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Characterizing the antitumor response in mice treated with antigen-loaded polyanhydride microparticles

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ABSTRACT

Delivery of vaccine antigens with an appropriate adjuvant can trigger potential immune responses against cancer leading to reduced tumor growth and improved survival. In this study, various formulations of a bioerodible amphiphilic polyanhydride copolymer based on 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) and 1,6-bis(*p*-carboxyphenoxy) hexane (CPH) with inherent adjuvant properties were evaluated for antigen-loading properties, immunogenicity and antitumor activity. Mice were vaccinated with 50:50 CPTEG:CPH microparticles encapsulating a model tumor antigen, ovalbumin (OVA), in combination with the Toll-like receptor-9 agonist, CpG oligonucleotide 1826 (CpG ODN). Mice treated with OVA-encapsulated CPTEG:CPH particles elicited the highest $CD8^+$ T cell responses on days 14 and 20 when compared to other treatment groups. This treatment group also displayed the most delayed tumor progression and the most extended survival times. Particles encapsulating OVA and CpG ODN generated the highest anti-OVA IgG_1 antibody responses in mice but these mice did not show significant tumor protection. These results suggest that antigen-loaded CPTEG:CPH microparticles can stimulate antigen-specific cellular responses and could therefore potentially be used to promote antitumor responses in cancer patients.

1. Introduction

Cancer is responsible for one in every four deaths in the USA and is still not effectively managed therapeutically [1]. The current paradigm of chemotherapy and surgery requires improvements so as to enhance the overall survival rate of cancer patients and to limit the toxic side effects of the current chemotherapeutic approaches. Therapeutic cancer vaccines have received substantial impetus through a succession of findings over the past two decades that include: (i) the discovery of tumor-associated antigens (TAAs) that potentially flag the presence of tumor cells to the host's immune system [2]; (ii) the finding that dendritic cells (DCs) orchestrate the course of immune responses [3]; and (iii) the observation that pathogen-associated molecular patterns derived from microbes are strong inducers of DC maturation and resultant cellular immune responses [4]. Most recently, US Food and Drug Administration approval of the first cancer vaccine, Sipuleucel-T (Provenge™), has given the field of cancer immunotherapy a further boost [5].

The delivery of a cancer vaccine through the use of nano- and microparticle-based vectors is showing promise in both clinical and preclinical settings [6]. Ideally, such vectors should possess a number of favorable traits that include: biocompatibility; being capable of efficient co-delivery of immunogen (e.g. TAAs) and bacterial adjuvants to DCs; possessing adjuvant properties; and being capable of being stably stored and inexpensively manufactured [7–9]. In this study we investigated the potential of amphiphilic polyanhydride microparticles based on 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) and 1,6-bis(*p*-carboxyphenoxy) hexane (CPH) to be used as a cancer vaccine delivery vehicle. We used 50:50 CPTEG:CPH (Fig. 1) microparticles, which have previously shown adjuvant properties in generating robust antigen-specific humoral responses and preferential uptake and activation of DCs and macrophages [10–14]. In addition, CPTEG:CPH copolymers have been shown to be non-toxic, stabilizing to antigens and biodegradable with CPTEG:CPH particles, providing a burst release of 10–30% of encapsulated antigen followed by zero-order release for 10–30 days [11,15–17]. Thus, these polyanhydride vectors possess many of the traits desirable for a cancer vaccine vehicle. When combined with a potent antigen delivery vehicle, the presence of a bacterial or viral adjuvant has shown to increase antigenicity. Many unmethylated CpG motifs of bacterial DNA act as immune stimulants which can bias immune responses to a Th1 type. The synthetic CpG-B oligonucleotide 1826 (CpG ODN) induces DC maturation and B cell activation through interaction with the Toll-like receptor-9, which leads to enhanced activation of cytotoxic T cell responses [18].

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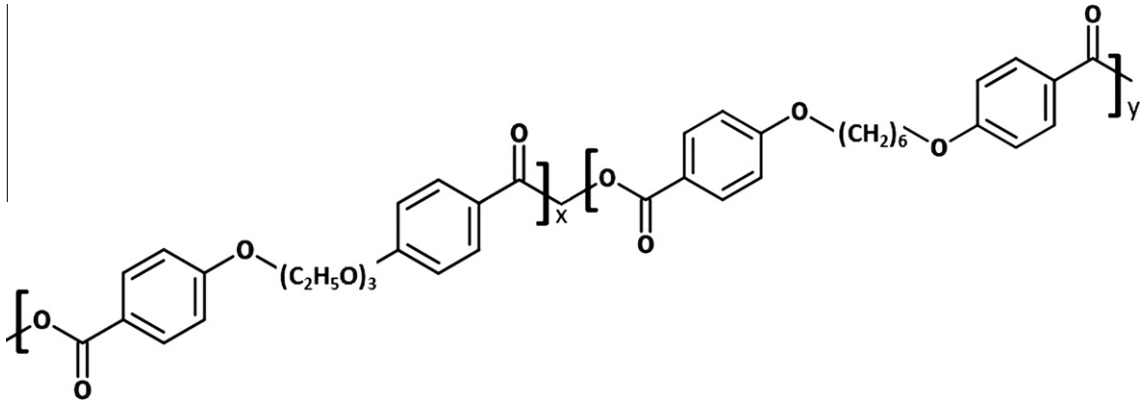

Fig. 1. Chemical structure of CPTEG:CPH polymer.

