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Transdermal immunization with low-pressure-gene-gun mediated chitosan-based DNA vaccines against Japanese encephalitis virus

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ABSTRACT

DNA vaccine is a milestone in contemporary vaccine development. It has considerably offset many shortcomings in conventional vaccines. Although DNA vaccines applied through 'traditional' high-pressure gene guns generally elicit high titers of protective immunity, such a practice however requires enormous investment in daunting instruments that often discourage vaccines due to an inevitable pain-eliciting effect. In this study, we exploited a less expensive yet low-pressure-gene-gun that can alleviate such phobia of pain. DNA vaccines were prepared by using the associative feature of cationic chitosan and anionic DNAs. The optimized *N*/*P* ratio is 3. The formulized complex sizes to nano-scale. The vaccine complexes were tested in C3H/HeN mice. The expression of GFP reporter gene was observable and traceable in epidermis and spleen over 3 days. The expressions of GFP and the activation of dendritic cells (DCs) were evident and co-localized in hair follicles and epidermis. C3H/HeN mice immunized with the developed chitosan-JEV DNA vaccines can elicit desired JEV specific antibodies, whereby the mice maintained high survival rates against $50 \times LD_{50}$ JEV challenge. The low-pressure-gene-gun mediated chitosan-based JEV DNA vaccines have proven to be convenient and efficacious, thereby with high capacity in deployment for future prophylaxis against JEV outbreaks.

1. Introduction

The developmental trends of contemporary vaccines invariably demand higher and long-lasting efficacy but as few as any adverse effect. DNA vaccine stands just as one of latest vaccine advances that utilizes bare DNAs rather than proteins as the direct source for antigenicity to elicit given somatic responses for desirable immunity [1,2]. DNA vaccination exceptionally becomes the most promising means other than non-virus immunization [3–5], which supersedes other conventional vaccines. This success may be attributed to such features as: (i) higher stability of plasmid DNA, (ii) lower costs of preparation, (iii) low risk of viral infection, (iv) high capacity for multiantigen, and (v) elicibility for humoral and cellular duo immune responses [6]. Vaccination with antigen-encoding plasmid DNAs has also been exemplified in such cases as influenza virus [2], rabies virus [7], malaria parasites [8] as well as *Mycobacterium tuberculosis* [9], wherein a broad range of immune responses is inducible, including humoral and cell-mediated immunities, capable of counteracting given antigen-bearing pathogens.

DNA vaccines are administered most commonly by either muscle injection or particle-bombardment (gene gun) dermal injection [10]. The former usually requires needle-mediated injections by medical personnel. However, needle-free delivery as a trend has loomed because needle-borne diseases are very often associated with the re-use and improper disposal of needles [11]. The gene gun-mediated transdermal delivery of DNA vaccine has been shown promising, because the skin is known to be a potent immunological site where it is fraught with antigen presenting cells (Langerhans cells) [12]. In general, DNAs are coated onto gold particles that are bombarded into cytoplasm and/or nuclei of cells to facilitate the protein expression of the corresponding gene. The DNA-coated gold particles usually are jetted by high-pressure helium, penetrating the stratum corneum of the epidermis [13]. The high-pressure gene gun (200–500 Psi) however inherits some uncorrected drawbacks such as expensive to operate and requiring skillful operators. By contrast, the low-pressure (30–80 Psi) gene gun was developed with features of low noise and less cell damage

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[13,14]. The most negative disadvantage has been that the gold particle is undegradable and difficult to excrete, which may result in unforeseen adverse effects when accumulated over time in body [14]. Biodegradable or bioabsorbable polymeric nanoparticles are thus much preferred and have been widely investigated to serve as the DNA carrier [15]. Chitosan, a non-toxic and biodegradable cationic polysaccharide that is comprised of primarily D-glucosamine repeating units, is a tempting material [16]. A collection of chitosan polymers and/or oligomers has been demonstrated to be able to efficiently condense plasmid DNAs in serum as salt-stable particles as well as in several distinct cell types or tissues or organs [17–20].

