Research Article

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Development and production of antibodies against gamma inactivated pathogenic bacterial spores

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Abstract

Objectives: Gram-positive sporulated bacilli can cause many different diseases and isolation from environmental samples is difficult. Therefore, the quick detection and diagnosis of these microorganisms have critical importance because of their potentially harmful situation. However, many accepted diagnostic methods exist, and future technology points to immunoassay systems. Immunological methods to detect biological microorganisms require antigen-specific high-affinity antibodies as key materials.

Methods: In this study, Bacillus anthracis (34F2 sterne) bacterium, which causes anthrax disease, was chosen as a model organism to develop antibodies against bacterial spores. The produced spores were inactivated with gamma irradiation, and the development of monoclonal antibodies against inactivated spores was performed using hybridoma technology. Also, the polyclonal antibody was successfully obtained by immunizing the rabbit. Indirect and sandwich ELISA tests were performed to determine the antigenic properties of inactivated spores and the specific affinity of the developed antibodies.

Results: The spores, inactivated with 15 kGy, have the best-preserved surface epitopic regions and were selected as immunogen. Developed monoclonal and polyclonal antibodies were shown that there was no cross-reaction with other Bacillus species. Also, it was demonstrated that these antibodies could detect inactivated spores at a concentration of 10² spores/mL in a sandwich ELISA assay.

Conclusions: These qualified antibodies obtained will be essential in developing antibody-based diagnostic systems for spore detection from various environmental samples. This study suggests that the inactivated spores are a decent immunogen for generation antibodies and may be a candidate component for live vaccine formulation.

Keywords: Bacillus anthracis spore; gamma inactivation; antibody; ELISA; immunoassay

Introduction

The spore-forming forms of Gram-positive bacteria have a double life cycle. They remain in vegetative form at suitable conditions, called bacilli and form spores when environmental conditions are disrupted [1, 2]. Since the spore form is highly resistant to harsh environmental conditions, various disinfectants, UV, and high temperatures, it can remain in nature for up to 90 years without damage [3–5]. Pathogenic bacterial spores can easily cause food poisoning, infection and serious health problems. These properties allow one of the members of these bacteria Bacillus anthracis, to cause the most famous disease, anthrax [6]. Once inhaled more than 10⁴ anthrax spores could be lethal, unless receiving medical treatment within 24–48 h [7]. The progression of the disease begins when spores in soil enter the bodies of feeding herbivorous animals. The spores are carried to the lymph nodes by alveoli, macrophages, and dendritic cells. After the spores become vegetative, anthrax toxin proteins and virulence factors are produced. The first symptoms begin 1–5 days after the bacterial infection and result in respiratory failure, sepsis, and shock, resulting in death [8, 9]. Although anthrax disease mainly affects herbivorous animals, it can also indirectly affect other animals and humans [10]. The environmental destruction it causes, the
disease of animals, and the fact that anthrax is also a biological threat necessitate the development of diagnostic/detection systems for this spore.

Conventional methods for bacteriological assays are reliable but time-consuming [11]. For this reason, alternative and advantageous methods such as molecular fingerprinting, mass spectrometry (MS), PCR-based methods, and immunological assays are being studied [12, 13]. There are differences in the target molecule in these test methods. Rapid and real-time testing makes it impossible to detect spores due to the complicated requirements of other diagnostic methods, such as cell lysis of the target molecule, nucleic acid extraction, and protein purification. Immunological assays stand out because they can detect bacterial spores without pre-treatment [14–18].

As bacterial spore B. anthracis, which was chosen as the model organism, is in category A in the classification determined by the Centers for Disease Control and Prevention (CDC) and requires Biosafety level 3 type laboratory to conduct studies [19, 20]. Bacterial spores must be inactivated to avoid this requirement. Spore inactivation can be accomplished by heat treatment, chemical treatment, and gamma irradiation methods. The most effective way to inactivate the spores by conserving its antigenic regions is the gamma irradiation at the appropriate dose. The gamma irradiation process mainly consists of high-energy gamma rays emitted from a Cobalt-60 source passing through the spore, removing electrons from organic molecules and causing ionization. Therefore, while protecting the spore surface, gamma rays destroy the metabolic structure and the spore’s ability to transform into an active form, even if appropriate conditions are provided. The energy unit used in gamma irradiation is rad or kilogram (kGy). One kilogram is equal to 100,000 rad. The dose and duration to be applied are determined according to the half-life of the Cobalt-60 source and the amount of energy (kGy/min) that the sample to be irradiated will absorb per minute [21].

