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Immunogenic properties of plant-derived recombinant smallpox vaccine candidate pB5

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ABSTRACT

The extracellular virion membrane protein B5 is a potent inducer of immune responses capable of protecting mice and primates against poxvirus infections. Here, we examined the antibody response induced in mice immunized intramuscularly (i.m.) or intranasally (i.n.) with plant-derived B5 (pB5) accompanied or not with plant total soluble protein (TSP) at various concentrations. Increasing amounts of TSP inhibited the pB5-specific response in both i.m.- and i.n.-immunized mice, with more dramatic effects in the latter. pB5 administered to mucosal surfaces induced specific IgG and IgA responses, whereas i.m. immunization produced high serum IgG titers and no IgA. A 6-fold increase in pB5 dosage administered i.n. led to an antibody response comparable to that obtained by i.m. injection. Our study addresses the quality/quantity issues of the pB5 subunit preparation and demonstrates the feasibility of mucosal administration of plant-derived smallpox subunit vaccine in obtaining a potent immune response. Overall, this work points to the practicability of needle-free mucosal administration of such vaccines in light of purity, dosage and adjuvant formulation.

1. Introduction

Advances in plant-based pharmaceutical production have led to significant improvements in the high-yield accumulation of functionally potent recombinant proteins [1–5]. Plant-derived vaccine candidates are currently moving from the lab bench to clinical trials and/or animal use [3–5]. These subunit vaccines are capable of inducing strong systemic and/or mucosal immunity against various pathogens [6–13].

Several candidate antigens can protect against smallpox disease [14,15]. The extracellular virus-specific membrane glycoprotein B5 is highly immunogenic and protects mice and primates against poxvirus infections [16–18]. Preparations of purified pB5 administered parenterally or i.n. induce a strong immune response and mice vaccinated i.m. with pB5 were protected from vaccinia virus challenge [7].

Plant-based production systems have obvious advantages over conventional expression systems, e.g., the absence of human pathogens, ease of scale-up and reduced production costs. Nonetheless, some plant cell components may interfere with the performance of recombinant products. One possible hurdle might rest in the differences in glycosylation patterns between plants and mammals [19,20], which could affect immune responses, stimulate allergic reactions, enhance clearance of pharmaceuticals, or be inert [21,22]. Another potential problem comes from plant-specific soluble proteins such as Rubisco, which account for >50% of total soluble proteins (TSP) and likely contaminates any recombinant preparation.

Here, we analyzed the impact of purity of plant-based pB5 smallpox subunit vaccine preparations, administered by different routes and accompanied or not with TSP contamination, on specific antibody responses in mice. Our results indicate a direct correlation between immune response and antigen purity, dosage and mode of application.

2. Materials and methods

2.1. Plant transformation

Six- to eight-week-old *Nicotiana benthamiana* (Tobacco) plants were used for production and purification of B5 antigen as described [7] using the “Magnifection” procedure [23,24]. Briefly, an expression cassette harboring the B5 coding region of the vaccinia virus antigenic domain was transformed into Agrobacterium strain GV3101 and used for transient plant transformation. Plant tissues were harvested 7–10 days post-infection, lyophilized and

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stored under vacuum at ambient temperatures until used for purification of pB5.

2.2. Plant protein preparations

The soluble form of pB5 protein was extracted and purified (>50%) from plant tissues essentially as described [7]. TSP from wild-type tobacco plants were extracted from lyophilized plant leaves (10 g) by grinding in mortars together with dry ice and 50 ml of frozen buffer (50 mM sodium phosphate, pH 8.0, 0.3 M NaCl, 0.1% Tween 20, 5 mM β-mercaptoethanol). After centrifugation at 12,000 × *g* for 20 min at 4 °C, supernatants were filtered through Miracloth tissue and dialyzed against PBS (pH 7.4) with 0.05% Tween-20. Samples were standardized to 1 mg/ml, aliquoted, frozen in liquid N_2 and stored at −80 °C. All protein extracts were analyzed by SDS-PAGE.

2.3. Immunization and sample collection

Six- to eight-week-old female BALB/c mice, 5 per group, total of ten groups, were used in all experiments. For i.m. immunization, mice were injected in the hind leg muscles with 1 μg of purified pB5 along with CpG-ODN (sequence no. 1826 [25], TCCATGACGTTCCTGACGTT; Operon Biotechnologies, Huntsville, AL) and alum 1.3% Alhydrogel (Accurate Chemical, Westbury, NY) as adjuvant (CpG/alum) at 50 μg each per mouse. For i.n. immunization mice were anesthetized with isoflurane, 1 μg of pB5 was administered with 1 μg of cholera toxin (CT) (MD Biosciences, San Diego, CA) as adjuvant at 15 μl per nostril. In some groups, antigen was supplemented with 20, 10 or 2 μg of TSP for i.n.-immunized mice, and with 50, 30, 10 and 2 μg for i.m. administration. Three additional groups of mice were immunized i.n. with 0.2, 1, and 6 μg of purified pB5 and 1 μg of CT.

