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Research article

Antibody Induction in Rabbits with *Blastomyces dermatitidis* Preparative Isoelectric Focusing (Rotofor) Fractions

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Abstract

The objective of this study was to evaluate *Blastomyces dermatitidis* preparative isoelectric focusing (Rotofor) fractions prepared from yeast phase lysate antigens for their ability to induce an antibody response in immunized rabbits. Pooled preparations from early (E: fractions 3,4,5,6) and late (L: fractions 17,18,19,20) from three isolates (B5896 and B5931; human and ERC-2; dog) were used as the immunizing antigens and antibody was detected using the indirect enzyme-linked immunosorbent assay (ELISA). In Trial 1, 8 human and animal *B. dermatitidis* lysates were used to evaluate antibody produced by the E and L Rotofor fractions with mean absorbance values with the B5896 fractons of 0.685 (E) and 0.513 (L); values of 0.972 (E) and 0.588 (L) with the ERC-2 fractons and values of 0.728 (E) and 0.536 (L) with the B5931 fractions. Comparable results were evidenced in Trial 2 when 9 human lysates were used for antibody detection with mean absorbance values with the B5896 fractions of 0.738 (E) and 0.474 (L); values of 0.871 (E) and 0.608 (L) with the ERC-2 fractions and values of 0.581 (E) and 0.507 (L) with the B5931 preparations. Therefore the results indicated that greater amounts of antibody were induced by the E fractions than by the L fractions with all three *B. dermatitidis* yeast phase lysate preparations.

Keywords: *Blastomyces dermatitidis*, isoelectric focusing (Rotofor) fractions, Lysate antigens, Indirect ELISA, Antibody induction, Serum specimens, blastomycosis

Introduction

Blastomycosis is a systemic fungal infection in humans and animals, caused by the dimorphic organism *Blastomyces dermatitidis*. In the United States, it is found in the Southeastern, South-Central and upper Midwestern states that border the Ohio and Mississippi Rivers, like Wisconsin and Minnesota. Recent studies have indicated that blastomycosis may be present in other regions with sporadic cases being reported in Colorado, Texas, Kansas and Nebraska [1, 2]. It is acquired by the inhalation of the infectious particles (mycelial phase spores). *B. dermatitidis* is thermally dimorphic and is acquired by inhalation of the infectious particle (mycelial phase spore) into the lung which then converts to a yeast cell resulting in a primary pulmonary acute infection. The disease may be self-resolving or it may be misdiagnosed as tuberculosis. If the disease goes undiagnosed or untreated while in the lungs, it may become invasive and disseminate to other organs, and possibly to the central nervous system where fatal meningitis may develop. Blastomycosis is cause for concern in individuals with AIDS or other deficiency diseases that compromise the immune system [3-5].

Researchers have begun to devote more attention to developing ways of diagnosing, preventing and treating these mycoses. Considerable progress with blastomycosis has been a concern because problems have existed with diagnosis of the disease and the misdiagnosis as some other infectious disease. Current laboratory diagnostic methods include culturing or histologic identification, but in many cases, these methods may not provide a reliable diagnosis or take a long amount of time which delays treatment. This has led to a ton more research being done to improve immunological assays which provides rapid diagnosis, but problems still exist with regard to the sensitivity and specificity of immunoassays [6-10].

To contribute to improve immunodiagnostic laboratory assays, our laboratory has been concerned with the preparation and comparative studies of *B. dermatitidis* yeast lysate antigens for the past several years. The thrust of research in our laboratory has been associated with studies on various strains of *B. dermatitidis* from human, animal or environmental specimens from many geographical locations in an effort to better understand how antigens prepared from these isolates might be useful as immunodiagnostic reagents [11-21]. These studies have only opened up new avenues of approach with regard to how we might improve immunodiagnostic methods in the future.

The objective of this study was to evaluate *Blastomyces dermatitidis* preparative isoelectric focusing (Rotofor) fractions prepared from yeast phase lysate antigens for their ability to induce an antibody response in immunized rabbits. We evaluated and compared both Trials 1 and 2.

Materials and Method

Yeast Lysate Antigens

Mycelial phase cultures were converted to yeast cells by culturing at 37°C on brain heart infusion agar. Yeast phase lysate reagents were prepared by a method similar to one that was previously used for the production of antigen from *Histoplasma capsulatum* [22-24] and modified in our laboratory for *B. dermatitidis* lysate antigen production [21]. The yeast phase cells were grown for 7 days at 37°C in a chemically defined medium in an incubator shaker, harvested by centrifugation (700 x g; 5 min), followed by washing with distilled water, re-suspended in distilled water and then allowed to lyse for either 1 or 7 days at 37°C in water with shaking. The preparations were centrifuged, filter sterilized, merthiolate added (1:10,000) and stored at 4°C for further use. Protein determinations

were performed on the lysates using the BCA Protein Assay Kit (Thermo-Fisher Pierce) and dilutions of the antigenic reagents used in the assays were based on protein concentration.

