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A plant based protective antigen [PA(dIV)] vaccine expressed in chloroplasts demonstrates protective immunity in mice against anthrax☆

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ABSTRACT

The currently available anthrax vaccines are limited by being incompletely characterized, potentially reactogenic and have an expanded dosage schedule. Plant based vaccines offer safe alternative for vaccine production. In the present study, we expressed domain IV of *Bacillus anthracis* protective antigen gene [PA(dIV)] in *planta* (by nuclear agrobacterium and chloroplast transformation) and *E. coli* [rPA(dIV)]. The presence of transgene and the expression of PA(dIV) in *planta* was confirmed by molecular analysis. Expression levels up to 5.3% of total soluble protein (TSP) were obtained with AT rich (71.8% AT content) PA(dIV) gene in transplastomic plants while 0.8% of TSP was obtained in nuclear transformants. Further, we investigated the protective response of plant and *E. coli* derived PA(dIV) in mice by intraperitoneal (i.p.) and oral immunizations with or without adjuvant. Antibody titers of $>10^4$ were induced upon i.p. and oral immunizations with plant derived PA(dIV) and oral immunization with *E. coli* derived PA(dIV). Intraperitoneal injections with adjuvanted *E. coli* derived PA(dIV), generated highest antibody titers of $>10^5$. All the immunized groups demonstrated predominant IgG1 titers over IgG2a indicating a polarized Th2 type response. We also evaluated the mucosal antibody response in orally immunized groups. When fecal extracts were analyzed, low sIgA titer was demonstrated in adjuvanted plant and *E. coli* derived PA(dIV) groups. Further, PA(dIV) antisera enhanced *B. anthracis* spore uptake by macrophages *in vitro* and also demonstrated an anti-germinating effect suggesting a potent role at mucosal surfaces. The antibodies from various groups were efficient in neutralizing the lethal toxin *in vitro*. When mice were challenged with *B. anthracis*, mice immunized with adjuvanted plant PA(dIV) imparted 60% and 40% protection while *E. coli* derived PA(dIV) conferred 100% and 80% protection upon i.p. and oral immunizations. Thus, our study is the first attempt in highlighting the efficacy of plant expressed PA(dIV) by oral immunization in murine model.

Abbreviations: AP, alkaline phosphatase; AVA, anthrax vaccine adsorbed; BAP, benzyl amino purine; BC, before challenge titers; cfu, colony forming units; CTAB, cetyltrimethyl ammonium bromide; CT, cholera toxin; DMEM, Dulbecco's modified eagle medium; EF, edema factor; ELISA, enzyme linked immunosorbent assay; HRP, horseradish peroxidase; HEPES, *N*-(2-hydroxyethyl) piperazine-*N*′-(2 ethanesulfonic acid); i.p., intraperitoneal; IPTG, isopropyl β-D-1-thiogalactopyranoside; LeTx, lethal toxin; LF, lethal factor; MAP kinase, mitogen activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-5 diphenyltetrazolium bromide; MTD, mean time death; MWCO, molecular weight cut-off; NAA, α-naphthalene acetic acid; NBT, nitroblue tetrazolium; (NTdIV Nu), domain 4 from nuclear transformed plants; PA(dIV), domain IV of protective antigen *B. anthracis*; PAGE, poly acrylamide gel electrophoresis; PBS, phosphate buffered saline; PGA, poly-D-glutamic acid; PMSF, phenyl methyl sulfonyl fluoride; PA, protective antigen; SDS, sodium dodecyl sulphate; SM, selection medium; TSP, total soluble protein; TMB, tetramethylbenzidine; RM, regeneration medium; UTR, untranslated region; WT, wild type.

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1. Introduction

Anthrax, a zoonotic disease is caused by the gram-positive spore-forming bacterium '*Bacillus anthracis*'. Recently, anthrax has attracted considerable attention due to malevolent use of its spores as a biological weapon. The anthrax bioterror attacks in late 2001 in US *via* postal mail affected 22 people of which 5 succumbed to fatal death [1]. Anthrax is considered as one of the most resilient agents; the spores survive for decades and easily disseminate in the environment [2,3]. Anthrax spores can enter into the host through skin, inhalation or ingestion. The disease can take its worse shape leading to toxemia and death within few days of defined clinical symptoms [4].

The pathogenesis of anthrax is attributed to its 'tripartite exotoxin' comprising of protective antigen (PA), lethal factor (LF) and edema factor (EF) that exert their toxic effects in binary combinations and also the 'poly-D-glutamic acid (PGA)' component of the capsule that inhibit phagocytosis [5]. PA is the central component of anthrax toxin and as the name suggests provides protection against the disease. LF and EF are enzymatic components and form lethal toxin and edema toxin respectively, when combined with PA. The intoxication process is mediated by the binding of PA83 to the mammalian host cell receptors [6]. Upon proteolytic cleavage of 20 kDa protein at the furin protease site, the PA63 protein gets activated and oligomerises to form a heptameric prepore complex which competitively binds EF and LF. The heptameric complex is internalized by receptor mediated endocytosis. Under the influence of acidic pH in the late endosome, EF and LF are released and translocated into cytosol where they exert their toxic effects. LF, a Zn metalloprotease disrupts mitogen activated protein kinase (MAPK) signal transduction pathway leading to shock like symptoms and death [7]. EF, a calmodulin dependent adenylate cyclase augments cellular cAMP levels leading to imbalance in water homeostasis and edema [8]. In concern with the biological threat and potential lethal effects of *B. anthracis*, it becomes imperative to prevent the disease.

