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MINI REVIEW

Recent advances in experimental polyphosphazene adjuvants and their mechanisms of action

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Abstract

Vaccination continues to be a very important public health intervention to control infectious diseases in the world. Subunit vaccines are generally poorly immunogenic and require the addition of adjuvants to induce protective immune responses. Despite their critical role in vaccines, adjuvant mechanism of action remains poorly understood, which is a barrier to the development of new, safe and effective vaccines. In the present review, we focus on recent progress in understanding the mechanisms of action of the experimental adjuvants poly[di(carboxylatophenoxy)phosphazene] (PCPP) and poly[di(sodiumcarboxylatoethylphenoxy)phosphazene] (PCEP) (in this review, adjuvants PCPP and PCEP are collectively referred to as PZ denoting polyphosphazenes). PZs are high molecular weight, water-soluble, synthetic polymers that have been shown to regulate innate immune response genes, induce cytokines and chemokines secretion at the site of injection and, also, induce immune cell recruitment to the site of injection to create a local immune-competent environment. There is an evidence that as well as its role as an immunoadjuvant (that activate innate immune responses), PZ can also act as a vaccine carrier. The mechanism of action that explains how PZ leads to these effects is not known and is a barrier to the development of designer vaccines.

Keywords Adjuvant · Vaccine · Mechanism of action · Polyphosphazene · Innate immunity

Introduction

Vaccination continues to be a very important public health tool in the control of infectious diseases as vaccines are estimated to prevent approximately 2.5 million deaths and many more illnesses worldwide each year (Andre et al. 2008). Vaccines mimic natural infection in the body leading to activation of the immune system so that future exposure to similar antigens will trigger the memory immune response. This response is much quicker than a primary immune response due to the generation and reactivation of long-lived memory plasma cells and memory helper T cells (Castellino et al. 2009; Pasquale et al. 2015; Sarkander et al. 2016). Subunit vaccines are safe but because they often contain highly purified antigens that tend to be poorly immunogenic, they require the addition of adjuvants to induce protective immunity (Ulmer et al. 2006). Effective adjuvants mediate their effects by one or more of the following: enhance the immunogenicity of highly purified or recombinant antigens; reduce the amount of antigen needed in a vaccine formulation without impacting efficacy; reduce the number of immunizations needed for protective immunity; improve the efficacy of vaccines in newborns, the elderly, or immune-compromised persons; enhance the speed and duration of the immune response; modulate antibody avidity, specificity, isotype, or subclass distribution; stimulate cell-mediated immunity; promote the induction of mucosal immunity; and help overcome antigen competition in combination vaccines (Singh and O'Hagan 2003; Rajput et al. 2007). Despite adjuvants being used in billions of doses of vaccines over many decades, how adjuvants function (i.e., their mechanisms of action (MOA)) remains poorly understood. This lack of clarity regarding adjuvant MOA is a barrier to the development of safe and effective designer vaccines.

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Generally, adjuvant MOA can be divided into two categories: (1) immune potentiators/immunoadjuvants that activate innate immune responses through pattern-recognition receptors (PRRs), which lead to increased immune cell recruitment and/or their immunomodulation (Pashine et al. 2005; Olive 2012; Apostólico et al. 2016; Haghparast et al. 2016; Wu 2016), or (2) delivery vehicle/carrier adjuvants that can bind or encapsulate antigen and bring it into association with immune cells (Edelman 1992; Sadeghinia et al. 2015; Liu et al. 2017). The choice of adjuvant combinations for any vaccine will have a direct effect on adaptive immune responses induced, which is a key component in the development of modern vaccines. We will detail how polyphosphazenes (PZs) function as an immunostimulant and as well as a delivery/carrier adjuvant.

Polyphosphazenes as immunostimulatory adjuvants

PZs are high molecular weight, water-soluble, synthetic polymers that have been shown to enhance the magnitude, quality and duration of immune responses when co-administered with bacterial and viral antigens in mice, pigs and cattle (McNeal et al. 1999; Andrianov et al. 2006; Mutwiri et al. 2007, 2008; Andrianov et al. 2009; Eng et al. 2010; Andrianov et al. 2011; Garlapati et al. 2011; Dar et al. 2012; Magiri et al. 2018). The two most investigated polyphosphazenes are poly[di(carboxylatophenoxy)phosphazene] (PCPP) and poly[di(sodiumcarboxylatoethylphenoxy)phosphazene] (PCEP) (Mutwiri and Babiuk 2009). Changes in synthesis (such as reduction in the reduction of acid groups) and formulation as a soluble adjuvant or microparticle impact how they influence the immune responses (Andrianov et al. 2004). PCEP has been shown to have a significantly higher adjuvant activity compared to PCPP (Mutwiri et al. 2008) and also induce 1000-fold higher antibody titres compared to alum when co-administered subcutaneously with an influenza antigen in mice (Mutwiri et al. 2007). Relative to PCPP, PCEP also promotes a significantly stronger mixed Th1/Th2 type of responses leading to a broad spectrum immunity (Mutwiri et al. 2007) (Fig. 1).

