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Interactions of poly (anhydride) nanoparticles with macrophages in light of their vaccine adjuvant properties

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ABSTRACT

Understanding how nanoparticles are formed and how those processes ultimately determine the nanoparticles' properties and their impact on their capture by immune cells is key in vaccination studies. Accordingly, we wanted to evaluate how the previously described poly (anhydride)-based nanoparticles of the copolymer of methyl vinyl ether and maleic anhydride (NP) interact with macrophages, and how this process depends on the physicochemical properties derived from the method of preparation. First, we studied the influence of the desolvation and drying processes used to obtain the nanoparticles. NP prepared by the desolvation of the polymers in acetone with a mixture of ethanol and water yielded higher mean diameters than those obtained in the presence of water (250 nm vs. 180 nm). In addition, nanoparticles dried by lyophilization presented higher negative zeta potentials than those dried by spray-drying (−47 mV vs. −35 mV). Second, the influence of the NP formulation on the phagocytosis by J774 murine macrophage-like cell line was investigated. The data indicated that NPs prepared in the presence of water were at least three-times more efficiently internalized by cells than NPs prepared with the mixture of ethanol and water. Besides, lyophilized nanoparticles appeared to be more efficiently taken up by J744 cells than those dried by spray-drying. To further understand the specific mechanisms involved in the cellular internalization of NPs, different pharmacological inhibitors were used to interfere with specific uptake pathways. Results suggest that the NP formulations, particularly, nanoparticles prepared by the addition of ethanol:water, are internalized by the clathrin-mediated endocytosis, rather than caveolae-mediated mechanisms, supporting their previously described vaccine adjuvant properties.

1. Introduction

Vaccine strategies can be grouped into broadly two fundamental approaches: live-attenuated and non-living vaccines (e.g., bacterins and subunits vaccines). Recent strategies involve the use of purified or recombinant subunit vaccines since they offer the safest alternative in vaccination. However, they suffer from a low immunogenicity and, in general additional components are required to confer protective immunity. This led us to the full concept of vaccine formulation, enclosing antigens but also other chemical substances with adjuvant properties (Brito et al., 2013).

The development of adjuvants has been mainly based on an empirical approach and, as a consequence, their mechanisms of action are still not completely known, precluding their extensive applicability. Furthermore, this erratic comprehension of their effects leads to several safety concerns that limit their use. The understanding of the immune mechanisms will support the rational design of adjuvants filling the still remaining gaps (Levitz and Golenbock, 2012). Among several strategies, the employment of nanoparticles appears as a potential tool since they put together immunological properties as well as technical and practical features. Nanoparticles offer several advantages, including their capability as a delivery system, offering protection and controlled release properties of the loaded antigen (Gamazo et al., 2015). In addition, the particulate nature of nanoparticles has some inherent ability to facilitate antigen cross-presentation by antigen presenting cells (APCs). In this context, poly(anhydride)-based nanoparticles of the copolymer of methyl vinyl ether and maleic anhydride have been shown to modulate the immune response (Gomez et al., 2006, 2007; Irache et al., 2010; Kipper et al., 2006;

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Mallapragada and Narasimhan, 2008; Ochoa et al., 2007; Salman et al., 2005, 2009). In fact, previous results in our group have shown that antigenic complexes loaded in poly(anhydride) nanoparticles shift the immune response from Th2 to Th1 (De S. Rebouças et al., 2012, 2014; Gomez et al., 2007; Irache et al., 2010; Ochoa et al., 2007). The mechanism of this immunomodulation has been partially elucidated, although there still remain gaps to full understanding. It has been demonstrated that these nanoparticles act as agonists of TLR2 and TLR4 (Tamayo et al., 2010) and they are capable of activating the complement system by targeting C3b (Camacho et al., 2011; Tamayo et al., 2010). Now, we wanted to go further in the understanding of how particle properties affect cellular uptake and/or interaction. To this respect, the rate of uptake and intracellular localization of a variety of nanoparticles has been studied by many research groups, and several review articles summarizing the published data are available (Hild et al., 2008; Hillaireau and Couvreur, 2009; Iversen et al., 2011; Mailander and Landfester, 2009; Maysinger et al., 2007; Delehanty et al., 2009; Sahay et al., 2010; Verma and Stellacci, 2010). These reviews expose the difficulty to draw general conclusions about how to produce particles for optimal cellular uptake, as the rate and mechanism of uptake turns out to be cell-type and density dependent (Kaplan, 1976; Snijder et al., 2009) and vary between nanoparticles with different size, surface charge, material compositions and other surface properties. Still, the study of how nanoparticles are captured by certain cells is of particular relevance considering that the different mechanisms of internalization may trigger different immune responses (Mogensen, 2009). Accordingly, we wanted to evaluate how the previously described poly(anhydride) nanoparticles interact with immune cells, such as macrophages, depending on the physicochemical properties derived from the method of preparation of these nanoparticles. Thus, the aspects studied here are the employment of different methods for nanoparticle preparation and the subsequent effect on size and zeta-potential. Subsequently, we explored the interaction between four different formulations based on the copolymer of methyl vinyl ether and maleic anhydride with J744 macrophage cell line by measuring cytotoxicity, the overall uptake kinetic, and the mechanisms of uptake by using selected uptake inhibitors.

