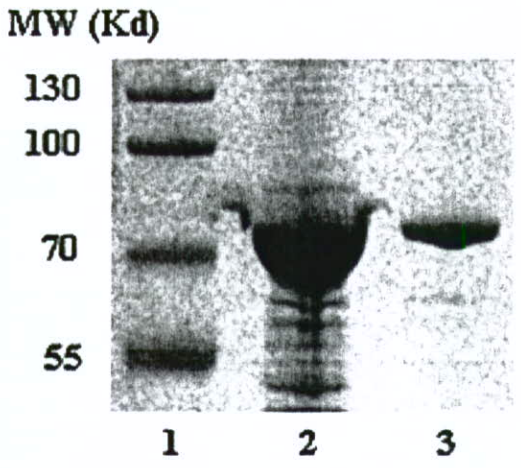

FIG. 2. SDS-PAGE analysis of the purified PA. Lane 1, molecular weight marker; lane 2, solubilized protein fraction; lane 3, purified fraction.

2/0 myeloma cells using a Clonacell-HY kit (StemCell Technologies, Vancouver, Canada) following the manufacturer's instruction. After 2 weeks in semisolid medium, single hybridoma clones were picked and transferred to 96-well tissue culture plates (Costar, Corning, NY) and grown for 3–4 days in Clonacell Medium E. Hybridoma supernatants were screened for anti-PA MAb production by ELISA.

ELISA

Detection of anti-PA antibodies in the immune sera from AVA-immunized mice to evaluate anti-PA immune responses and in the culture supernatants from hybridoma-containing wells to screen anti-PA hybridoma clones were performed by ELISA as follows. Ninety six-well ELISA plates (Nunc maxisorp, Canadian Life Technologies, Burlington, Canada) were coated overnight at 4°C with the recombinant PA at 10 μg/mL in carbonate bicarbonate buffer (pH 9.6). After blocking, the plates were incubated with 100 μL of serum dilutions or culture supernatants for 2 h at room temperature. Anti-PA antibodies were detected by incubation with 1:3000 diluted HRP-goat anti-mouse IgG (Caltag Laboratories, Burlingame, CA) followed by the addition of a tetramethylbenzidine peroxidase substrate (Kirkegaard and Perry Laboratories, Gathersburg, MD). Absorbance was measured at 635 nm by a microplate autoreader (Molecular Devices, Sunnyvale, CA).

Production and purification of MAbs

All the hybridoma clones secreting anti-PA MAbs were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Invitrogen) initially, then adapted gradually to and finally expanded in hybridoma serum-free-medium (SFM, Invitrogen). The anti-PA MAbs were purified from supernatants by a Melon gel monoclonal IgG purification kit (Pierce Biotechnology, Rockford, IL). The purities of anti-PA MAbs were assessed by SDS-PAGE.

Characterization of MAbs

The immunoreactivities of the purified anti-PA MAbs were analyzed by ELISA, as described above with the following exceptions. Coating antigens included the recombinant PA at 10 μg/mL or AVA at 20 μL/mL in carbonate bicarbonate buffer. Plates were incubated for 2 h at 37°C with various concentrations of 0.001–100 μg/mL anti-PA MAbs.

Isotype determination

All the purified anti-PA MAbs were isotyped using a mouse IsoStrip kit from Roche Diagnostics (Laval, Canada), following the manufacturer's instruction.

Results

Cloning, expression, purification, and refolding of the recombinant PA

The PA gene of *B. anthracis* was amplified by PCR. The amplified PCR product was then cloned into pET19b plasmid. The DNA sequence of the PA gene with N-terminal

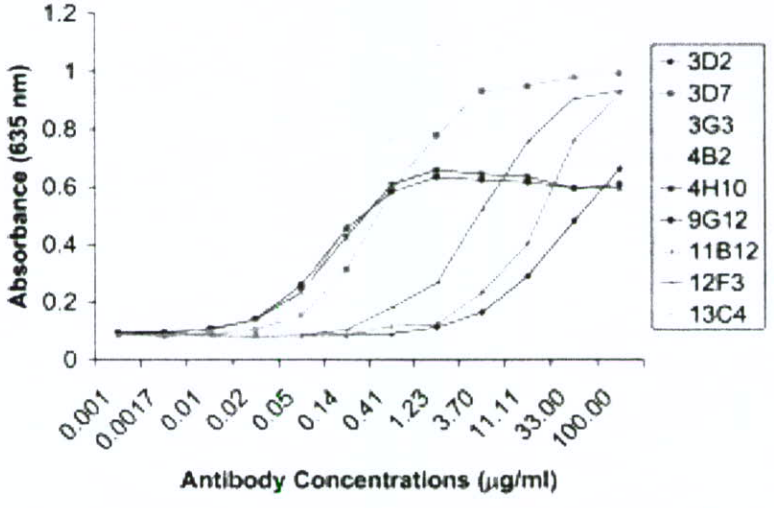

FIG. 3. The immunoreactivities of anti-PA MAbs by ELISA.

poly-His tag and enterokinase cleavage site in pET19b-PA expression plasmid was 2208 bp in length, encoding 736 residues with an estimated molecular weight of 83 kDa. The poly-His-tagged PA was expressed in *E. coli* cells. The bacterial lysate was analyzed by Western blotting probed by an anti-poly-His MAb. As shown in Figure 1, one band was visible from the pET19b-PA plasmid-transformed *E. coli*, while no band was evident from mock *E. coli* (negative control). Its molecular weight was around 80 kDa, corresponding to the predicted size (83 kDa) of the recombinant PA, indicating the recombinant PA was successfully expressed in *E. coli*. The recombinant PA was mainly expressed in the form of inclusion bodies. The inclusion bodies were then denatured in an urea buffer, purified by IMAC resin, and refolded in the buffer containing glycerol and non-detergent sulfobetaines. The purity of the recombinant PA was about 90% based on SDS-PAGE (Fig. 2).