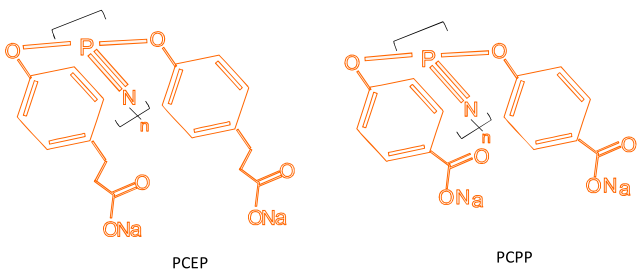

Fig. 1 The structures of the polyphosphazene polyelectrolytes, PCEP and PCPP (Andrianov et al. 2006; Teasdale and Brüggemann 2013). Image recreated from Motifolio.com

Regulation of innate immune response genes, induction of cytokines and chemokines and recruitment of immune cells to the site of injection

Studies with mice and pigs revealed species-specific differences in how PZs induce stimulation of innate immune responses (Awate et al. 2012; Magiri et al. 2016). Intramuscular injection of PCEP induced time-dependent changes in the gene expression of many "adjuvant core response genes" (Mosca et al. 2008) such as chemokine genes CCL-2, CCL-4, CCL-5, CCL-12 and CXCL-10 in mice (Awate et al. 2012) and CCL-2 and CXCL-10 (but not CCL-5) in pigs (Magiri et al. 2016). Major transcription factor NF-κB gene and the inflammatory cytokine TNF-α genes were upregulated in response to PCEP in mice (Awate et al. 2012) but not in pigs (Magiri et al. 2016). At the protein level, PCEP promoted significant local production of Th1-type proinflammatory cytokines (IL-1β, Il-6, IL-18 IFN-γ and TNF-α) and Th2-type cytokines (IL-4 and monocyte chemoattractants CCL-2 and CXCL-10) at the site of injection in mice but not systemically (Awate et al. 2012). Further, in vitro studies showed that PCEP activated the NLRP3 inflammasome in a caspase 1-depedent manner, which leads to the processing of interleukin IL-1β-, IL-18- and IL-33-stimulated splenic dendritic cells (DCs) in mice (Awate et al. 2014a, b). However, in pigs, PCEP induced IL-6 gene expression but not IL-10, IL-17, or IFN-α (Magiri et al. 2016). PCEP injection in mice increased the expression of TLR4 and TLR9 at the site of injection (Awate et al. 2012) whereas PCEP did not induce any significant expression of the TLR genes in pigs suggesting differences in activation of immune responses in different animal species (Magiri et al. 2016). These results suggest that PCEP may modulate antigen-specific immune responses by activating early innate immune responses and promoting a strong immunostimulatory environment at the site of injection. Our studies provide evidence that the effect that adjuvants have on the innate immune response can differ remarkably between species.

Intramuscular (i.m.) injection of PCEP promoted recruitment of largely neutrophils but also macrophages, CD4+ T cells, CD8+ T cells and CD19+ B cells, monocytes and DCs to the injection site and the draining lymph nodes in mice (Awate et al. 2014a, b). Confocal analysis revealed that many recruited myeloid cells (but only a few lymphocytes) showed evidence of intracytoplasmic lysosomal localization of PCEP (Awate et al. 2014a, b). These findings suggest that the recruitment of distinct immune cells to the site of injection site may be an important mechanism by which PCEP potentiates immune responses.