Japanese encephalitis virus (JEV) belongs to flavivirus family that causes serious encephalitis resulting in considerable mortality in southern and eastern Asia annually [21]. The currently used inactivated [22] and live-attenuated [23] JEV vaccines have been known bearing some unavoidable jeopardy that has become less and less welcome [21,24]. Our previous reports have demonstrated that the plasmid (pCJ-3/ME) encoding the membrane (M) and envelope (E) proteins of JEV are able to elicit desired protective immunity [25]. While, the feasibility of using chitosan nanoparticles as a JEV DNA vaccine carrier for transdermal vaccination in conjunction with a low-pressure gene gun has never been reported. In this study, we focused on developing chitosan nanoparticle JEV DNA vaccine on the bases of the known M- and E-coding genes. The constructed plasmid DNAs were first encapsulated into chitosan nanoparticles. The physical/chemical properties of the chitosan/DNA complexes were then characterized by photon correlation spectroscopy (PCS) and scanning electron microscopy (SEM). The chitosan/DNA complexed nanoparticles were delivered into skin by using a low-pressure gene gun. The resulted efficacy and protectiveness for the provoked immunological responses in a mouse model were evaluated.

2. Materials and methods

2.1. Plasmid DNA

pGFP-N1 and pCMVβ vectors (Clontech, Palo Alto, CA) encode a green fluorescent protein (GFP) and β-galactosidase genes that all are driven by a cytomegalovirus promoter. pCJ-3/ME that was constructed and characterized to be an effective JEV DNA vaccine has been reported previously [25]. A commercial kit (Qiagen, Hilden, Germany) was used to purify the above vectors that were multiplied in *Escherchia coli* DH5α; procedures followed were according to the manufacturers' instruction.

2.2. Preparation of chitosan nanoparticles and chitosan/DNA complex

Chitosan (deacetylation degree was 85%, 800 kDa) from crab shells was purchased from Sigma (St. Louis, MO). Briefly, 1% (w/v) chitosan was dissolved in an acetic acid solution (167 mM) under gentle heating to form a chitosan stock solution; the pH of the stock solution was adjusted to 5.5. The chitosan nanoparticles were prepared by ionic gelation. In brief, the chitosan solution (20 ml) was gently added into an 8 ml of tripolyphosphate (TPP) solution (0.84 mg/ml). This solution immediately was subjected to ultrasonication (29 W, 4 min) at room temperature, and followed by centrifugation at 12,000*g* for 45 min to remove pelleted particles [26]. The clear supernatant contained chitosan nanoparticles. The chitosan/DNA complexes were prepared according to the method reported by Sato et al. [27]. The chitosan/DNA complexes were obtained by adding a 10 μl of plasmid DNA solution (1 mg/ml) into varying amounts of the prepared chitosan solution. These mixtures were vortexed at maximum speed 20 s at room temperature. The ratios of the chitosan's amino group (N) to the plasmid's phosphate anion (P) were in the range of 0.25–5.

2.3. Physical property characterization for the chitosan/DNA complex

Particle size was measured by photon correlation spectroscopy (PCS; Malvern 4700 (Malvern instrument, U.K.)) with scattering light at 90° in 120 s of duration at 25 °C (sample volume: 3 ml). The zeta potentials of the particles were measured by Zetasizer 3000 (Malvern Instruments, Southborough, MA). The size and morphology of chitosan nanoparticles or chitosan/DNA complexes were observed using scanning electron microscopy (SEM) (Hitachi S-4800, Japan). SEM samples were prepared by placing a drop of the prepared particle suspension on a carbon conductive tape (Ted Pella Inc., Redding, CA, USA). The grids were allowed to dry for another 1 day and then were examined under the electron microscope.

2.4. Viruses and animals

Female C3H/HeN mice were purchased from the National Laboratory Animal Breeding and Research Center, Taipei, Taiwan; they were housed at the Laboratory Animal Facility, College of Life Sciences, National Taiwan Ocean University, Keelung, Taiwan. The Beijing-1 strain JEV was maintained in suckling mouse brains for the preparation of virus stocks and the experiments of lethal dose challenging. The immunized C3H/HeN mice were treated sequentially with an intraperitoneal injection of JEV with a dose of 50 times the LD_{50} and an intracerebral injection of PBS. The symptoms of the test animals were recorded every day for 15 days.