All mice were immunized three times at 2-week intervals. Blood and stool samples were collected 10 days after each immunization. Mice were sacrificed 10 days after the last immunization.

2.3.1. Sera

Serum was collected through the orbital sinus of anesthetized animals. At time of sacrifice, mice were sedated with pentobarbital and serum was collected by cardiac puncture. Sera were stored at −20 °C until analysis by ELISA and Western blotting.

2.3.2. Broncheo-alveolar lavage (BAL)

After exsanguination, BAL fluid was collected as described [26] with minor modifications. Briefly, each animal was incised ventrally along the midline and trachea exposed. A 21-g blunt needle hub was introduced into the trachea and 0.6 ml of 1N NaCl injected into the lungs. After 1 min, the fluid was aspirated and frozen at −20 °C. Prior to analysis, samples were centrifuged and cleared of cell debris. Total protein content was quantified using Nanodrop measurements at 280 nm (Thermo Fisher Scientific, Wilmington, DE). Total protein concentration in BAL was used for sample standardization.

2.3.3. Stool extracts

Fresh fecal pellets were collected and extracted in PBS (10 vol/wt) supplemented with 1% BSA and protease inhibitors as described [27]. Solids were removed by centrifugation and supernatants frozen at −20 °C until analysis.

All animal experiments were conducted in accordance with the institutional guidelines for animal welfare.

2.4. Immunological analyses

ELISA was performed as described [7] on 96-well MaxiSorp plates (Nalgen Nunc, Rochester, NY) coated overnight with one of the following antigens: 1 μg/ml of B5 purified from *E. coli* or derived from baculovirus (BEI Resources, Manassas, VA); 10 μg/ml of TSP; or 1 μg/ml of CT in PBS. Sera were tittered as 4-fold dilutions. Antibody subclasses were quantified with goat anti-mouse IgG (BD Biosciences, San Diego, CA), goat anti-mouse IgA (Sigma, St. Louis, MO), rat anti-mouse IgG1 (BD Biosciences), rat anti-mouse IgG2a (BD Biosciences) or goat anti-mouse IgE (Immunology Consultants Laboratory, Inc., Newberg, OR). Antibody titers are shown as optical density at 450 nm in serial dilutions. Results are expressed as the mean ± S.D. for groups of five mice. Differences were evaluated using Student's *t*-test and considered significant at $p < 0.05$. Percent response was calculated as log 2 of the reciprocal of dilutions where titer was 0.1 units above background.

For Western analysis, purified pB5 samples were electrophoresed on 12% SDS-polyacrylamide gels loaded at 0.25, 1.25 and 2.5 μg per lane, while TSP extracts were loaded at 2.5, 5 and 10 μg per lane (Supplementary Fig. S1A); blots on PVDF membranes were blocked with Iblock (Tropix, Bedford, MA) in PBST and probed with c-myc mAb (Supplementary Fig. S1B) as in [7] or the corresponding mouse sera (see Fig. 1, right) at a dilution of 1:1000 followed by goat anti-mouse horseradish peroxidase-conjugate (Upstate, Temecula, CA) at 1:10,000 dilutions. Immunoreactive bands were visualized by chemiluminescent substrate (Pierce, Rockford, IL).

3. Results

3.1. IgG response depends on pB5 purity and administration route

Purified pB5 elicited a specific and strong serum IgG, especially when administered i.m. (Fig. 1A), consistent with our previous observations [7]. The response after i.n. immunization at the same dose of pB5 (1 μg) was lower (Fig. 1B, left). When administered at 6 μg, pB5 induced IgG titers comparable to those obtained by i.m. immunization with 1 μg of pB5 (Fig. 1C, left). Western analysis of mouse sera with the highest IgG titers (Fig. 1C, right) revealed no plant-specific IgG accompanying the pB5-specific response after i.n. immunization.

Addition of plant TSP at increasing concentrations to the i.m. immunizations with pB5 at 1 μg had no effect on pB5-specific IgG titers at TSP doses of 2 and 10 μg, whereas addition of 30 and 50 μg TSP led to a ∼25% decline in titers (Fig. 1A, left). The titers of pB5-specific IgG after i.n. immunization were negatively affected by TSP at 20 μg (Fig. 1B, left). Western analysis (Fig. 1A, B right) of representative serum samples obtained after i.m. immunization (indicated by asterisk) confirmed the presence of antigen-specific antibodies as well as antibodies against some plant proteins.

3.2. IgA response detected after i.n. immunization with pB5

Titers of B5-specific IgA in BAL after i.n. immunization were more sensitive to the presence of TSP than IgG titers in serum (compare Fig. 2A with Fig. 1B, left). While B5-specific antibody levels in the respiratory tract were not affected by addition of TSP at 2 μg, addition of 10 and 20 μg TSP led to a 50–60% decline in IgA as compared to levels after immunization with pB5 alone ($p < 0.005$); note that i.m. immunization with pB5 did not induce IgA responses in the respiratory tract (Fig. 2A, i.m. group). When 6 μg of pB5 was administered i.n., the absolute amounts of IgA