Vaccination is the most reliable means of prophylaxis against anthrax. All the current human anthrax vaccines have PA as the predominant or only component. The current licensed vaccines, the US based anthrax vaccine adsorbed (AVA) and the British vaccines are derived from the culture filtrates of acapsular, toxigenic strains of *B. anthracis* adsorbed to aluminum adjuvant [9,10]. Although, these vaccines proved to be protective in various animal models and humans [11–13] questions related to the characterization of vaccine components for each batch, reactogenicity, length of immunization schedule, route of administration remain a matter of concern [10]. Thus, the next generation vaccines focused on the development of subunit vaccines. The subunit vaccines since, free of pathogens are considered to be more safe and least reactogenic [14]. Since, PA is the most immunogenic component of anthrax toxin, the primary subunit vaccines focused on development of PA based vaccines.

Immunization with PA provides complete protection against anthrax spore challenge [11–13,15,16]. However, recent reports indicate that not all the antibodies generated against PA are protective. In fact, a subset of antibodies promote lethal toxin mediated killing [17]. The degree of immune response and the level of protection with recombinant PA vaccine was also not consistent in various animal models [18]. Moreover, these vaccines were prone to rapid degradation of protein thus, questioning the stability of the molecule for long term storage [19].

Studies have shown that 'Domain IV' (596–735 aa of PA) [PA(dIV)], the receptor binding element of PA, is sufficient to generate complete protection against toxin and spore challenge suggesting that dominant protective epitopes are present in this region [20,21]. Epitopes mapped to this region have shown to be protective in mice model [22,23]. Genetic deletion of PA(dIV) from *B. anthracis* genome showed a drastic decline in the virulence [24]. Also, monoclonal antibodies against this molecule are efficient in blocking PA from binding to the receptor [20,25]. Therefore, PA(dIV) can be used in effective vaccination against anthrax.

In order to overcome the problems associated with the stability and safety, vaccine antigens can be successfully expressed in plant system. Plant based vaccines are advantageous in being cost effective, provide easy scale up and are devoid of bacterial contaminants. Several studies have demonstrated the feasibility of expression of vaccine candidates in plant system. A plant produced poultry vaccine against Newcastle disease virus has already been commercialized (www.dowagrosciences.com) and many antigens expressed in plants, e.g.: HBSAg, Norwalk virus capsid protein, *E. coli* heat labile toxin, rabies glycoprotein have shown to be protective in clinical trials [26,27].

Most of the current approaches used in generating plant based vaccines relied on agrobacterium mediated transformation, that target the genes to nucleus. The impinging problems of nuclear transformation associated with low expression levels, position effects due to random gene integration, and safety due to environmental dissemination of genes by pollen has hampered its expediency for commercialization [26,28]. Chloroplast transformation technology has offered immense relief in this regard by generating enormous expression levels [29]. Chloroplast transcription machinery is akin to the prokaryotic system. Therefore, multiple genes can be processed thus, facilitating the expression of polyvalent vaccines. Other significant attributes include targeted gene integration that precludes position effects and the containment of transgenes due to maternal inheritance [30]. More importantly, the proteins expressed in plastids were found to be stable in the gut and were efficiently transported to the circulatory system [31]. Previous studies have demonstrated successful expression of antigens against cholera [32], tetanus [33], anthrax [34,35], plague [36], ameobiasis [30], canine parvovirus [37], HIV [38] and malaria [39] in chloroplasts. These studies have opened new avenues for development of effective plant based vaccines. The present study explores the feasibility of PA(dIV) expression in plant system by nuclear and chloroplast transformation. The study also compares the protective efficacy of plant expressed PA(dIV) with that of recombinant domain IV i.e.: rPA(dIV) derived from expression in *E. coli*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

E. coli strains, DH5α and XL Blue were used for the maintenance and propagation of plasmids. Luria Bertani (LB) + ampicillin (100 mg/l) or kanamycin (50 mg/l) or spectinomycin (100 mg/l) was used to grow bacteria depending on the antibiotic resistance gene in the constructs. Agrobacterium strain GV2260 (procured from lab stock) was cultured in yeast extract medium (YEM) supplemented with kanamycin (50 mg/l) or rifampicin (10 mg/l). All the room temperature chemicals and plant tissue culture media components and hormones were procured from Sigma–Aldrich (USA). Murashige and Skoog (MS) media was purchased from Himedia Laboratories (India).

2.2. Cloning of PA(dIV) gene in pCAMBIA vector

pCAMBIA-1303 vector purchased from Cambia (Australia) was used for cloning PA(dIV) gene. The vector is marked by a constitutive CaMv 35S promoter, *npt*II (neomycin phosphotransferase) gene for bacterial selection, *hpt*II (hygromycin phosphotransferase) gene