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Identification and characterization of immunodominant B-cell epitope of the C-terminus of protective antigen of *Bacillus anthracis*

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ABSTRACT

Bacillus anthracis is the etiological agent of anthrax. Protective antigen (PA) has been established as the key protective immunogen and is the major component of anthrax vaccine. Prior studies have indicated that C-terminus host cell receptor binding region contains dominant protective epitopes of PA. In the present study, we focused our attention on determining B-cell epitopes from this region, which could be employed as a vaccine. Using B-cell epitope prediction systems, three regions were identified; ID-I: 604–622, ID-II: 626–676 and ID-III: 707–723 aa residues. These epitopes elicited potent B-cell response in BALB/c mice. ID-II in particular was found to be highly immunogenic in terms of IgG antibody titre, with a predominantly IgG1/IgG2a subclass distribution indicating Th2 bias and high affinity/avidity index. Effective cellular immunity was additionally generated which also signified its Th2 bias. Further, ID-II induced high level of lethal toxin neutralizing antibodies and robust protective immunity (66%) against *in vivo* lethal toxin challenge. Thus, ID-II can be classified as an immunodominant B-cell epitope and may prove significant in the development of an effective immunoprophylactic strategy against anthrax.

1. Introduction

Anthrax is an epizootic disease mainly affecting cattle and wild bovidae worldwide (Mock and Fouet, 2001). It is rare in humans but can occasionally be caused through contact with contaminated farm animals and animal products (Leppla et al., 2002). Of potential concern is the use of anthrax spores as biowarfare agents. Although there have been several earlier hoaxes or failed attempts, 2001 saw the first effective anthrax terror attack, infecting 11 and killing 5 people in US (Jernigan et al., 2001). Pathogenesis of *Bacillus anthracis* is mediated by two plasmids, pXO1 and pXO2, which encode for primary virulence factors—toxins and capsule, respectively (Brey, 2005). pXO1 encodes protective antigen (PA), lethal factor (LF); and edema factor (EF). They act in binary combinations (Stanley and Smith, 1961) to produce exotoxins; Lethal Toxin (LeTx) and Edema Toxin (EdTx) comprising of PA with LF and EF respectively. Prior to exotoxin formation, PA binds to cellular receptors, undergoes proteolytic cleavage and forms heptameric oligomers. The heptamer competitively binds LF and EF, which are then translocated into the cytosol. LF is a zinc dependent protease that cleaves mitogen-activated protein kinases (MAPKKs) leading to toxic shock and death (Vitale et al., 1998). EF is an adenylate cyclase converting intracellular ATP into cAMP, therefore provoking a substantial increase in intracellular cAMP levels leading to edema (Leppla, 1982). pXO2 encoded capsule enhances virulence *in vivo* by inhibiting phagocytosis of the organism (Little and Ivins, 1999).

Induction of neutralizing antibodies to PA is considered the key to protection against anthrax (McBride et al., 1998; Brossier et al., 2000). Anthrax lethality is primarily attributed to toxemia (Mock and Fouet, 2001) and PA is essential for host cell intoxication as PA contains the host cell receptor binding site (Escuyer and Collier, 1991), the cell binding component for both EF and LF (Elliott et al., 2000) and facilitates the entry of the toxin complex into the host cell (Flick-Smith et al., 2002). PA is also the dominant antigen in both natural and vaccine-induced immunity against anthrax (Flick-Smith et al., 2002). However, the extent of immune response and protection conferred by PA-based vaccines against lethal anthrax

Abbreviations: AP, alkaline phosphatase; ELISA, enzyme-linked immuno sorbent assay; FBS, fetal bovine serum; HEPES, N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) sodium salt; HRP, horse radish peroxidase; ID, immunodominant; LF, lethal factor; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PA, protective antigen; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate.

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infection in different experimental models is variable (Brahmbhatt et al., 2007).

PA is subdivided into 4 domains (Mock and Fouet, 2001). The C-terminus; domain 4 (aa 596–735) contains the host cell receptor binding region (Little et al., 1996), which has been identified as being in and near a small loop located between 679 and 693 aa residues (Varughese et al., 1999). It is essential for host cell intoxication, as previous studies have demonstrated that expressed forms of PA containing mutations (Varughese et al., 1999) or deletions (Brossier et al., 1999) in this region are nontoxic. Flick-Smith et al. (2002) showed that domain 4 contains the dominant protective epitopes of PA. Park et al. (2008) demonstrated that DNA vaccine based on domain IV linked to CRT generated potent humoral response and conferred significant protection against LeTx challenge.

Therefore, in the present study, we focused on identification of B-cell epitopes within domain 4 of PA and determination of their immunogenic potential. B-cell epitopes can be exploited in the development of epitope-based marker vaccines and diagnostic tools for various diseases (Peng et al., 2008). A number of such vaccines are currently under clinical trials against viruses (El Kasmi and Muller, 2001), bacteria (Sabhnani et al., 2003) and cancer (Kieber-Emmons et al., 1999). These epitopes are also important for allergy research and in determining cross-reactivity of IgE-type epitopes of allergens (Selo et al., 1999).