In this study, we report on the successful fabrication of CPTEG:CPH microparticles encapsulating a model TAA, ovalbumin (OVA), either alone (CPTEG:CPH–OVA), or co-encapsulated with CpG ODN (CPTEG:CPH–OVA/CpG). We also further demonstrate the immunogenicity and anticancer potential of these microparticles in a prophylactic mouse tumor model.

2. Materials and methods

2.1. Polymer synthesis

Synthesis of CPTEG:CPH copolymer was carried out by melt polycondensation, as described previously [19]. In brief, CPTEG and CPH monomers were mixed in a round-bottom flask at a 50:50 M ratio to provide a total of 2 g of monomers. Next, 100 ml of acetic anhydride was added to the monomer mixture and reacted for 30 min at 125 °C. The acetic anhydride was then removed in the rotary evaporator, and the resulting viscous liquid was polymerized in an oil bath at 140 °C under a vacuum (<0.03 torr) for 90 min. The resulting polymer was dissolved in methylene chloride and isolated by precipitation into cold hexane in a 1:15 ratio. The purity of the polymer and the number average molecular weights were verified and estimated using ^{1}H nuclear magnetic resonance (^{1}H NMR) spectra obtained from a Varian VXR-300 MHz NMR spectrometer (Varian Inc., Palo Alto, CA). In addition, gel permeation chromatography (GPC; Waters HPLC 277 System, Milford, MA using Varian Inc. GPC columns) was performed to determine the polymer's molecular weight.

2.2. Fabrication and characterization of microparticles loaded with CpG ODN and OVA

Microparticles were prepared using a double emulsion solvent evaporation method derived from Intra and Salem [20]. Briefly, purified endotoxin-free chicken egg white OVA (Sigma, St. Louis, MO) and endotoxin-free CpG ODN (Integrated DNA Technologies, Coralville, IA) were dissolved in 100 μl of 1% poly(vinyl alcohol) (PVA, Mowiol®; Sigma, Allentown, PA) solution. This solution was sonicated for 30 s on power setting #10, (Sonic Dismembrator Model 100, Fisher Scientific, Pittsburgh, PA) in 1.5 ml of dichloromethane (DCM) containing 200 mg of 50:50 CPTEG:CPH copolymer. This primary emulsion was then sonicated in 8 ml of 1% PVA solution to generate a secondary emulsion, which was then added to 22 ml of 1% PVA solution. The secondary emulsions were stirred in a fume hood for 2 h to allow evaporation of DCM. Microparticles were centrifuged at 2880 *g* for 5 min. The pellets obtained were washed twice with distilled water, followed by freeze drying using FreeZone 4.5 (Labconco Corporation, Kansas City, MO). Particles were stored in sealed containers at −20 °C. Size distribution and zeta potential were measured using a Zetasizer Nano ZS (Malvern, Southborough, MA).

To estimate the loading of OVA and CpG ODN, 20 mg of microparticles from each batch was incubated with 0.2 N NaOH for approximately 12 h at room temperature or until microparticles had fully degraded. This solution was then neutralized using 1 N HCl and loading was calculated using Eq. (1). The percentage encapsulation efficiency (EE) of the fabrication process was calculated as described in Eq. (2).

$$\text{loading} = (\text{concentration} \times \text{volume})/\text{weight of particles (mg)} \quad (1)$$

$$\text{EE} = (\text{weight of particles (mg)} \times \text{loading} \times 100)/\text{initial weight}\}\ \text{of drug (μg)} \quad (2)$$

Here, loading = μg of OVA or CpG ODN encapsulated per mg of particles, concentration = calculated concentration of OVA or CpG from the standard curve (μg ml^{-1}), and volume = volume of OVA or CpG ODN solution (ml).

The surface morphology and shape of microparticles were examined using scanning electron microscopy (SEM). Briefly, a suspension of particles was plated onto a silicon wafer mounted on a scanning electron microscope stub. This was then coated with gold–palladium by an argon beam K550 sputter coater (Emitech Ltd., Kent, England). Images were captured using a Hitachi S-4800 scanning electron microscope (Hitachi High-Technologies, Ontario, Canada) at 5 kV accelerating voltage.

2.3. Prophylactic murine tumor model

Eight- to twelve week-old male wild-type C57BL/6 mice (Jackson Laboratory, Bar Harbor, Maine; $n = 4$ per group) were treated with intraperitoneal injections of the following six groups of treatments: (i) OVA and CpG ODN encapsulated in 50:50 CPTEG:CPH microparticles (CPTEG:CPH–OVA/CpG); (ii) OVA encapsulated in 50:50 CPTEG:CPH microparticles (CPTEG:CPH–OVA); (iii) OVA encapsulated in 50:50 CPTEG:CPH microparticles with soluble CpG ODN; (iv) blank 50:50 CPTEG:CPH microparticles at an equivalent dose to the OVA particles; (v) soluble OVA and CpG ODN; and (vi) naive (untreated). For mice treated with soluble CpG, the particles or solution of OVA was admixed with CpG solution immediately prior to injections. Each mouse was primed on day 0 and similarly boosted on day 7 with the indicated treatments. Doses of 100 μg of OVA and 50 μg of CpG ODN per mouse were consistently used. On day 21 OVA-specific $CD8^+$ $CD3^+$ T lymphocyte levels were determined from peripheral blood harvested by