Hybridoma technology is one of the most common methods to develop monoclonal antibodies and was first applied in 1975 by researchers Georges Köhler and Cesar Milstein [22, 23]. The method briefly proceeds: BALB/c mice are immunized periodically with the relevant antigen. After each immunization process, the antibody response in the mouse serum is controlled, immortal myeloma cells are fused with the antibody-producing B cells of the mouse with sufficient antibody titration, and hybrid cells are formed. After the culture studies, a stable cell line with immortal properties and the ability to develop antibodies is obtained. Also, polyclonal antibodies have become the preferred detection molecules due to their biophysical diversity, high affinity for their antigens, and the fact that they can be obtained in a shorter time than hybridoma-based monoclonal antibodies [24].

This study aimed to develop antibodies against the whole spore structure of B. anthracis, a model organism selected for immunological diagnostic methods developed as an alternative to conventional detection methods for bacteriological assays from different environmental samples. For this purpose, to be used as an immunogen, the bacteria were sporulated and then inactivated by gamma irradiation. Using hybridoma technology, a high-affinity monoclonal antibody against the inactive spore was developed and named anti-BAs MAM02. At the same time, an anti-BAs polyclonal antibody has been developed. As a foremost step, performing a sandwich Enzyme-Linked Immunosorbent Assay (ELISA) demonstrated the potential of these antibodies to be used in immunological diagnostic assays.

Materials and methods

Sporulation of B. anthracis and characterization of spores

The supplied bacterial stock was cultured on typical nutrient agar plates prepared with 10 g/L biological peptone, 3 g/L beef extract, 5 g/L NaCl, and 15 g/L agar and incubated at 37°C overnight to obtain a single colony. Subsequently, a single colony was grown in Tryptic Soy Broth (TSB). The bacterial suspension achieved from single colony cultured in sporulation media prepared with 0.5% peptone, 0.3% beef extract, 0.5% NaCl, and 1.5% agar, which had relatively reduced nutritional properties, and was incubated for 48 h at 28°C. Then, plates were incubated at room temperature for 24 h. Visible spore colonies on the plates were collected and suspended in dH2O. Suspension was incubated for 48 h at room temperature to remove forms that had not transformed into spores. After 48 h, the spores were incubated at 60°C for 1 h and washed thrice with cold 1x phosphate buffer saline (PBS). Centrifugation was done at 7000 rpm for 10 min.

To control the quality of the spores before inactivation, 50 µL of the spore suspension was spread on a glass slide and allowed to dry. Then, malachite green dye (Sigma, cat no: 2437-29-8) was added and held over the fire with forceps. The dye was heated until steam came out without boiling. As the dye evaporated, it was added again, and this process was repeated three times. Then, the coverslip was washed with dH2O. Safranin dye (Sigma, cat no: 477-73-6) was added and left for 1 min. After washing abundantly with dH2O, it was dried, and a coverslip was covered by dropping immersion oil and examined under a microscope.

Inactivation of spores by gamma irradiation

The spore suspension was washed three times with cold 1x-PBS before irradiation, and counting was performed using the culturing method [25]. This method created stocks of different concentrations by serial dilution of the spore suspension. Spores at each concentration were cultured three times repetitively on nutrient agar plates, and the stock was determined by calculating over two consecutive concentrations. Twenty milliliters of spore samples were divided into falcon tubes to be 10⁷ spores/mL and irradiated with five different gamma doses: 5, 10, 15, 20, and 30 kGy (Nordion JS9600, GammaPak Sterilization Company, Nordion).
Monoclonal antibody (mAb) production by hybridoma technology and cross-reactivity-test