Generation of anti-PA MAbs

BALB/c mice were immunized with AVA. Following the last booster, mice were sacrificed and splenocytes were prepared and fused with myeloma cells in a standard hybridoma fusion protocol. After growth in semisolid medium, hybridoma clones were transferred to individual wells of 96-well plates and cultured, and supernatants were screened for specific reactivity with the recombinant PA. A panel of nine hybridoma clones (3D2, 3D7, 3G3, 4B2, 4H10, 9G12, 11B12, 12F3, and 13C4) with specific reactivity for the recombinant PA was identified by ELISA and expanded.

Production, purification, and characterization of anti-PA MAbs

The nine hybridoma clones were respectively cultured in DMEM supplemented with 10% FBS initially and then adapted into hybridoma SFM. The MAbs were purified from supernatants by melon affinity chromatography. The purities of the MAbs were 85–90% assessed by SDS-PAGE (data not shown). The purified MAbs were isotyped using a mouse IsoStrip kit. All the MAbs showed the same subtype of heavy chain, gamma 1, and the same type of light chain, kappa. The immunoreactivities of these MAbs to the PA were investigated by ELISA. All the MAbs bound to both the AVA (data not shown) and the recombinant PA (Fig. 3) in a dose-dependent manner.

Discussion

AVA, a PA-based vaccine is a cell-free culture filtrate prepared from microaerophilic cultures of the attenuated, non-encapsulated strain of *B. anthracis*.[22] Therefore, AVA was used to immunize mice to elicit anti-PA antibodies in mice. However, AVA was not an appropriate screening antigen for detection of anti-PA MAbs to screen anti-PA hybridoma clones, since AVA is a mixture of proteins and contains all three components of the anthrax toxin (PA, LF, and EF), although it contains mainly PA.[23] In addition, the native PA purified from *B. anthracis* could not be used as a screening antigen, because even the highly purified native PA was found to be contaminated with small amounts of EF and LF.[24,25] In order to get pure PA without any trace of the other two components (LF and EF), we planned to produce PA using a gene engineering approach and then to use the recombinant PA as an antigen to screen anti-PA hybridoma clones. We did not choose the recombinant PA as an immunogen to immunize mice, because the conformation of the recombinant PA might be altered slightly compared to that of the native PA and consequently some epitopes of the recombinant PA might not be the same as those of the native PA. The hybridoma clones secreting MAbs specific to the changed epitopes of the recombinant PA would probably be selected if the recombinant PA was used as both an immunogen and a screening antigen. Obviously, these MAbs would not bind to the native PA. That is why we used AVA as an immunogen to elicit anti-PA antibodies in mice and the recombinant PA as a screening antigen to select anti-PA hybridoma clones. In this way, only the hybridoma clones secreting the MAbs elicited by the native PA and bound by the recombinant PA could be selected, eliminating any possibility of selection of false positive anti-PA hybridoma clones. To the best of our knowledge, this is the first report to use an appropriate pair of the roughly purified native antigen as an immunogen and the recombinant antigen as a screening antigen to prepare MAbs without any concern about false selection.

Recombinant proteins can be produced in various expression systems, such as bacterial, mammalian, insect, yeast, and plant cells. Bacterial expression system is very popular due to its fast, convenient, and high yield. However, bacteria are not capable of glycosylating proteins. Since PA is not a glycosylated protein, we cloned the PA gene fragment into the pET19b bacterial expression vector for the production of the recombinant PA. The large amount of the expressed PA appeared in the form of inclusion bodies. After the inclusion bodies were denatured, the PA was purified by IMAC resin and then refolded.

Nine anti-PA MAbs were obtained by using the recombinant PA as an antigen to screen the hybridoma clones derived from the AVA-immunized mice. These MAbs were produced in SFM and purified by melon gel. The melon gel binds to non-antibody proteins, like albumin and transferrin, allowing the antibodies to flow through unlike traditional affinity purification which binds to the antibodies, resulting in a fast, easy, and gentle approach to antibody recovery and purification. Since this gel allows any antibody to flow through regardless of species, SFM was used to culture hybridoma cells in order to avoid co-purifying bovine immunoglobulins from FBS.

The ELISA showed that all nine of the MAbs bound to the PA and AVA in a dose-dependent manner. The anti-PA MAbs have the potential to be developed as diagnostic and therapeutic agents for diagnosis, prevention, and treatment of anthrax. Most importantly, this novel approach to the development of MAbs using an appropriate pair of the native antigen as an immunogen and the recombinant antigen as a screening antigen without any possibility of false selection should be applicable to the production of MAbs to other microbes, especially to those from which antigens can hardly be purified to a high degree.

Acknowledgment

This study was supported by the Canadian Chemical, Biological, Radiological, and Nuclear Research and Technology Initiative (CRTI), grant no. 01-0091RD.

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Received: February 27, 2008
Accepted: March 28, 2008