Serum Specimens

Serum specimens were obtained from rabbits immunized with pooled Rotofor fractions of lysate antigens produced from *B. dermatitidis* isolates B5896, ERC-2 and B5931 (E: fractions 3,4,5,6) and late (L: fractions 17,18,19,20). The animals were housed in accordance to the NIH Guide for Care and Use of Laboratory Animals with approval from the Idaho State University IACUC.

Prepatrative Isoelectric Focusing (Rotofor)

Isoelectric focusing was performed using the BIO-RAD Rotofor apparatus (BIO-RAD, Hercules, CA). Ampholytes (BIO-RAD) were added to the yeast phase lysate in a 2% to 98% ratio. These small charged molecules create a pH gradient in solution from a pH of 3 to 10 when electrical current is applied so that proteins can be separated based on their isoelectric point. All proteins have a unique net charge that will force the proteins to move through the pH gradient until their net charge becomes zero (the isoelectric point). When proteins reach their unique isoelectric point in this pH gradient they are no longer able to migrate and forced to remain where their net charge is zero by the established pH gradient. Twenty protein fractions were collected after focusing (15 watts constant current) for approximately four hours at 4°C to ensure that no denaturing of the proteins occurred. The focusing was stopped when the voltage stopped fluctuating [15,16]. After collection of the fractions the pH was measured and adjusted to return the proteins to their physiologically active pH. This was accomplished by the addition of HCl or NaOH to either lower or raise the pH as required. Protein determinations were performed on the fractions using the Pierce BCA Protein Assay, as above. The 20 fractions were used to detect antibody in immunized rabbits using the ELISA and it was determined that the early fractions (3-6) were considerably more reactive than the late fractions (17-20) with mean absorbance values of 1.017 and 0.687 with the B5896 lysate preparation, 2.068 and 0.383 with the ERC-2 lysate and 1.304 and 0.685 with the B5931 lysate respectively. The pooled preparations (100 ug/ml) of the three lysate reagents (early:E and late:L fractions)were then used to immunize rabbits (2ml intramuscular and 1 ml subcutaneous).

Enzyme-Linked Immunosorbent Assay (ELISA)

The ability of each yeast lysate reagent to detect antibodies in the above serum specimens was determined using the indirect enzyme-linked immunosorbent assay (ELISA). Each lysate antigen was diluted (2000 ng/ml of protein) in a carbonate-bicarbonate coating buffer (pH 9.6) and then added to triplicate wells (100 µl) of a NUNC 96-well microplate (Thermo-Fisher). The plates were then incubated overnight at 4°C in a humid chamber followed by washing three times with phosphate buffered saline containing 0.15% Tween 20 (PBS-T). The serum specimens (1:2500 dilution; 100 µl) were added to the microplate wells and incubated for 30 min at 37°C in a humid chamber. Following this incubation the wells were washed as above and 100 µl of goat anti- rabbit) IgG (H & L) peroxidase conjugate (Kirkegaard and Perry, KPL) was added to each well and incubated for 30 min at 37°C. The plates were again washed as above and 100 µl of SureBlue Reserve TMB Microwell Peroxidase Substrate (KPL) was added to each well and incubated for approximately 2 min at room temperature. The reaction will be stopped by the addition of TMB Stop Solution (KPL) and the absorbance read at 450 nm using a BIO-RAD 2550 EIA reader.

Results and Discussion

The results of Trial 1, as shown in Figure 1, indicated that all three (B5896, ERC-2 and B5931) pooled E fractions induced antibody to a greater extent than the L fractions with an ELISA mean absorbance value obtained with all three preparations of 0.795 with the E pooled fractions to 0.546 with the L fractions. Comparable reactivity results

were obtained in Trial 2 (Figure 2) with a mean absorbance value with the three lysate fractions of 0.730 (E) versus 0.530 with the L fractions.