10^6 pure inactivated *B. anthracis* spores were mixed 1:1 with Freund’s complete adjuvant (Sigma, cat no: F5881) and injected subcutaneously into 6–8-week-old BALB/c mice in a volume of 200 μL. The following injections were performed with Freund’s incomplete adjuvant (Sigma, cat no: F5506) once a month, and one mouse was kept non-immunized as a control. Mice displaying a high immune response were selected for the fusion study. Spleen cells and F0 myeloma cells (American Type Culture Collection, USA) were combined at a ratio of 3:1 through fusion. After the 10-day incubation period, the hybrids were screened under a microscope, and the initial ELISA screening against the spores was performed. After all limited dilutions and screening ELISA tests, we generated a hybridoma cell line that produced monoclonal antibodies with high affinity against spores. A cross-reactivity test was performed as the last step to measure the specificity of the MAM02 hybrid. For this purpose, *Bacillus cereus*, *Bacillus subtilis*, and *Bacillus thuringiensis* bacteria, closely related strains of *B. anthracis*, were obtained, sporulated and an indirect ELISA test was applied.

mAb purification and characterization

The subisotype of the MAM02 was determined to choose which chromatographic method must be applied to purification. The commercially obtained kit (BD Pharmingen Mouse Immunoglobulin Isotyping ELISA kit) has been used for this. A high volume of culture supernatant was collected to purify the MAM02 cell line. After increasing the concentration through ammonium sulfate precipitation and desalting through dialysis, the protein mixture containing the target antibody was subjected to affinity chromatography on a Hitrap Protein A column (Cytiva, cat no: 17040201) using the NGC (Next Generation Chromatography, BIORAD) device by using the recommended buffers by Cytiva. The purity of the obtained antibody fractions was demonstrated using SDS-PAGE gel electrophoresis followed by silver staining. The SDS-PAGE has been performed by following the instructions of BIORAD MiniPROTEAN Tetra Cell System. The silver staining procedure was applied by following the specified protocol [26]. Additionally, the affinity of the fractions to the antigen was confirmed through indirect ELISA testing. The fraction containing antibodies was purified through dialysis, and characterization processes were completed.

Rabbit polyclonal antibody production

New Zealand rabbits were immunized with inactivated spores at 10^7 spores/mL once a month, and antibody titration in blood serum was routinely checked using the indirect ELISA method. After reaching proper antibody titration, a high volume of blood serum was collected. Purification and characterization procedures were carried out by following the same protocol with mAb purification [27].

Horseradish peroxidase (HRP) – anti-BAs pAb conjugation

1 mg anti-BAs pAb antibody was conjugated with Horseradish peroxidase (HRP) (Roche, cat no: 1108913001). This process was carried out by following the relevant protocol steps [28]. As different from the protocol followed, Vivaspin 500 columns (Sartorius, cat no: VS0172) were used instead of dialysis procedures. After conjugation, the usage concentration of HRP-labeled antibody was tested by direct ELISA.

Indirect ELISA test

In the indirect ELISA test to detect the inactive spore, 10^5 spores/100 μL were coated on the bottom of the 96 well plates in 1×PBS solution and incubated at 37 °C overnight. The wells were blocked with 1.5 % skim milk powder for 1 h at 37 °C, and an anti-spore polyclonal antibody commercially obtained was used as recommended at a ratio of 1:1000. After using HRP-labeled anti-rabbit antibody (Sigma, A0545) at a ratio of 1:2000, TMB (3,3′,5,5′-Tetramethylbenzidine) substrate was added, and absorbance was measured at a wavelength of 450 nm by using plate reader (BIOTEK, Synergy HT). The wells were washed three times with 1×PBST after each step and five times before the substrate by micropore washer (BIOTEK, 405 LS) [29]. This method was applied to control the interaction of the epitopic regions of the inactivated spore with the antibody, to control the response of the developed antibody to spores of other *Bacillus* species, and to all stages of the antibody development process.

Spore detection by sandwich ELISA assay

To be a primitive study on the use of the produced antibodies in diagnostic tests for spore detection, a sandwich ELISA assay was performed. For this purpose, anti-BAs MAM02 was coated as a capture antibody at 4°C. After washing with 1×PBST and blocking with 1.5 % skim milk powder, different concentrations of inactive spores were used as intermediate antigens, and HRP labeled anti-BAs pAb was used as the detection antibody. After the TMB substrate was added, absorbance was measured at a wavelength of 450 nm [30].

Statistical analysis

Statistical analysis was performed using one-way and two-way ANOVA tests, and data are presented as mean±standard error. Data with a p-value less than 0.05 were considered statistically significant. GraphPad Prism 5 was used to visualize data.