2.5. Transdermal immunization using a low-pressure gene gun & antibody assays

6-week-old female C3H/HeN mice were anesthetized using acepromazine maleate (i.p.) and the hairs covering the areas of abdomen skin were removed with a shaver. Residual hairs were removed by hair-remove-cream (Yanagiya, Japan). The shaved mice were then washed to remove stratum corneum for subsequent topical administration. Chitosan/DNA complex (10 μg DNAs) or chitosan/DNA complex in combination with 10 μl ImmunEasy™ mouse adjuvants (CpG adjuvants) (Qiagen, Hilden, Germany) in sterile DI water (200 μl) was loaded in a low-pressure gene gun (Sondlin Technology Co., Taipei, Taiwan). The low-pressure gene gun injection was triggered with the nitrogen pressure of 60 Psi onto abdominal epidermis three times at 2-week intervals. For the challenge experiments, the immunized mice were challenged with the 50 times LD_{50} dose of JEV at the second week after the third immunization (week 6). Serum samples were collected by tail bleeding every other week (at the 0, 2nd and 4th week) before each immunization. The samples were analyzed by ELISA using anti-E antibodies by the methods described previously [10,28]. Briefly, serum samples were added into microtiter plates coated with live JEV virions that were produced in Vero cell cultures. The bound antibodies were detected by using horseradish peroxidase-conjugated goat anti-mouse IgG Fc (1:1000; Chemicon, Temecula, CA) and *o*-phenylenediamine dihydrochloride (OPD) (Sigma, St. Louis, MO). Absorbance readouts were recorded at 405 nm by an ELISA reader. These readouts were referenced to a standard serum curve, and the results were expressed by arbitrary units per milliliter (U/ml; 1U = 50% maximal optical density); 1 U/ml is roughly equal to 22 ng/ml of anti-E antibody.

2.6. Immunofluorescence staining and dot-blot assay for the gene expression

After 72 h post transdermal delivery by the low-pressure gene gun, mice were sacrificed. The treated skins were dissected out and immediately frozen (−20 °C) in 1.5-ml conical screw cap microtubes (Quality Scientific Plastics). The skin tissues treated with the pGFP/N1 plasmids (10 μg) alone or the chitosan/pGFP/N1 complexes were sequentially embedded in O.C.T. Embedding Medium (Sakura Finetek, CA, USA) was sectioned into 10 μm thickness and stained with an immunofluorescence staining solution. These section slides were fixed with a 3.7% paraformaldehyde solution, and were washed three times with PBS and then permeabilized with a 0.2% Triton X-100 solution for 15 min at 37 °C. This washing procedure was repeated once. The skin tissues were then blocked with a 5% normal bovine serum PBS buffer solution for 60 min at 37 °C. The skin tissues then were treated with PE-anti-mouse CD11c mAb (BioLegend, San Diego, CA) in 1:50 dilution for 60 min at 37 °C. After washing three times with PBS, the stained skin tissues were observed under fluorescence microscopy (BX-51, Olympus, Japan) that was equipped with a digital camera. For dot-blot assays, the skin tissues were grounded with mortar and pestle in liquid nitrogen, and followed by adding 500 μl of a Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris, pH 8.0, and protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany)). After 10 min centrifugation at 4 °C at 12,000 rpm, the amount of total proteins from the supernatant was measured by BCA protein assay (Pierce, USA). Proteins in given amounts (25 μg) were applied to the nitrocellulose membrane with vacuum. The membrane was reacted for 2 h at room temperature with a blocking buffer (5% skimmed milk, 150 mM NaCl, 50 mM Tris, pH 8.0), and then incubated for 1 h at room temperature in PBS containing 1% BSA (PBSB) containing the E3 monoclonal antibody (1 μg/ml) [29]. After 4 × 5 min washing with a washing buffer solution (0.05% Tween-20, 1% skimmed milk, 150 mM Tris, pH 8.0), the membrane was incubated for 1 h in PBSB containing the horseradish peroxidase-conjugated goat anti-mouse IgG Fc (1:1000; Cappel, Organon Teknika, Veedijk, Belgium). After 6 × 5 min washing, the blots were developed using an enhanced chemiluminescence Western blot detection system (Amersham, Little Chalfont, UK) and exposed to X-ray film. The data were analyzed by the UVP VisionWorks Software (5.5.3) (UVP, CA, USA).

2.7. FDG^+ cells migration assay

Mice of 6–8 weeks old were treated with a single transdermal immunization by the low-pressure gene gun with pCMV-β. The cells of lymph nodes and spleens were isolated at various time points (0, 24, 48, 72, 96 h) after immunization. These cell