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Protective antigen and extractable antigen 1 based chimeric protein confers protection against *Bacillus anthracis* in mouse model

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ABSTRACT

Recombinant bivalent chimeric protein was generated comprising of domain 4 of protective antigen (PA4) and carboxy terminal region of extractable antigen 1 (EA1C) by overlap extension PCR. The immunogenicity and protective efficacy of recombinant chimeric protein (PE) and protein mixture (PAEA) along with the individual components, PA4 and EA1C were evaluated in this study. We found that PE and PAEA exhibited higher endpoint titer and elevated IgG1 response. Compared to PA4 and EA1C, the chimeric protein PE and protein mixture PAEA exhibited 1.52 and 1.39 times more proliferative effect on lymphocytes in vitro. The spore uptake by anti-PE and anti-PAEA antibodies was significantly more than the individual components. We further evaluated the effects of antisera on the toxins in vitro and in vivo. Anti-PE and anti-PAEA antibodies displayed nearly 80% protection against crude toxin activity on RAW 264.7 cell lines. We further demonstrated that the anti-PE and anti-PAEA antibodies displayed better protection in controlling the edema induced by crude toxin. Passive immunization with anti-PE and anti-PAEA provided protection against toxin challenge in mice. The present study reveals that the chimeric protein consisting of heterologous regions of PA and EA1 can render better protection than PA4 or EA1C alone against toxins and bacilli.

1. Introduction

Anthrax, a highly lethal disease caused by *Bacillus anthracis*, has emerged as a serious biological threat agent, especially after Amerithrax attacks in 2001 (Wang and Roehrl, 2005). The spores of the pathogen are highly resilient to extreme environmental conditions, temperature and chemical disinfectants and can survive for years. There are three types of anthrax infection. Inhalational anthrax being the most serious form has high mortality rate mostly caused by occupational exposure to spores or intentional exposure in cases of biological terror attack. It is followed by ingestional exposure or gastrointestinal anthrax caused by consumption of meat contaminated with anthrax spores. Ingestional anthrax is difficult diagnose due to the non-specific symptoms and may result in 25–60% fatality if ignored. And another type of anthrax infection encountered is cutaneous anthrax which is by far the most common type caused by the contact of skin with the infected animals or their body parts such as skin, hooves and horns, and it can be easily cured by antibiotic intervention (Cote et al., 2011). But ingestional and inhalational anthrax are often fatal. Initial symptoms of the disease are similar to flu-like illness and can proceed to toxemia and death within a few days after incurring the infection.

The major virulence factors of the pathogen – toxins and poly-D-glutamic acid (PGA) capsule are encoded in the plasmids pXO1 and pXO2, respectively. The toxin complex is composed of three different proteins: protective antigen (PA), edema factor (EF) and lethal factor (LF). Protective antigen is the key molecule of anthrax toxin complex. PA has inherent ability to ferry the enzymatically active LF and EF into a host cytosol where they execute their functions. At present, only two licensed vaccines which are PA-based cell-free components, anthrax vaccine adsorbed (AVA) in USA and anthrax vaccine precipitated (AVP) in UK, are available for human use globally. Though these vaccines are proved to be protective, some serious limitations have been pointed out such as

Abbreviations: AVA, anthrax vaccine adsorbed; AVP, anthrax toxin precipitated; DMEM, Dulbeccos Modified Eagle's medium; DMSO, dimethyl sulphoxide; EA1, extractable antigen 1; EA1C, C terminal region of extractable antigen 1; EF, edema factor; LeTx, lethal toxin; LF, lethal factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OPD, o-phenylenediamine; PA, protective antigen; PA4, domain 4 of protective antigen; PAEA, mixture of PA4 and EA1C; PBS, phosphate buffered saline; PE, chimeric protein consisting of PA4 and EA1C; SDS, sodium dodecyl sulphate.

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(a) presence of sub optimal levels of EF and LF, (b) require multiple boosters, (c) fail to provide complete protection against inhalational challenge, (d) cannot provide protection against spores when present in high numbers (Aulinger et al., 2005; Wang and Roehrl, 2005).

Live attenuated vaccine candidates, on the other hand, were proven to be more protective than PA based vaccines hinting that the components other than PA may also have significant role in eliciting better immune response (Cohen et al., 2000; Rhie et al., 2003; Cote et al., 2012; Uchida et al., 2012; Vergis et al., 2013). Though effective, live attenuated vaccines possess residual virulence which led to the death of animals (Mock and Fouet, 2001). Number of reports have also shown that recombinant PA can protect animals from lethal toxin challenge (Koya et al., 2005; Cote et al., 2012) and the anti-rPA antibodies inhibits the spore germination (Welkos et al., 2001). However, Cote et al. (2008) opined that naturally existing or genetically modified strains of the pathogen may gain resistance to antibiotics and/or may not be responsive to PA based vaccines. Therefore, introduction of other molecules such as PGA (Rhie et al., 2003; Schneerson et al., 2003; Chabot et al., 2004; Wang et al., 2004), spore antigens (Brahmbhatt et al., 2007; Cote et al., 2012) or surface layer proteins (Baillie et al., 2003; Uchida et al., 2012) along with well established PA can confer improved protection by destroying the bacilli at the early stage and also abrogate the toxemia. The antibodies raised against the capsule prevent bacterial replication thereby reducing the levels of toxins secreted into the host. It, therefore, augments the protection conferred by anti-PA antibodies (Sloat et al., 2008). But, conjugation of PGA to PA involves additional steps of synthesizing/purifying the PGA and chemically linking it to PA. Protein based bivalent vaccine molecules could be better choice especially when they are spliced to make fusion/chimeric protein. It has already been established that domain 4 of PA contains the dominant epitopes of PA which were proven to be sufficient enough to protect mice against toxin and spore challenge. The C terminal region of PA is the host receptor binding region. Earlier workers have shown that mutations or deletions in PA in the region especially between 679 and 693 aa residues render it non-toxic (Varughese et al., 1999; Brossier et al., 1999). Many researchers have also demonstrated that domain 4 of PA generates potent humoral response and confer significant protection against LeTx challenge (Flick-Smith et al., 2002; McConnell et al., 2006; Chichester et al., 2007; Park et al., 2008; Kaur et al., 2009). In 2009, Kelly-Cirino and Mantis generated monoclonal antibodies against some epitopes of PA domain 4. They observed that these mAbs were able to confer significant protection against toxin challenge by inhibiting the toxin binding to the receptor. Later, Kaur et al. (2009) evaluated B cell epitopes against anthrax. They showed that epitopes residing in domain 4 of PA (626–676 aa residues) generate more potent neutralizing antibodies and protective response at par with the whole molecule (PA). In line with the above findings, we considered that inclusion of domain 4 of PA rather than whole molecule for developing vaccine molecule against anthrax might be suitable approach.

