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Research paper

Detection of anthrax protective antigen (PA) using europium labeled anti-PA monoclonal antibody and time-resolved fluorescence ☆

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ABSTRACT

Inhalation anthrax is a rare but acute infectious disease following adsorption of *Bacillus anthracis* spores through the lungs. The disease has a high fatality rate if untreated, but early and correct diagnosis has a significant impact on case patient recovery. The early symptoms of inhalation anthrax are, however, non-specific and current anthrax diagnostics are primarily dependent upon culture and confirmatory real-time PCR. Consequently, there may be a significant delay in diagnosis and targeted treatment. Rapid, culture-independent diagnostic tests are therefore needed, particularly in the context of a large scale emergency response.

The aim of this study was to evaluate the ability of monoclonal antibodies to detect anthrax toxin proteins that are secreted early in the course of *B. anthracis* infection using a time-resolved fluorescence (TRF) immunoassay. We selected monoclonal antibodies that could detect protective antigen (PA), as PA83 and also PA63 and LF in the lethal toxin complex. The assay reliable detection limit (RDL) was 6.63×10^{-6} μM (0.551 ng/ml) for PA83 and 2.51×10^{-5} μM (1.58 ng/ml) for PA63. Despite variable precision and accuracy of the assay, PA was detected in 9 out of 10 sera samples from anthrax confirmed case patients with cutaneous ($n = 7$), inhalation ($n = 2$), and gastrointestinal ($n = 1$) disease. Anthrax Immune Globulin (AIG), which has been used in treatment of clinical anthrax, interfered with detection of PA. This study demonstrates a culture-independent method of diagnosing anthrax through the use of monoclonal antibodies to detect PA and LF in the lethal toxin complex.

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1. Introduction

Bacillus anthracis is an aerobic spore-forming gram-positive bacterium that is the causative agent of anthrax. Anthrax in humans can manifest in four different forms: cutaneous, gastrointestinal, inhalation or injection (Logan et al., 2011; Palmateer et al., 2013). Cutaneous anthrax is the most common form of the disease, accounting for 99% of cases worldwide but with a low fatality if treatment is available (CDC, 2001; Logan et al., 2011). Ingestion of *B. anthracis* can result in either oropharangeal or gastrointestinal disease, with a variable mortality rate depending on how quickly treatment is started (Logan et al.,

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2011). Inhalation anthrax is rare but has a high mortality rate (89%) if not diagnosed early and treated promptly (Logan et al., 2011). In 2001 anthrax spores were intentionally released in mailed letters in the United States, resulting in 22 cases (Logan et al., 2011). The mortality rate of inhalation anthrax was as high as 89% before 2001, but with advanced treatment and supportive care, the mortality rate was only 45% in 2001 (Jernigan et al., 2002). Injection anthrax is a more recent type of infection associated with intravenous drug users (Palmateer et al., 2013). Symptoms of injection anthrax is similar to cutaneous, but the infection may be in deeper tissues such as muscle and it can go systemic quickly (CDC, 2013).

Toxins released by *B. anthracis* play a major role in establishing and maintaining infection. Anthrax toxins consist of protective antigen (PA), lethal factor (LF), and edema factor (EF). Native PA is produced as a 83-kDa protein (PA83) that binds to host cell receptors, is cleaved and activated by cellular proteases to release a 20-kDa segment, leaving PA63 to form an oligomeric complex at the cell membrane (Young and Collier, 2007; Kintzer et al., 2009). The PA63 complex binds up to four LF and EF molecules to form lethal toxin (LTx; PA63 + LF) or edema toxin (ETx; PA63 + EF) which may then be internalized into the cell to cause a cascade of cytotoxic effects (Young and Collier, 2007).

Anthrax is diagnosed by a variety of methods including: staining of specimens to visualize the organism, culture, PCR, and serology (Logan et al., 2011). Other methodologies for diagnosing anthrax have been reported in the literature and include those that detect anthrax toxins instead of the organism itself (Kobiler et al., 2006; Boyer et al., 2007; Rossi et al., 2008; Tang et al., 2009; Oh et al., 2011; Dragan et al., 2012). Anthrax toxins are secreted early during the course of infection and therefore provide a more timely diagnosis than the use of immunoserology, which requires the production of antibodies by the immune system, or culture, which may take several days and requires appropriate laboratory facilities (Logan et al., 2011). Tang et al. previously described an immunoassay using both polyclonal and monoclonal antibodies in time-resolved fluorescence (TRF) immunoassay, a method that utilizes a high fluorescent nanoparticle (europium), to detect PA in sera to aid in diagnosis of anthrax (Tang et al., 2009).

