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Catalytically inactive anthrax toxin(s) are potential prophylactic agents

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Abstract

The anthrax exotoxin, which is a key mediator of anthrax related pathogenesis, is composed of two separate toxins formed by pairwise combinations of three proteins that are encoded on the pXO1 plasmid of *Bacillus anthracis*. Lethal toxin is composed of protective antigen (PA) combined with lethal factor (LF) while edema toxin is composed of PA and edema factor (EF). The present study found that the catalytic mutants of LF (LF_{E687A}) and EF (EF_{H351A}) competitively inhibited lethal toxin and edema toxin-mediated activity in vitro and lethality in vivo and were non-toxic to sensitive cell lines when combined with PA. While PA combined with EF_{H351A} was non-lethal in mice, PA combined with LF_{E687A} was of reduced virulence. Full protection of mice against a lethal toxin challenge required injection of mice with PA combined with both LF_{E687A} and EF_{H351A}. The potential use of these full-length, biologically inactive mutant proteins combined with PA as prophylactics or therapeutics is discussed.
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Keywords: Edema factor; Lethal factor; Active site mutants

1. Introduction

Anthrax is caused by toxigenic, encapsulated strains of *Bacillus anthracis*. Pathogenesis of this disease is primarily attributed to the tripartite exotoxins, lethal toxin (LeTx) and edema toxin (EdTx), which are formed by pairwise binding of PA to LF or EF, respectively. Before formation of the exotoxins, PA binds to cellular receptors, undergoes proteolysis, and forms heptameric oligomers. LeTx and EdTx are endocytosed by the cell and LF and EF are translocated into the cytosol by PA [1]. LF is a zinc-dependent protease that targets MAPKK [2,3]; while EF is a calmodulin-dependent adenylate cyclase that causes a dramatic elevation of cAMP in cells [4]. The pathogenesis of anthrax includes bacterial evasion accomplished through the capsule, and lethality and immune suppression by the exotoxins [5–11]. Rapid disease progression severely limits the effectiveness of post-exposure antimicrobial therapies. Vaccination against anthrax has been considered as one of the most effective prophylactic measures. Vaccine formulations that neutralize anthrax toxin might not only limit its cytotoxic effects but might also augment the clearance of *B. anthracis*. Noticeably, strategies that provide antitoxin immunity can be exploited for effective treatment of anthrax supplementing antibiotic therapy. Therefore, reducing the toxin effects is thought to be central in host protection, and a body of evidence indicates that protection is mediated by antibody responses, whether actively induced or passively administered [12,13].

PA is the principal immunogen in all the currently licensed anthrax vaccines for human use [14]. Clinical trials with FDA-licensed PA-based vaccine, anthrax vaccine adsorbed (AVA), indicate that a critical level of serum anti-PA antibodies confers immunity to both cutaneous and inhalational anthrax [15]. However, the inherent limitations of this vaccine such as reactogenecity and requirement for multiple

Abbreviations: PA, protective antigen; LF, lethal factor; EF, edema factor; LeTx, lethal toxin; EdTx, edema toxin; Ab, antibody; Ig, immunoglobulin; LPS, lipopolysaccharide; ELISA, enzyme-linked immunosorbent assay.

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immunizations underscore the need to develop new, improved cell-free anthrax vaccines [16]. Several immunization studies point toward the importance of LF and EF in eliciting protective immunity against anthrax [17–20]. Besides anti-PA immunoglobulins (Igs), Abs to LF and EF would also augment toxin neutralization by preventing the association of PA with LF and EF. Previously, the interaction among toxin components has been speculated in attaining heightened humoral response [21]. The importance of toxin-mediated immunity is reiterated by the fact that immunization of animals with spores deficient in producing all three exotoxins of *B. anthracis* did not impart protection against anthrax [17].

Wild-type LF or EF cannot be used as supplementary immunogens in PA-based vaccines due to their toxic effects. Hitherto, mutational studies have identified several residues of LF and EF that are central to their biological activity [22–27]. Studies have highlighted the need of using mutants of anthrax toxin components in vaccine preparations [17,21]. A comprehensive structural and biochemical analysis of EF mutants established the important role played by H351 in enzymatic activity of EF [28,29]. Mutations in HEXXH motif present in the catalytic domain of LF have been shown to drastically reduce its activity [27,30]. A detailed analysis of such catalytically inactive mutants ought to be the coherent approach for finding new antidotes to the disease. Herein we report that the structurally conserved, active site variants of LF and EF, LF_{E687A} and EF_{H351A}, respectively are non-toxic but potentially immunogenic. Our results provide a rationale for design of safer and efficacious vaccine and therapeutic molecules against anthrax.