Results

Preparation of spores, staining, and inactivation

The supplied *B. anthracis* bacteria were sporulated, and 40 mL of spores were obtained at a concentration of 2.5×10^15 spores/mL. Spores were stained with malachite green for
characterization, and the remaining vegetative bacteria were stained with safranin. Spores were stained green and vegetative bacteria stained red (Figure 1A). The spores were radiated with 5, 10, 15, 20, and 30 kGy gamma irradiation doses respectively. As a result of inactivation control, it was observed that spore colonies did not occur at any dose except for spores exposed to 5 and 10 kGy irradiation after incubation. An indirect ELISA test was performed, and it was determined that surface antigenic regions of the spores exposed to 15 kGy gamma dose best reacted with spore specific polyclonal antibody (Figure 1B). In another ELISA study, the antibody responses of spores inactivated with 15 kGy were tested at doses of $10^3$–$10^9$ spores/well, and $10^6$ spores/well were selected as the coating concentration for future assays (Figure 1C).

After obtaining inactive spores that retained their antigenic properties, they were used to develop antibodies.

**Production of monoclonal antibodies**

After immunizations periodically, indirect ELISA was performed to check the antibody titration, and after fifth immunization, second mouse was boosted intraperitoneally with a half dose of the spores (Figure 2A). 10 days after cell fusion, limited dilutions, subcultured hybridomas, and with continuous screening ELISA tests, it has been shown that one of the hybridomas preserved its affinity for spores (Figure 2B). The monoclonal antibody cell line named as anti-BAs MAM02 has been obtained. To determine the specificity of the anti-BAs MAM02, a cross ELISA test was performed against *B. cereus*, *B. subtilis*, and *B. thuringiensis* spores. The results showed that the produced antibody did not recognize any other spore and was specific to its antigen (Figure 2C).

After being developed and ensured that it does not respond to any related strain spores, characterization studies have begun for the use of the anti-BAs MAM02 antibody in immunological tests.

**Purification and characterization of anti-BAs MAM02**

The subisotype of the anti-BAs MAM02 was determined as IgG1, Kappa by commercially obtained ELISA kit (Figure 3A). As binding buffer 3 M NaCl, 1.5 M Glycine pH:8 and as elution buffer 0.1 M Glycine pH:2.7 were used for purification, and affinity chromatography was performed on the HiTrap protein A column using the NGC device. The purity was determined using SDS-PAGE gel electrophoresis followed by

Figure 1: Examination of staining and antigenic properties of gamma irradiated spore samples by ELISA. (A) Staining with malachite green, the green dots present spore forms, and the vegetative forms stained with safranin dye are present as red chains. (B) The gamma irradiation doses that spores absorb, active *Bacillus* anthracis spores (aBAs) are non-inactivated spores. (C) Usage concentration of spores exposed to 15 kGy gamma irradiation. **Means both active BAs and gamma inactivated BAs show the same antigenic properties at the $10^6$ spores.
Figure 2: Immunizations of mice, immune response stability, and cross ELISA tests of candidate monoclonal antibody. (A) The immune responses of 5 mice after immunizations. The no-immune mouse is kept as a control group. p<0.05. (B) The antibody response stability of MAM02 cell line. (C) The cross-reaction of MAM02 cell line with other species of *Bacillus* group. p<0.0001.

Figure 3: Characterization of anti-BAcs MAM02. (A) IgG1 heavy chain, Kappa light chain determination of sub isotypes produced anti-BAcs MAM02 p<0.0001. (B) The silver staining gel of the fractions containing purified antibodies. S, sample fraction; SW, sample wash fractions; E, elution fractions; EW, elution wash fraction. (C) The immune response of anti-BAcs MAM02 at different concentrations against $10^5$ spores/well p<0.001.
silver staining, and the affinity was confirmed through an ELISA test (Figure 3B). Following these controls, called anti-BAs MAM02, was obtained at a concentration of 2.2 mg/mL. The affinity of anti-BAs MAM02 at different concentrations to the antigen was evaluated, and it was shown that it can recognize even at 0.25 μg/mL concentration (Figure 3C).

The anti-BAs MAM02 antibody with a very high affinity for its antigen has been successfully developed and characterized.

