Results in Immunology 4 (2014) 46–53

Contents lists available at ScienceDirect

Results in Immunology

journal homepage: www.elsevier.com/locate/rinim

DNA-encapsulated magnesium phosphate nanoparticles elicit both humoral and cellular immune responses in mice

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ARTICLE INFO

Article history:
Received 5 November 2013
Received in revised form
7 April 2014
Accepted 14 April 2014
Available online 18 April 2014

Keywords:
Inorganic phosphate nanoparticles
Microemulsion
pEGFP
Vaccine
Antigen-presenting cells

ABSTRACT

The efficacy of pEGFP (plasmid expressing enhanced green fluorescent protein)-encapsulated PEGylated (meaning polyethylene glycol coated) magnesium phosphate nanoparticles (referred to as MgPi-pEGFP nanoparticles) for the induction of immune responses was investigated in a mouse model. MgPi-pEGFP nanoparticles induced enhanced serum antibody and antigen-specific T-lymphocyte responses, as well as increased IFN-γ and IL-12 levels compared to naked pEGFP when administered *via* intravenous, intraperitoneal or intramuscular routes. A significant macrophage response, both in size and activity, was also observed when mice were immunized with the nanoparticle formulation. The response was highly specific for the antigen, as the increase in interaction between macrophages and lymphocytes as well as lymphocyte proliferation took place only when they were re-stimulated with recombinant green fluorescence protein (rGFP). Thus the nanoparticle formulation elicited both humoral as well as cellular responses. Cytokine profiling revealed the induction of Th-1 type responses. The results suggest DNA-encapsulated magnesium phosphate (MgPi) nanoparticles may constitute a safer, more stable and cost-efficient DNA vaccine formulation.

1. Introduction

DNA vaccines are promising vehicles for immunization against a variety of human pathogens, including HIV [1], *Mycobacterium tuberculosis* [2] and malarial parasites [3]. Such immunization with DNA can elicit both cellular and humoral immune responses [4,5], and can be administered repeatedly without inducing any anti-vector immunity. Other benefits of a DNA based vaccine include its ability to polarize T-cells, especially to a Th1 immunological response. DNA vaccine formulations are generally more stable and possess longer shelf-life, which in turn facilitates their cheaper manufacturing, storage, and shipping compared to that of protein-based vaccines. Nonetheless, the immunogenicity of DNA vaccines has been limited by several problems associated with their delivery, such as poor cellular uptake of DNA, degradation of the DNA by DNases and lysosomes, and transient DNA expression. A number of strategies have been used to improve their potency, including, electroporation, infusion, sonication and the gene gun [6,7]. Microparticles and nanoparticles that have been exploited as carriers for such DNAs include polylactidecoglycolide (PLGA) [8,9], alginate microparticles [10], chitosan nanoparticles [11,12], liposomes [13,14], and virosomes [15]. These methods are, however, not acceptable in practice because of a number of crucial limitations, including the requirement for large amounts of DNA, as well as their low expression levels and cytotoxicity. As a result, current non-viral genetic vaccine systems do not efficiently activate antigen-presenting cells (APCs) [16], and so lack the equivalent potency of viral vectors.

It has been suggested that the use of inorganic nanoparticles, such as phosphates of Ca^{2+}, Mg^{2+}, Mn^{2+}, Ba^{2+}, Sr^{2+}, might eliminate these limitations, yet they remain largely unexplored. Bulk-precipitated complexes using these ions have been shown to stimulate varying degrees of DNA transfer efficiency across the cell membrane [17]. Calcium phosphate (CaPi) nanoparticles of average diameters greater than 400 nm have already been reported to serve as non-toxic, biocompatible carriers for DNA delivery [18,19] notwithstanding these particles are too large for efficient intracellular uptake. Our group has previously demonstrated the potential of ultra low size (< 100 nm diameter) CaPi nanoparticles as efficient vectors for gene delivery *in vitro* [20–22]. Moreover, in relation to the induction of immune responses, it has been observed that smaller particles (< 300 nm), when complexed with DNA, induced better immune responses than did larger

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http://dx.doi.org/10.1016/j.rinim.2014.04.001

microparticles (~1 μm) [23]; this could be partially attributed to the ability of smaller particles to be taken up more readily by APCs. There is also evidence that particle size plays a critical role in the transfer of nanoparticles in the lymphatic system [24,25]. Our observations of the greater transfection efficiency, *in vitro* as well as *in vivo*, of DNA-encapsulated ultra-low size magnesium phosphate nanoparticles [26,27] prompted us to further investigate the potential of these nanoparticles as DNA vaccine carriers.

Here, we report an investigation of the levels of immunogenicity triggered by either a naked pEGFP, or MgPi-pEGFP nanoparticles, *via* intramuscular (i.m.), intraperitoneal (i.p.) or intravenous administrations (i.v.) in BALB/c mice. The immune response to the expressed antigen was studied through a combination of antibody (IgG) titration, cytokine profile measurement, macrophage (antigen-presenting cell) activation, and lymphocyte proliferation upon *in vitro* re-stimulation with recombinant green fluorescence protein (rGFP). The immune response so induced was markedly superior to that triggered by either naked pEGFP.

2. Materials and methods

2.1. Materials

All reagents and chemicals were purchased from Sigma unless otherwise stated. Anti-mouse IgG antibody was obtained from Bangalore Genei, India. Interleukin-12 (IL-12) and Interferon-γ (IFN-γ) were procured from Promega, USA. pEGFP was a gift of Prof. Debi P. Sarcar, Department of Biochemistry, University of Delhi, India. Recombinant green fluorescence protein was a gift of Prof. Anirban Maitra, Department of Pathology, Johns Hopkins Medical Institute, Baltimore, USA.