Activation of immune cells by polyphosphazenes

Even in the absence of antigens, PCPP and PCEP have strong avidity to soluble immune receptor proteins such as mannose receptor (MR) and endolysosome membrane-associated PRRs such as TLR-7, TLR-8 and TLR-9 (Sasai and Yamamoto 2013;

Andrianov et al. 2016a, b). Other studies revealed direct activation of immune cells by PCPP and PCEP through the TLR signaling pathway, both on the external cell surface (TLR-4) and endosome (TLR-3 and TLR-9) (Reed et al. 2013; Sasai and Yamamoto 2013). Incubation of primary mouse splenocytes with PCEP or PCPP triggered production of IL-4 and IL-12 but only PCEP induced significant IFN-γ production suggesting that activation of innate immunity may be important in mediating PZ adjuvant activity (Mutwiri et al. 2008). Others have demonstrated that PCPP induced activation and maturation of DCs (Andrianov et al. 2006; Andrianov et al. 2016a, b). In the presence of antigen, PCPP has been shown to promote activation and maturation of human adult and newborn DCs by upregulating co-stimulatory molecules and cytokine production *and induction of* an innate immune transcriptome (Palmer et al. 2014), which may suggest that PZ may be an appropriate adjuvant to include in early life immunization.

Vaccine carrier adjuvants

Vaccine carriers have been traditionally viewed as particulate delivery vehicles capable of facilitating physical uptake of the antigen by antigen-presenting cells (Storni et al. 2005; De Temmerman et al. 2011). Generally, it was thought that delivery systems tend to induce Th2-type immune responses that are not effective against many intracellular pathogens, while immunostimulatory adjuvants were traditionally thought to induce Th1-type immune responses by strongly activating the innate immune system (Ryan et al. 2001). However, these classifications are no longer appropriate since there is growing evidence that some delivery systems can activate innate immunity as well.

Polyphosphazenes as vaccine carriers

Polyphosphazenes have been exploited as protein carriers due to their versatile molecular structures and wide spectrum of chemical and physical properties including biodegradability and matrix permeability (Andrianov and Payne 1998; Teasdale and Brüggemann 2013). PZ can bind vaccine antigens as well as TLR ligands or other sites on immune cells leading to cell maturation and more effective antigen processing, which supports the idea that polyphosphazenes macromolecules have dual antigen carrier and immunostimulant functions (Andrianov et al. 2005; Palmer et al. 2014; Andrianov et al. 2016a, b). Further, PZ can form stable water-soluble, non-covalent complexes with antigenic molecules spontaneously and, thus, do not require chemical conjugation (Andrianov et al. 2005; Palmer et al. 2014). Non-covalent interactions with proteins have been correlated with immunoadjuvant activity, as well as the ability to stabilize proteins in solution and during drying processes (Andrianov et al. 2005; Marin et al. 2010).

Aqueous PZs can be transformed to microparticles by cross-linking them with divalent cations. Microencapsulation of antigens by PZ can be achieved under remarkably mild physiological conditions (which avoid denaturation or loss of biological activity of encapsulated material) giving them tremendous potential as matrices for sustained antigen release (Andrianov and Payne 1998). For example, immunogenicity of influenza antigen and tetanus toxoid were dramatically enhanced when microencapsulated in PCPP microparticles (Payne et al. 1995). Further, by varying polymer ratios and using PZ of reduced molecular weight, it can form macromolecular assemblies at the nanoscale level to cross-linked hydrogels while maintaining protein-binding ability (Andrianov et al. 2016a, b). Microparticles are more effective in mucosal delivery of antigens (Shim et al. 2010), which should be taken into consideration for vaccine development.

Adjuvant potential of polyphosphazene in combination with other adjuvants

Because of challenges in vaccine development and regulatory hurdles and/for purely economic reasons, the vaccine industry has historically used one adjuvant per vaccine. However, evidence has accumulated over the last decades showing that multiple adjuvant components in the same vaccine may act synergistically (Kindrachuk et al. 2009; Mutwiri et al. 2011; Salvador et al. 2012; Mount et al. 2013; Levast et al. 2014; Ciabattini et al. 2016; Didierlaurent et al. 2017; Madan-Lala et al. 2017). Combination adjuvants are particularly suited to only enhance and/or direct the immune responses towards Th1-, Th2- or Th17-type responses (Kindrachuk et al. 2009; Salvador et al. 2012; Levast et al. 2014).