2. Material and methods

2.1. Chemicals

Poly(methyl vinyl ether-co-maleic anhydride) or poly(anhydride) (Gantrez® AN 119; MW 200,000) was kindly gifted by ISP (Barcelona, Spain). Lumogen red was supplied by Kremer Pigmente (Germany). MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide), phorbol-12-myristate-13-acetate (PMA), chlorpromazine hydrochloride, sodium azide, genestein and cytochalasin D were provided by Sigma-Life Science (Germany). Wortmannin and LY94002 were obtained from Cell Signaling Technology (USA). Dihydrorhodamine (DHR) from Life Technologies (USA). Dimethylsulfoxid from Panreac (Spain). Saccharose and mannitol were supplied by Guinama (Spain).

2.2. Preparation of poly(anhydride) nanoparticles

Poly(anhydride) nanoparticles were obtained by a modification of the solvent displacement method previously described (Arbós et al., 2002), followed by a purification step by ultracentrifugation and, finally dried by either lyophilization or spray-drying.

Briefly, a 2% w/v solution of the copolymer of methyl vinyl ether and maleic anhydride (PVM/MA) in acetone was prepared under magnetic stirring at room temperature. Nanoparticles were formed by the addition of 2 volumes of acetone to either water or to a mixture of ethanol and water (1:1 by volume). Freshly formed nanoparticles were collected by centrifugation (27.000 × *g*, 20 min, 4 °C). Supernatants were discarded and, depending on the drying procedure, the pellets redispersed in an aqueous solution containing either sucrose (5% w/v) or mannitol (5% w/v). Then, nanoparticles dispersed in the sucrose solution were dried using a freeze–dryer apparatus (VirTis, New York, U.S.A.). On the other hand, nanoparticles dispersed in the mannitol solution were dried in a Büchi Mini Spray Drier B-290 apparatus (BüchiLabortechnik AG, Switzerland) under the following experimental conditions: (i) inlet temperature of 90 °C, (ii) outlet temperature 45–50 °C, (iii) air pressure: 2–5 bar, (iv) pumping rate of 2–6 mL/min, (v) aspirator of 100% and (vi) air flow at 900 L/h.

Nanoparticles were also fluorescently labeled with Lumogen red. For this purpose, Lumogen was dissolved in the acetone phase (0.02% w/v) containing the polymer. Then, nanoparticles were obtained, purified and dried as described above. The different sets of nanoparticles are identified in Table 1.

2.3. Characterization of nanoparticles

The particle size and the zeta potential of nanoparticles were determined by photon correlation spectroscopy (PCS) and electrophoretic laser Doppler anemometry, respectively, using a Zetamaster analyzer system (Malvern Instruments Ltd., Worcestershire, UK). The diameter of the nanoparticles was determined after dispersion in ultrapure water and measured at 25 °C by dynamic light scattering angle of 90°. The zeta potential was determined as follows: 200 μL of the samples was diluted in 2 mL of a 0.1 mM KCl solution adjusted to pH 7.4.

2.4. Cell culture

Mouse macrophage J744 cells (passage 4–10 from freezing stocks in liquid nitrogen) were grown at 37 °C (5% CO_2 and 95% humidified air) in cell medium [RPMI 1640 supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/ streptomycin (invitrogen)].

2.5. MTT-cytotoxicity assay

J774 cells were used for in vitro cytotoxicity analysis of the prepared PVM/MA nanoparticles using the MTT-colorimetric monocyte mediated cytotoxicity assay, based upon the ability of living cells to reduce 3-[4,5-dimethylthiazol-2-yl]-2,

Table 1
Nanoparticles used in this study.

	Empty nanoparticles	Lumogen-loaded nanoparticles
Spray-dried nanoparticles prepared in an ethanol:water mixture	SD EtOH	SD EtOH L
Spray-dried nanoparticles prepared in water	SD H_2O	SD H_2O L
Lyophilized nanoparticles prepared in an ethanol:water mixture	LF EtOH	LF EtOH L
Lyophilized Gantrez™ nanoparticles prepared in water	LF H_2O	LF H_2O L