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ScienceDirect
Biologicals 35 (2007) 123–129
IABS BIOLOGICALS
www.elsevier.com/locate/biologicals

Qualification and performance characteristics of a quantitative enzyme-linked immunosorbent assay for human lgG antibodies to anthrax lethal factor antigen

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Received 14 March 2006; revised 7 July 2006; accepted 13 July 2006

Abstract

The contribution of *Bacillus anthracis* lethal factor (LF)-specific immune responses to protection against anthrax disease in humans remains incompletely defined due, in part, to a paucity of qualified reagents and a lack of standardized serological assays. Toward this end, we have identified and characterized suitable positive quality control and standard reference sera and developed, optimized, and qualified an enzyme-linked immunosorbent assay (ELISA) to measure LF-binding IgG. Herein we describe the performance characteristics of this ELISA and propose criteria for its use in the detection and quantification of anti-LF IgG in human serum.

Keywords: Bioterrorism; Anthrax; Lethal factor; ELISA

1. Introduction

The extensive pathology and rapid lethality associated with *Bacillus anthracis* infection is primarily due to the actions of two protein toxins secreted by the organism [1]. The binary exotoxins, edema toxin (ETx) and lethal toxin (LTx), are formed through competitive binding of protective antigen (PA) to edema factor (EF) and lethal factor (LF), respectively [2]. Together, the cellular activities of ETx and LTx produce edema and local necrosis during cutaneous anthrax and tissue hypoxia and pleural edema during inhalation anthrax [3,4]. Although limited data are available regarding the nature of human immune responses following infection with *B. anthracis*, a strong IgG response to PA, the common component of both anthrax exotoxins, appears to provide protection against the disease [5]. Therefore, current strategies for vaccination or therapeutic intervention against anthrax have focused on neutralization of the PA component of the toxins.

Active immunization with PA-containing vaccines, or passive administration of anti-PA antibodies, has proven sufficient to protect mice, guinea pigs, rabbits, and non-human primates from an aerosol challenge with *B. anthracis* [6–11]. Additional factors, however, likely contribute to the development and maintenance of protective immunity to this pathogen [8,9,11,12]. Price et al. demonstrated that a plasmid DNA (pDNA) vaccine encoding a truncated form of LF constituting the PA binding site protects mice from LTx challenge [13], an observation subsequently extended in an inhalation spore challenge study in rabbits vaccinated with a LF pDNA prime,

Abbreviations: AP, affinity purified; CV, coefficient of variation; ETx, edema toxin; ELISA, enzyme-linked immunosorbent assay; LF, lethal factor; LTx, lethal toxin; MDC, minimal detectable concentration; PA, protective antigen; pDNA, plasmid DNA; QC, quality control; RID, radial immunodiffusion; RDC, reliable detectable limit; RT, reactivity threshold; TNA, toxin neutralizing activity.

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1045-1056/06/$32.00
doi:10.1016/j.biologicals.2006.07.003

recombinant protein boost regimen [14]. Similarly, vaccination with a pDNA encoding a detoxified, truncated form of LF (domains I–III) elicited neutralizing antibody that protected some anthrax spore-challenged rabbits and significantly delayed the time to death in others [15]. These data, coupled with the potential threat for release of *B. anthracis* containing genetically engineered forms of PA as bioterrorist or biowarfare agents, warrant the evaluation of LF for incorporation in next generation anthrax vaccines.

In individuals diagnosed in 2001 with bioterrorism-related cutaneous or inhalation anthrax, the US Centers for Disease Control and Prevention (CDC, Atlanta, GA) demonstrated a strong correlation between anti-PA IgG levels and serum LTx neutralizing activity [5]. The absence of suitable standards and quantitative immunoassays, however, has limited any detailed evaluation of the correlation between anti-LF IgG levels and serum LTx neutralizing activity, despite observations that clinical anthrax elicits strong anti-LF IgG responses in humans [16]. In an effort to examine the contribution of anti-LF IgG to serum LTx neutralizing activity and protective immunity against anthrax, we have characterized standard reference and positive quality control (QC) sera and have developed an anti-LF IgG enzyme-linked immunosorbent assay (ELISA) for the quantification of LF-specific antibody levels in human serum. Herein we describe the performance of the qualified ELISA and assign mass values to the standard reference and positive QC sera for use in anthrax serological assays.