Production of polyclonal antibody and HRP conjugation

Antibody titration of immunized New Zealand rabbit was checked by indirect ELISA and a high volume of blood serum was collected after the third immunization (Figure 4A). All the characterization steps, including cross-ELISA tests, were carried out (data not presented). As a result, anti-BAs pAb was obtained at a concentration of 2.4 mg/mL. The usage of HRP labeled anti-BAs pAb was checked at 1:250, 1:500, 1:1000, 1:2000, and 1:4000 ratios against two different spore concentrations, 10^7 and 10^8 spores/well. When the results were evaluated, a 1:1000 ratio was chosen (Figure 4B).

The anti-BAs pAb antibody has been developed successfully and conjugated with HRP. The studies have planned to use HRP-labeled anti-BAs pAb antibodies at the selected concentration as a couple with anti-BAs MAM02.

Inactive spore detection by sandwich ELISA as a preliminary diagnostic assay

To determine the potential usage in immunologic assays, both produced antibodies were tested as both capture and detection antibodies at different concentrations in the sandwich ELISA test (data not presented). In conclusion, it was shown that inactive spore detection could be achieved at different concentrations ranging from 10^7 to 5×10^4 spores/well, in the combination of which the 400 ng/well anti-BAs MAM02 was the capture antibody and at a ratio 1:1000 HRP labeled anti-BAs pAb was the detection antibody (Figure 5).

Discussion

The antibodies binds to their antigenic material with high affinity so they widely used in medicine, detection, and diagnosis of animal diseases, environmental contamination, and biological threats [31]. These qualities make them preferable critical materials for developing immunologic bacteriological diagnostic assays that detect the causative agent of anthrax, B. anthracis. Significantly, few monoclonal antibodies are explicitly developed for anthrax spores on the market. There is currently a need to develop high-affinity antibodies for use in diagnostic technologies. Therefore, developing antibodies that effectively recognize the spore is a complicated process. In recent studies, researchers have produced the antigenic surface proteins of the spore recombinantly and used them as immunogens, as they require a biosafety level 3 type laboratory [32]. Although we produced recombinant Bacillus collagen-like protein of anthracis (rBclA) and used it in antibody production in our previous studies, cross-reactions were seen in ELISA due to rBclA being found on the surface of other Bacillus species.

We inactivated the spore in this study and obtained antibodies through this immunogen.

Inactivation with heat treatment and chemical treatment causes changes in the epitopic regions in the antigen, causing the natural form to deteriorate [33]. Although these two methods can provide spore decontamination, they make it impossible to use spores in immunological assays [34]. Its high
binding capacity and selective effectiveness in macromolecules make the gamma irradiation method preferable and the most reliable method [35]. According to the related studies, measuring the conservation status of spore surface by PCR-based methods or controlling the antibody response was possible [36]. The success of inactivation was checked by the culturing method, and whether the surface antigenic regions of the spore were damaged or not was determined by an indirect ELISA test. As a result, it was shown that spore samples exposed to 15 kGy gamma dose were successfully inactivated, and there was no damage to the surface areas.

Using these inactivated spores, a high-affinity monoclonal antibody with the IgG1, K isotype was developed and named anti-BAs MAM02. The indirect ELISA test demonstrated the affinity of the antibody to its antigen, and it was able to detect spores even at a low concentration of 0.25 μg/mL. At the same time, a rabbit polyclonal antibody that can recognize inactive spores was developed and conjugated with the HRP enzyme. Both developed antibodies were tested in the sandwich ELISA system as a scope of preliminary study for use in antigen-antibody-based immunological tests. According to the results, 400 ng/well anti-BAs MAM02 antibody was chosen as the capture, and 1:1000 diluted HRP labeled anti-BAs pAb was used as the detecting antibody while they could recognize 5×10^4–10^7 spores/well inactivated spores.

**Conclusions**

The necessity of rapid and reliable detection of bacterial spores, which pose a direct danger to livestock and the environment and an indirect danger to human health, increases the need for immunological diagnostic tests. The monoclonal and polyclonal antibodies developed in this study are essential key materials for generating antibody-based diagnostic tests. At the same time, their ability to detect inactive spores in the sandwich ELISA system measured their potential for use in any other diagnostic tests, such as ELISA kits, lateral flow assays (LFA), and biosensor applications. Also, produced inactivated spores are a decent immunogen for generating antibodies and may be a candidate component of live vaccine formulation.

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