Thereby, using B-cell epitope prediction softwares, three regions spanning the C-terminus region of PA were identified. Their ability to confer protective humoral immunity against anthrax was demonstrated experimentally, both *in vitro* and *in vivo*. Thus, our results provide a rationale for the design of efficacious epitope-based vaccine against anthrax.

2. Materials and methods

2.1. Epitope prediction and synthesis of candidate peptides

Using BCPred (EL-Manzalawy et al., 2008), BcePRED (Saha and Raghava, 2004) and Emboss (http://Bioinfo.bgu.ac.il/EMBOSS); three regions (ID-I, ID-II and ID-III) spanning Domain IV of PA were selected. ID-I and ID-III were synthesized by Peptron Inc. (Daejeon, South Korea) on an automatic peptide synthesizer, PeptrEX™ and had >95% purity. Quality was evaluated by reverse-phase HPLC. Poly(DL-co-glycolide) [PLGA]-based microsphere delivery system was formulated for them employing a water-in-oil-in-water emulsion–solvent evaporation technique. This was followed by their calcium pectate gel (CPG) entrapment. ID-II was PCR-amplified using pMW1 (Chauhan et al., 2001) as the template and cloned in pET28a vector (Novagen) at BamHI and HindIII restriction sites. This construct designated as pET.ID-II was confirmed by sequencing and has been submitted to GenBank (EU828661). For purification, pET.ID-II was transformed into *Escherichia coli* BL 21 (DE3) cells and culture was induced at $\lambda_{600} \sim 0.8$, by addition of 1 mM isopropyl β-D-thiogalactoside for 4 h. ID-II protein was purified under denaturing conditions using metal-chelate affinity chromatography. Purified protein was analyzed on 15% SDS-PAGE and dialyzed against 10 mM HEPES buffer containing 10% glycerol.

PA and LF were purified as described by Chauhan et al. (2001) and Gupta et al. (1998) respectively.

2.2. Animal, immunization, sera collection

Four to six week old female BALB/c mice were procured from NIN, Hyderabad and maintained in pathogen free environment. All experiments were performed in accordance with 'Indian Animal Ethics Committee' regulations. A group of 10 mice were injected intraperitoneally with: 50 μg-peptide equivalent of ID-I and ID-III microspheres; and 50 μg protein of ID-II and PA; suspended in 100 μl of PBS and emulsified with complete Freund's adjuvant on day 0. Same dose of booster was given on days 15 and 29 with incomplete adjuvant. Blood was collected on days 0, 14, 28 and 42. The sera were separated and stored at −80 °C.

2.3. Serum antibody titers and estimation of IgG subclass

Antigen-specific IgG and isotypes (IgG1, IgG2a, IgG2b) levels in the sera were determined by ELISA. Wells were coated with respective antigens; overnight at 4 °C. After washing and blocking, serial dilutions of antisera were added in triplicates and incubated for 2 h at 37 °C. Secondary antibodies, HRP conjugated anti-mouse IgG or its isotypes (Santa-Cruz) were incubated for 1 h at 37 °C. Estimation of the enzymatic activity was carried out with TMB as the substrate. The reaction was stopped with l M H_3PO_4 and the absorbance was measured at 450 nm using Microplate Reader (BioRad).

2.4. Direct binding assay

Reactivity of each peptide antisera with PA was studied by direct binding assay. ELISA plates were coated with PA, after subsequent blocking and washing, respective antisera dilutions were incubated at 37 °C for 2 h. After three washings with PBST, HRP conjugated anti-mouse IgG was incubated for 1 h at 37 °C. Alternatively, reactivity of PA antisera with different peptides was also performed, wherein; ELISA plates were coated with peptides and incubated with PA antisera. Binding of PA with antisera raised against it was considered as the reference binding in both the assays.

2.5. Western blotting

Five micrograms of PA was resolved on 12% SDS-PAGE and electroblotted onto nitrocellulose membrane. The membrane was blocked in 5% skimmed milk powder in PBS at 4 °C overnight. After three washings with PBST, it was incubated with peptide and PA antisera for 2 h at 37 °C. After washing, bound antibodies were detected with AP conjugated goat anti-mouse IgG and NBT-BCIP substrate (Sigma).

2.6. Antigen binding characteristics

2.6.1. Affinity

The affinity of antibodies raised against different peptides was measured by estimating the disassociation constant (K_d). In brief, mice antisera were incubated with gradient of peptides (1–100 nM) for 16 h at 25 °C so as to attain antigen–antibody equilibrium. These complexes were transferred onto the wells of the microtitre plates previously coated with the respective peptide and blocked. The plates were incubated for 2 h at 37 °C. After three washings with PBST, HRP conjugated anti-mouse IgG was added and incubated for 1 h at 37 °C. Color was developed as described. K_d was calculated using the following equation derived from Scatchard and Klotz (Friguet et al., 1985):

$$\frac{A_0}{A_0 - A} = \frac{1 + K_d}{a_0}$$

wherein A_0 and A: absorbance measured for antibody in absence and presence of peptide respectively; K_d: disassociation constant; a_0: total peptide concentration.

2.6.2. Avidity

Avidity describes the collective interactions between antibodies and a multivalent antigen. For its determination, peptides were coated onto the wells of ELISA plates. After washing and blocking, peptide antiserum from each group of mice was incubated for 2 h