2.2. Mice

Inbred strains of pathogen-free female BALB/c mice (6–8 weeks old; 20–25 g) were obtained from the Animal House Facility, Department of Zoology, University of Delhi, India. The animals were reared in uniform hygienic conditions under a controlled environment (at 20–25 °C and 12 h dark/light cycle) following the guidelines of the Animal Ethics Committee, University of Delhi, India. The animal experiments were also executed in strict accordance to guidelines approved by the Animal Ethics Committee of the university.

2.3. Preparation of pEGFP-encapsulated MgPi nanoparticles

pEGFP-encapsulated MgPi nanoparticles were prepared using a water-in-oil microemulsion method exactly as reported in our previous work [26,27]. Briefly, 25 ml of an AOT (Aerosol OT or sodium bis (2-ethylhexyl) sulfosuccinate) in hexane solution (0.1 M) was prepared, into which 70 μl of an aqueous solution of magnesium chloride (1.0 M) and 2.94 μg of pEGFP were dissolved by continuous stirring for 12 h to form microemulsion A. In another 25 ml of AOT in hexane solution, 70 μl of aqueous solution of $(NH_4)_2HPO_4$ (1.0 M) and 2.94 μg of pEGFP, were dissolved by continuous stirring for 12 h to form microemulsion B. Additional buffer (0.1 M Tris HCl buffer, pH 8) was added to both microemulsions before stirring so that the aqueous volume in each microemulsion could reach 450 μl so as to adjust the W_o (the molar ratio of water to AOT) of each microemulsion to 10. W_o governs the size of aqueous core in such microemulsion systems and thus govern the size of the particle formed in these microemulsions. Both the microemulsions were optically clear solutions after 12 h stirring. Microemulsion B was then slowly added to microemulsion A at a rate of 4 ml/h with continuous stirring at 4 °C. The resulting solution was further stirred for another 12 h. The development of translucency indicated magnesium phosphate nanoparticle formation within its aqueous core. Dry ethanol (2 ml) was then added to break the microemulsion. The mixture was centrifuged for 30 min at 13,000 rpm at 4 °C. The pelleted nanoparticles were washed (4 ×) with 15 ml n-hexane and the particles dispersed in PBS (pH 7.2) by vortexing. The dispersed nanoparticles were dialyzed for 12 h in a 12 kD cut-off dialysis membrane bag to yield a clear dispersion. The dispersed nanoparticles were characterized by particle size determination. The void (placebo) nanoparticles were also prepared using exactly the same protocol without adding pEGFP solution.

2.4. Tagging of methoxy-PEGamine to pEGFP-encapsulated MgPi nanoparticles

In order to render the pEGFP-encapsulated MgPi nanoparticles long circulating inside the body upon their administration *via* the different routes, their surfaces were modified to acquire polyethylene glycol (PEG) terminals. This process is referred to as "PEGylation" of the surface. To obtain PEGylated nanoparticles, both void as well as pEGFP-encapsulated MgPi nanoparticles were first coated with the highly adhesive polymer, polyacrylic acid (PAA). Acid-coated MgPi nanoparticles were then conjugated with methoxy PEG-amine (Mol Wt 5000) to create the PEGylated nanoparticles. Briefly, a 10 ml dispersion of MgPi nanoparticles in PBS (pH 7.4) obtained from the above process was incubated with 10 μl of acid neutralized (pH 8) PAA (5 kD, 0.5% V/V) for 2–3 h with stirring, followed by a dialysis (12 kD membrane) to remove excess polymer. The carboxylate groups of PAA were conjugated to amine groups of methoxy PEG-amine using EDCI (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride). Methoxy PEG-amine (50 μl of 40 mg/ml) was added to the nanoparticle suspension with continuous stirring and to this, 50 μl of EDCI (20 mg/ml) was added. Stirring was continued for 8 h, followed by 2–3 h of dialysis to remove all the unconjugated molecules. The particle size of these PEGylated nanoparticles was again measured by DLS to reconfirm whether the PEGylation process had caused any change in the nanoparticles sizes. Lyophilized product was stored at 4 °C until further use. The PEGylated nanoparticle formulation was readily dispersible in an appropriate injectable volume of PBS (pH 7.4). We refer pEGFP-encapsulated PEGylated MgPi nanoparticles to as MgPi-pEGFP nanoparticles in this study.

2.5. Determination of the size of the nanoparticles

The particle sizes of both the void as well as the pEGFP-encapsulated nanoparticles in water-in-oil microemulsions as well as in aqueous solutions were determined by a dynamic light scattering (DLS) technique. Briefly, the measurements were done with a Brookhaven BI8000 instrument fitted with a BI200SM goniometer. An argon-ion air-cooled laser was operated at 488 nm as the light source and the intensity of scattered light were recorded on a scattering angle of 90°. The time-dependent autocorrelation function was derived using a 136-channel digital photon correlator. The particle size was calculated from the auto correlation function using the Stokes–Einstein equation: $d = kt/3\pi\eta D$, where D is the translational diffusion coefficient, d is the particle diameter, η is the viscosity of the liquid in which particles are suspended, k is Boltzmann's constant and T is absolute temperature.

2.6. Entrapment efficiency (E%)

The pEGFP-encapsulated nanoparticles in AOT microemulsion were separated after ultracentrifugation (40,000 rpm for 4 h at 4 °C) and the pellet, after washing with hexane, was dissolved in acidic buffer (pH 3). The amount of DNA released from the nanoparticles, $[DNA]_r$, was estimated spectrophotometrically by measuring the optical density at λ_{260nm}. The entrapment efficiency (E) was then calculated from the amount of DNA originally added