2. Materials and methods

2.1. Preparation of proteins

PA, EF and EF_{H351A} were expressed and purified as previously described [28,31]. PCR cloning of LF gene from pXO1 was done in pET28a (Novagen). E687A mutation was introduced into LF gene using site directed mutagenesis. LF_{WT} and LF_{E687A}, which expressed in cytosolic fractions of *E. coli* BL21 (DE3), were purified as described for LF_N, N-terminal of LF [32]. In brief, cultures grown in LB-kanamycin (25 μg/ml) to an OD_{600} of 0.6–0.8 were induced with 1 mM isopropyl-1-thiogalactopyranoside (IPTG). Cells were harvested after 4 h of induction. All purification procedures were carried out at 4 °C. Cell pellet obtained from 1 L culture was washed with sonication buffer (50 mM potassium phosphate, pH 8.0, 0.5 M NaCl, 2 mM β-ME, 1 mM PMSF) and suspended in 50 ml lysis buffer (sonication buffer containing 1 mg/ml lysozyme). Cell suspension was shaken on ice for 30 min. The cells were disrupted by pulse sonication and clarified supernatant obtained after centrifugation was mixed with 5 ml of 50% Ni–NTA resin, preequilibriated with sonication buffer. The matrix was washed with 20 mM imidazole in elution buffer (20 mM potassium phosphate, pH 8.0, 1 M NaCl, 2 mM β-ME, 1 mM PMSF, and 1% glycerol) and protein was eluted with 300 mM imidazole prepared in elution buffer. Protein fractions were pooled and dialyzed overnight against buffer A (10 mM Tris, pH 8.0, 1 mM EDTA, 2 mM β-ME, 1% glycerol, and 1 mM PMSF). The dialyzed protein was then applied to Mono Q Sepharose column previously equilibriated with buffer A. Protein was eluted with a linear gradient of 0–500 mM NaCl prepared in buffer A. Purity of the fractions was analyzed by SDS–PAGE. The fractions with highest purity were pooled and dialyzed against 10 mM potassium phosphate, pH 8.0. Protein concentrations were determined using the Bradford reagent (Bio-Rad). For immunizations, all proteins were dialyzed against phosphate-buffered saline (PBS, pH 7.4) and dilutions were made in PBS. Polyclonal antisera were raised against recombinant wild-type LF and EF in New Zealand White rabbits as done previously for EF [28].

2.2. Binding of mutants to PA in solution

Purified wild-type and mutant LF and EF were checked for PA binding in solution as described previously [31]. Briefly, PA was incubated with trypsin (1 ng/μg of PA) for 30 min at 30 °C in nicking buffer (25 mM Hepes, 1 mM $CaCl_2$ and 0.5 mM EDTA). Trypsin was inactivated by addition of 1 mM PMSF. Nicked PA (PA63) was incubated with wild-type proteins or mutants in equimolar ratio in binding buffer (20 mM Tris, pH 9.0 containing 2 mg/ml CHAPS) for 15 min at 30 °C. Samples were analyzed on 6 % non-denaturing polyacrylamide gel after silver staining.

2.3. In vitro assays

2.3.1. Cell culture

Murine macrophage-like cell line (J774A.1), and Chinese Hamster Ovary (CHO-K1) cells (ATCC) were maintained in RPMI 1640 and EMEM media, respectively; supplemented with 10% fetal bovine serum (FBS, Biological Industries), 25 mM HEPES, 2mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Spleens were removed aseptically from naive mice, and splenocytes were obtained by gently teasing the organ in RPMI. After lysis of erythrocytes with 0.85% NH_4Cl, splenocytes were washed thrice and resuspended in RPMI along with the above-mentioned supplements.

Cell culture and all in vitro assays were carried out at 37 °C in presence of 5% CO_2 and 95% humidity.

2.3.2. Macrophage lysis assay

Viability of J774A.1 cells exposed to 0.001–10 μg/ml of LF or LF_{E687A} and 1 μg/ml of PA was assessed as described earlier [33]. Briefly, 96-well plates were seeded with 5×10^4 cells per well and incubated overnight. Later, the spent medium was replaced with test reagents followed by incubation for 3 h. Subsequently, MTT dissolved in RPMI was added to each well at a final concentration of 0.5 mg/ml

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