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Humoral immune response after genetic immunization is consistently improved by electroporation

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ABSTRACT

Aiming to evaluate some parameters to influence the immune response to DNA vaccination, we compare three protocols of DNA immunization (i.m. injections, i.m. injections followed by electroporation, and the effect of i.p. injection of stably antigen-transfected cells before DNA administration), using three different antigens. Statistical analyses showed that electroporation after intramuscular injections provided an immune response comparable to that obtained by pre-treatment with antigen-transfected cells and similar to that obtained by protein immunization. The results allowed us selecting a protocol that worked well for all three antigens and reinforced the idea that high level of gene expression is essential to get good immunization.

1. Introduction

After immunization, it is expected an immune response able to specifically identify the antigen and eventually to neutralize it. However, it is not unusual that the reactivity of the products of the immune response to the native antigens come upon trouble after protein immunization. The main concerns at this point are the presence of contaminants and the alteration of antigen conformation during the processes of purification and immunization [1–3]. Vaccination with DNA may bypass some of those difficulties because the protein antigen is produced by the host organism and, in addition, is an alternative in those cases that the obtainment of antigen represents an obstacle.

For gene immunization, DNA encoding a desired antigen is inserted into eukaryotic plasmid expression vector and the purified plasmid DNA is inoculated directly into the host to transfect cells. The immunizing protein is produced in transfected cells *in vivo* under the control of the plasmid expression vector promoter [4,5], utilizing the host cells' transcriptional machinery. Consequently, an immune response is elicited by the protein produced with the appropriate post-translational modifications and forming suitable tertiary or quaternary structure. Thus, unlike immunization with proteins, the intracellular synthesis of antigen in host cells results in its native conformation, favoring the production of specific antibodies. For generation of antibodies against either highly hydrophobic proteins, like membrane-bound receptors, or difficult to obtain in large scale from the source, such as growth factors, the idea of using DNA for immunization is much more favorable to reach success than the use of protein.

Thus, genetic immunization represents a novel means to stimulate immune response [6] and has provided effective protective immunity in various animal models [1,7,8]. Furthermore, gene delivery by plasmid DNA injection is safer, cheaper and usually easier to be prepared.

However, the magnitude of the immune responses induced by DNA immunization varies to a great extent. Kasinrerk et al. [9] showed that multiple intramuscular (i.m.) DNA immunizations were necessary to elicit specific antibodies against different leukocyte surface antigens, which is according to that described by others [7,10–14]. One injection with either protein or protein-producing cells has been described as necessary when the goal was the production of monoclonal antibodies [10–12]. The variable levels of gene expression after gene administration have been pointed out as an important limiting factor of the genetic immunization approach.

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Table 1
Protocols of immunization

Protocol	Time (days)				
	0	14	21	35	42
I	i.m.	i.m.		i.m.	
II	i.m. + ep	i.m. + ep		i.m. + ep	
III	i.p.		i.m. + ep		i.m. + ep

i.m., Intramuscular (DNA 100 μg/100 μl), i.m. + ep, intramuscular and electraporation (DNA 50 μg/50 μl), i.p., intraperitoneal (5×10^6 cells/500 μl).

The electroporation after gene injection has been used to improve the DNA transfection, working as an effective adjuvant resource [15,16].

Considering the potential of the DNA vaccine technology, it is important to establish the immunization conditions. Doses, injection route, number of immunizations, among other factors greatly affect the efficacy of DNA vaccines. Aiming to analyze the ability of some parameters to influence the immune response to DNA immunization, in the present study, we compare three commonly used protocols of DNA immunization, using three different antigens: two of them highly conservative, human vascular endothelial growth factor (hVEGF) and human fibroblast growth factor (hFGF), and one from phylogenetically distant origin, Kunitz-type serine protease inhibitor from *Bauhinia bauhinioides* (BbKi) [17].

2. Material and methods

2.1. Animals

Eight-week-old female Balb/c mice were immunized according to the protocols described in Table 1 or as otherwise specified. Blood samples were collected from immunized mice by tail bleeding 7–10 days after each immunization. Sera were separated and stored at −20 °C. Always when necessary, mice were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg). All animals were purchased from the Center of Development of Experimental Models for Medicine and Biology – CEDEME – of Federal University of São Paulo (UNIFESP) and maintained in our animal facilities. All animal experiments were done in compliance with the NIH-Guidelines for the care and use of laboratory animals, and approved by the Animal Ethics Committee of the UNIFESP (CEP 181/06).