The aim of this study was to evaluate antigen-specific monoclonal antibodies for use in culture independent assays capable of detecting PA83, PA63 and LTx in the early and convalescent stages of infection following treatment with antibiotics and immunotherapy. TRF was chosen to evaluate our collection monoclonal antibodies because of its higher sensitivity compared to ELISA.

2. Materials and methods

2.1. Materials

Purified recombinant native protective antigen [83 kDa (1 μM = 83 μg/ml); PA83] was obtained from BEI Resources (Manassas, VA). Activated protective antigen [63 kDa (1 μM = 63 μg/ml); PA63] and recombinant lethal factor [90 kDa (1 μM = 90 μg/ml); LF] were obtained from List Biological Laboratories (Campbell, CA). Dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA) buffer, wash concentrate, enhancement solution, Streptavidin Microtitration 96-well plates, Platewash, Plateshake, and Victor™ X4 Multilabel Plate Reader were from Perkin-Elmer Life Sciences (Shelton, CT). Lethal toxin (LTx) was prepared by adding 1.75 X more PA63 to LF. Anthrax Immune Globulin (AIG), an investigational product for anthrax treatment consisting primarily of anti-PA antibody, was previously acquired from Cangene (Winnipeg, MB, Canada).

2.2. Monoclonal antibody preparation, selection, and labeling

Mouse monoclonal anti-PA IgG antibodies (1 μM = 150 μg/ml) were prepared in the Division of Scientific Resources at CDC as previously described (Boyer et al., 2007). Monoclonal anti-PA IgG AVR1046 was selected as the detector antibody (Li et al., 2008) and monoclonal anti-LF antibody, AVR1674, was selected for experiments for detection of LF in the LTx complex (Boyer et al., 2007). AVR1046 and AVR1674 were europium (Eu) labeled (Eu-AVR1046 and Eu-AVR1674) by Perkin-Elmer BioSignal Inc. (Montreal, QC, Canada). Four anti-PA mouse monoclonal antibodies were tested and AVR1162 was selected as the capture antibody based on its ability to bind PA83 and PA63 without competing with AVR1046, the detector antibody (data not shown). AVR1162 was biotinylated (Bio-AVR1162) in the Division of Scientific Resources at CDC with EZ-Link® Sulfo-NHS-Biotin (Pierce Biotechnology, Rockford, IL) following the manufacturer's instructions.

2.3. Human sera

Serum from 10 healthy human donors that had not been previously diagnosed with anthrax or received the anthrax vaccine was obtained from Tennessee Blood Services (Memphis, TN) for use in assay development. Sera were confirmed non-reactive for anti-PA IgG by ELISA before spiking experiments (Quinn et al., 2002; Semenova et al., 2012).

Serum samples from 10 healthy unvaccinated volunteers enrolled in an anthrax vaccine human clinical trial (Marano et al., 2008) and 10 patients confirmed with anthrax were tested to determine performance on clinical samples. Of the 10 human patients confirmed with disease, seven of the cases were cutaneous anthrax with sera samples drawn between 1 and 8 days after symptom onset (Boyer et al., 2011b). Two of the sera were from inhalation anthrax cases and the samples were collected two to eight days after symptom onset. The sample from the patient with gastrointestinal anthrax was drawn 11 days after symptom onset. All samples were taken prior to treatment with AIG. Methods for detection of LF and anti-PA IgG have been previously described (Boyer et al., 2011a; Semenova et al., 2012).

The of human serum in the study was approved by the Centers for Disease Control and Prevention (CDC) Human Subjects Institutional Review Board (IRB).

2.4. Optimization of LTx complex detection assay

Ratios of capture and detector antibody pairs for PA were optimized using standard checkerboard titrations. Capture