On the other hand, extractable antigen 1 (EA1) has also been shown to control infection in mice by effectively eliminating bacteria from infected organs (Uchida et al., 2012). Further, they have shown that EA1 in combination with PA delayed the onset of the disease in comparison to PA alone. EA1 is a major cell associated S layer protein, comprising of 15–30% total cell mass. It is also an immuno dominant protein present in copious in vegetative cells and also on the spore surfaces as vegetative cell contaminant (Williams and Turnbough, 2004). EA1 is a bimodular protein comprising of two domains: first, made of three SLH motifs situated at the N-terminal region which anchor EA1 to the peptidoglycan cell wall through cell wall associated polymers (Mesnage et al., 2000; Fouet, 2009). The remaining region of EA1 forms the second domain which is situated between the capsule and the peptidoglycan layer. The carboxy terminal region of EA1 (EA1C) was assumed to be exposed to the environment. Being an immunodominant protein exposed to the host immune system, carboxy terminal domain of EA1 can possibly elicit better antibody response. Hence, in the present study, we generated a chimeric protein (PE) consisting of protective domain IV of PA (PA4) and C terminal region of EA1 (EA1C) to provide dual protection against toxin and bacteria.

We evaluated the antibody response and lymphocyte proliferation ability of the chimeric protein; and opsonophagocytic ability and toxin neutralization efficacy of antisera of the chimeric protein in comparison with recombinant subunit proteins, and mixture of subunit proteins in mouse model. Overall we present the evidence that the chimeric protein confers better protection in vitro studies against the pathogen and toxins and against the toxins in mouse model in comparison to PA4 alone or EA1C.

2. Materials and methods

2.1. Bacterial strains, reagents and chemicals

In the present study *Bacillus anthracis* Sterne strain harboring pXO1 plasmid was used. The bacterial spores were prepared following the protocol described by Enkhtuya et al. (2006). The *B. anthracis* Sterne and *Escherichia coli* cells were cultivated in Brain Heart Infusion broth (BHI) and Luria Bertani broth (LB), respectively. *E. coli* strains, BL21DE3 and DH5α were used for cloning and maintaining the plasmids, respectively. The handling of the pathogen was done in Biosafety level 3. All the experiments conducted were approved by Institutional Biosafety Regulations Committee. All the microbiology media were purchased from Himedia Laboratories, India and all the chemicals, reagents and cell culture media were purchased from Sigma–Aldrich, USA unless mentioned otherwise.

2.2. Animal maintenance

Healthy female Balb/C mice were employed in all the experiments in the present study. The animals were procured from in-house Institute Animal House. During the experiments, the animals were fed with food and water ad libitum. All the experiments conducted were approved by Institutional Animal Ethics Committee. The toxin challenging experiments in mice were strictly done in Biosafety level 2 facility.

2.3. Construction of chimeric gene

The construction of the fusion gene, PE was performed by overlap extension PCR (OE PCR) by joining domain IV of protective antigen (PA4) and carboxy terminal region of extractable antigen 1 (EA1C) of *B. anthracis*. The DNA of *B. anthracis* was extracted by phenol-chloroform method. The individual gene fragments *pa4* and *ea1c* were amplified from the extracted DNA using the primers shown in Table 1. The PCR reactions were carried out in 20 μl reaction volume consisting of 200 μM each dNTP, 10 pM each primer [*pa4* – fPA kpn and rPA hind; *ea1c* – fEA kpn and rEA1C hind], 1 U pfu polymerase enzyme (Fermentas), 1.5 mM $MgSO_4$, 1× pfu polymerase buffer (Fermentas) and 10 ng genomic DNA. Similarly, the individual genes were amplified for OE PCR. For this, the reverse primer of *pa4* gene and forward primer of *ea1c* gene were modified to suit the overlapping of the two genes [*pa4* – fPA kpn and rPA (PAEA); *ea1c* – fEA (PAEA) and rEA1C hind]. The PCR program for both PCRs mentioned above was optimized to 30 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 60 s and extension at 72 °C for 2 min followed by final extension of 6 min. Then, the PCR products were purified and subjected to OE PCR. The OE PCR