2.2. Antigens and vectors

The plasmid employed along the experiments for *in vivo* immunization was the uP expression vector, elaborated for *in vivo* transfection, as described recently [18]. The uP vector was constructed by insertion of a sequence of DNA containing CMV intron 1 with splicing signals between the CMV promoter and polycloning sites of the pVAX (Invitrogen). Therefore, all features of the pVAX vector were maintained including the cloning sites.

Three antigen genes were used: hVEGF165 (human vascular endothelial growth factor 165), hFGF-2 (human fibroblast growth factor 2) and BbKi (Kunitz-type serine protease inhibitor from *B. bauhinioides*) [17]. cDNA of those genes were inserted between Eco RI and Eco RV sites of uP expression vector. The vector was produced in large scale, purified by chromatography (Qiagen Gigaprep, São Paulo, Brazil) and dissolved in sterile phosphate-buffered saline (PBS, 150 mM NaCl and 10 mM phosphate, pH 7.4) then used as DNA vaccine.

For transformation of A293T cells with the hVEGF165, hFGF-2 and BbKi genes, the pcDNA3 expression vector (Invitrogen) was opened at the Eco RI and Eco RV sites, which are located in the cloning sites, and those cDNA were inserted in the vector after digestion with the same enzymes. For transfection, calcium phosphate method was used [19].

2.3. Intradermic immunization (i.d.)

Female Balb/c mice were immunized three times with 50 μg of uP-hVEGF in 50 μl of PBS. The immunogen was intradermally injected into shaved thigh of anesthetized mice, using an insulin syringe with an attached 28-gauge needle.

2.4. Intramuscular immunization (i.m.)

Mice were anesthetized as described above. Using a 1 ml insulin syringe, 50 μg of plasmid DNA in 50 μl of PBS were delivered into each of the anterior tibialis muscle (100 μg of DNA per mouse).

2.5. Intramuscular immunization plus electroporation (i.m. + E)

The intramuscular injection was performed as described above, except that it was made just at one hind leg and 50 μg of plasmid were injected per mouse. Soon after the DNA injection, electroporation was performed using needle electrode of 0.5 cm needles of 0.5 mm thickness and 5 mm distance between them. Three electric pulses (field strength = 100 V/cm; pulse length = 50 ms; ECM 830 field generator, BTX Division, Genetronix, San Diego, CA, USA) were delivered at 1 s interval.

2.6. Injection of transformed cells

A293T cells were transfected with pcDNA3/hVEGF, pcDNA3/hFGF2 or pcDNA3/BbKi by calcium phosphate method [19]. The transfected cells were selected with G418 (1 mg/ml). Five millions of stably antigen-transfected A293T cells were resuspended in 500 μl of PBS and administered to each animal by intraperitoneal injection. Protein production was evaluated in the supernatant of cell cultures by ELISA.

2.7. Vaccination

The immunization protocols I, II and III were performed as indicated in Table 1. To monitor the humoral immune responses, anti-hVEGF, anti-hFGF-2 and anti-BbKI antibodies were detected by enzyme immunoassay (ELISA) from blood samples. For this, 100 μl of a solution containing 0.5 μg ml^{-1} (w/v) of antigen {hVEGF165 (PeproTech Mexico, Ver., Mexico), hFGF-2 [20] or BbKi [17]} in PBS were applied to each well to coat 96-well polyvinyl plates (Corning, USA). Blocking of the remaining active sites on plastic was made with 1% (w/v) bovine serum albumin (BSA, Sigma) in PBS (PBS/BSA). Sera from immunized mice were diluted in 0.1% (w/v) BSA/PBS, added to the coated wells and incubated for 4 h at 4 °C. After three washes with PBS containing 0.05% (v/v) Tween 20 (PBS-T), plates were incubated for 1 h at 4 °C with biotin-conjugated goat anti-mouse IgG (Bio-Rad) diluted 1:5000 in 0.1% BSA/PBS.

After washing, horseradish peroxidase–streptavidin (1:1000) (Bio-Rad) was added to the plates and incubated for 30 min at room temperature. After thorough washing, the reactions were developed with *ortho*-phenylenediamine (3 mg ml^{-1}) in 0.1 M acetate buffer, pH 5.8, containing 0.03% H_2O_2, and interrupted with 4N H_2SO_4. The absorbance at 492 nm was determined in an ELISA reader (EL 808 Ultra Microplate Reader, Bio-Tek Instruments, Inc., Winooski, VT, USA). Pre-immune mouse serum was used as negative control. Each sample was analyzed in triplicate.