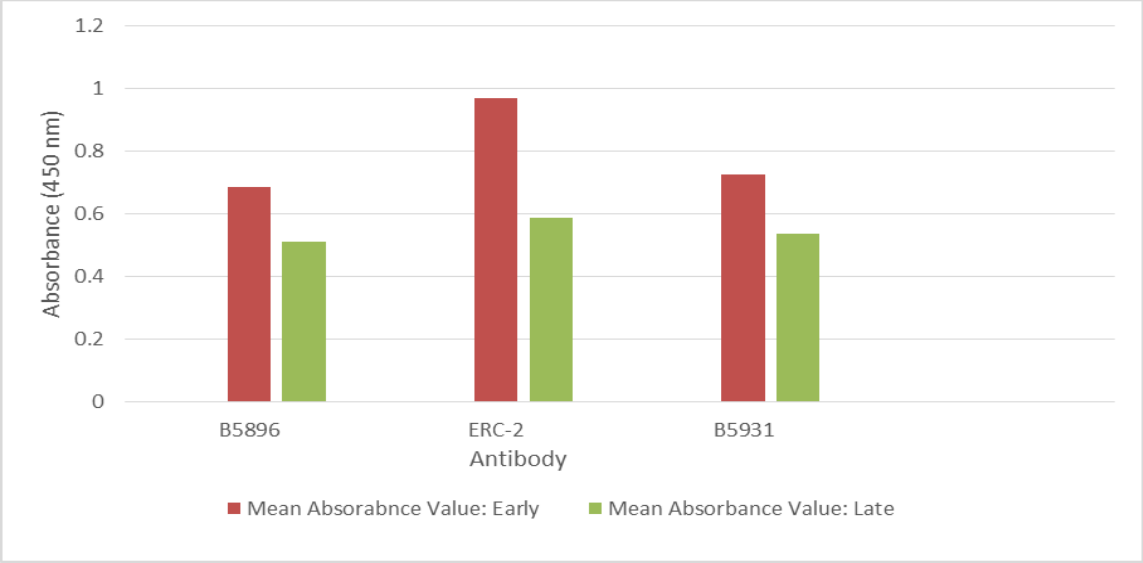

Figure 1.Trial 1: Rabbit serum specimen mean absorbance's of early and late antibodies.

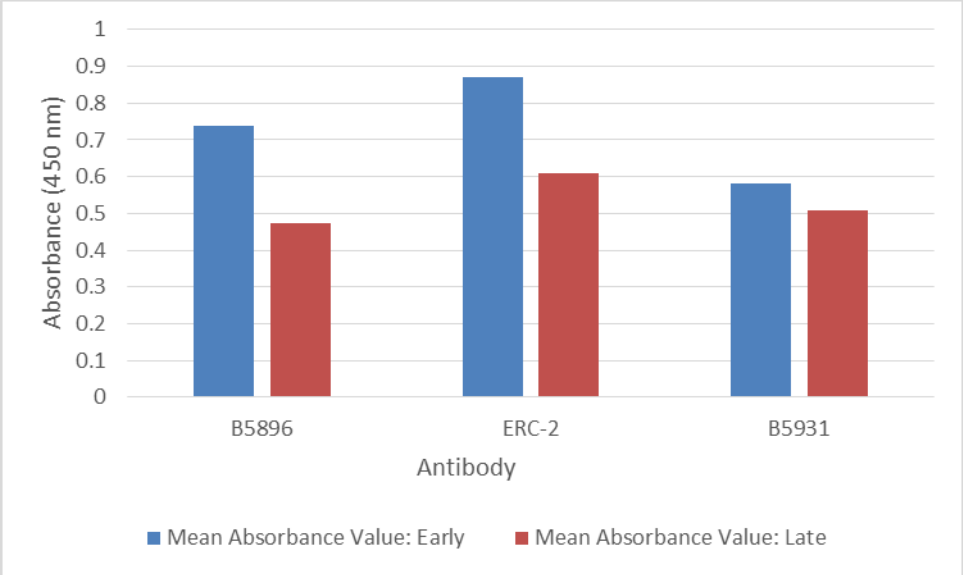

Figure 2. Trial 2: Rabbit serum specimen mean absorbance's of early and late antibodies.

As determined in a previous study the early (3,4,5,6) Rotofor fractions were able to detect antibody in rabbit sera using the ELISA to a much greater degree than the late (17,18,19,20) fractions. This current study provided data that indicated that the early Rotofor fractions were also more efficacious with regard to inducing antibody in immunized rabbits. Thus the Rotofor purification process seems to produce antigens obtained in the early fractions that are considerably more immunoreactive than the antigens in the late fractions. This study has now provided data

on the potential use of the antibodies produced by the early Rotofor fractions as antibodies in a competitive ELISA for the detection of *B. dermatitidis* antigen in animals or humans with blastomycosis.

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