2. Materials and methods

2.1. Recombinant PA and LF

Lyophilized *B. anthracis* PA and LF proteins were purchased from List Biological Laboratories (Campbell, CA). The vials were stored at 4 °C until reconstituted in sterile water to 1 mg/mL. Aliquots were stored at −70 °C.

2.2. Human standard reference, positive QC, and negative control sera preparation

After obtaining Institutional Review Board approval and informed consent, sera designated VCT530 and VCT905 were obtained on two separate occasions from a healthy volunteer who had received the initial priming course (0, 3, 6, and 24 weeks) of the licensed UK anthrax vaccine followed by booster vaccinations administered annually beginning in 1991. The UK anthrax vaccine consists of a protein preparation precipitated from supernatant fluid of cultures of *B. anthracis* Sterne strain [17]. Based on its ability to elicit LF-specific humoral immune responses, the UK vaccine is distinguished from the vaccine licensed for use in the US (Anthrax Vaccine Adsorbed, AVA, Biothrax™, Bioport Corp. Lansing, MI) [11]. VCT530 was collected approximately two months prior to the annual booster vaccination while VCT905 was collected approximately two months post-boost. A negative control serum, VCT190, was prepared by pooling a panel of 52 serum samples obtained from the San Diego Blood Bank. *B. anthracis* exposure and anthrax vaccination history of the donors are unknown; however, the sera of these individuals, as well as the serum pool, exhibit minimal reactivity (<0.1 optical density (OD) units) in the anti-LF IgG ELISA. Sera were stored frozen at −70 °C and thawed at 4 °C prior to testing.

2.3. Radial immunodiffusion (RID) and nephelometry

Mass values (in mg/mL) of total IgG and each IgG subclass in VCT530, VCT905, and anti-LF IgG purified from VCT905 were measured by two independent methods. First, RID values were assigned using the BINDARID kit (The Binding Site, San Diego, CA). Mass values were calculated from two separate RID determinations performed on non-consecutive days using the manufacturer-supplied calibrator as a standard. Concentrations of IgG were interpolated from a standard curve generated on the same plate. Second, nephelometry was performed by the Mayo Central Laboratory for Clinical Trials (Rochester, MN), and the concentrations were compared to the RID values.

2.4. Affinity purification of human anti-LF IgG

Because there is no established LF reference anti-serum, anti-LF IgG was affinity purified from VCT905 for use as a working reference standard. Total IgG was purified from VCT905 using standard protein G chromatography methods. Eluted fractions containing protein, as identified by the BCA protein assay (Pierce Biotechnology, Rockford, IL) or by absorption at 280 nm (A_{280}, extinction coefficient of 1.35 [18]), were pooled and dialyzed against phosphate-buffered saline (PBS), pH 7.4. LF-specific IgG then was purified from the protein G-purified total IgG by affinity adsorption to recombinant LF immobilized on CNBr-activated Sepharose 4B (Amersham Biosciences Corp/GE Healthcare, Piscataway, NJ) according to the manufacturer's specifications. The relative purity of the eluted protein was >95% as determined by silver-stained SDS-PAGE. The final concentration of purified anti-LF IgG was obtained by averaging the concentrations determined by BCA and A_{280} using an extinction coefficient of 1.35 [18].

To determine the concentration of LF-specific IgG in the serum samples, dilutions of VCT530 and VCT905 were assayed by ELISA in parallel with the purified standard, which was diluted to the appropriate starting dilution in negative control serum. Concentrations of LF-specific IgG were interpolated for each serum dilution from a standard calibration curve using a four-parameter logistic log (4-PL) model [19] and the ELISA for Windows software (version 2.0) developed by the CDC [20]. VCT530 and VCT905 then were used as the positive QC and standard reference sera, respectively, for all subsequent assays.

2.5. Anti-PA IgG ELISA

The ELISA to quantify PA-specific IgG in human serum followed the method of Quinn et al. [21] with recombinant PA as a solid phase immobilized antigen and horseradish peroxidase (HRP)-conjugated mouse anti-human gamma chain-specific