Due to the short half-life of most immunostimulatory adjuvants in vivo, combining a delivery vehicle adjuvant with an immunostimulatory adjuvant may increase the magnitude and modulate the quality of immune responses (Weiner et al. 1997). Mice vaccinated subcutaneously with PCPP microparticles encapsulating OVA and CpG ODN generated higher antigen-specific antibody responses compared to antigen alone (Garlapati et al. 2010; Wilson et al. 2010). Studies by several investigators at VIDO-InterVac demonstrated that PZ as part of a triple adjuvant combination (TriAdj) consisting of PCEP or PCPP plus TLR agonist (CpG or poly I:C) plus Host Defense Peptide (HDP) is a robust adjuvant combination in multiple species and multiple routes of delivery. For example, subcutaneous immunization of mice with HBsAg plus TriAdj resulted in enhanced production of HBsAg-specific antibody responses compared with the mice immunized with HBsAg plus any of the three adjuvants alone (Mutwiri et al. 2008). Relative to mice immunized with OVA plus the adjuvants alone, mice vaccinated with OVA plus TriAdj showed enhanced antibody and cell-mediated responses via both MHCI and II pathways, promoting a more balanced

antibody-mediated and type1-biased cell-mediated immune response (Kovacs-Nolan et al. 2009a, b, c). Mice vaccinated subcutaneously with Bordetella pertussis antigen plus TriAdj had a significantly reduced bacterial load after challenge and increased antigen-specific IL-17-secreting cells relative to vaccine comprised of one or two adjuvants alone (Garlapati et al. 2011). Formulation of pertussis toxoid (PTd) with TriAdj increased IgG1 responses in adult mice and induced superior serum IgG2a antibody titers in both adult and neonatal mice compared to mice immunized with each adjuvant alone (Gracia et al. 2011). Recombinant-truncated bovine respiratory syncytial virus (bRSV) fusion protein (DeltaF) plus TriAdj showed enhanced secretion of antigen-specific serum antibody titres when compared with mice immunized with antigen alone (Kovacs-Nolan et al. 2009a, b, c). Intranasal vaccination with a formalin-inactivated bRSV vaccine plus TriAdj resulted in induced systemic and mucosal immunity in mice (Mapletoft et al. 2010) and a significant reduction in viral replication upon bRSV virus challenge (Mapletoft et al. 2008). Cattle immunized subcutaneously on days 0 and 90 with hen egg lysozyme antigen plus TriAdj produced superior antigen-specific humoral responses and cell-mediated immune responses relative to cattle immunized with Emulsigen (Kovacs-Nolan et al. 2009a, b, c). Intramuscular or intrauterine immunization of rabbits with a single dose of OVA, truncated glycoprotein D (tGD) from bovine herpesvirus and a fusion protein of porcine parvovirus protein VP2 and bacterial thioredoxin (rVP2-TrX) formulated with TriAdj induced antigen-specific humoral responses systemically and within the local (uterus) and distal mucosa (lungs and vagina) (Pasternak et al. 2017, 2018). Thus, PZ as part of the TriAdj combination contributes to the robust immune responses and results in a balanced immunity for broader protection.

Antigen-dose-sparing effect of polyphosphazene adjuvants

The implementation of antigen stabilization and dose-sparing technologies is an important step in improving availability of vaccines and is a critical feature of effective vaccines at the time of a pandemic outbreak. PZ have the potential to significantly reduce the cost of vaccination by reducing the number of immunizations or reducing the minimal doses of antigen required to induce significant immunity. Indeed, lethal challenge studies in ferrets demonstrated 100% protection for low-antigen dose PCPP-adjuvanted formulations and at least a tenfold antigen-sparing effect with improved thermal stability of H5N1 influenza vaccine in solution (Andrianov et al. 2011). Additionally, reducing the dose of antigen by 25-fold had no effect on antibody responses in mice immunized with PCPP and PCEP in mice (Mutwiri et al. 2007). When used as part of an intradermal delivery system for hepatitis B surface antigen, PCPP demonstrated superior induction of immunity in pigs compared to i.m. administration and significant antigen sparing potential (Andrianov et al. 2009). Further development of PZ as an adjuvant may therefore have a great economic impact in the vaccine industry.

The safety profile of polyphosphazene adjuvants

Many potential immunological adjuvants are not licensed for use in humans or veterinary species due to safety and/or toxicity concerns (Eng et al. 2010; Sivakumar et al. 2011; Petrovsky 2015). At doses up to 1 mg, PZs have been shown to be a safe and effective adjuvant when injected in sheep and cattle (Kovacs-Nolan et al. 2009a, b, c) without triggering adverse reactions such as pathological inflammatory reactions e.g., swelling or pain (Kovacs-Nolan et al. 2009a, b, c; Mutwiri and Babiuk 2009). In pigs, up to 500 μg PCEP was tolerated with few injection site reactions and reduced delayed type hypersensitivity (Dar et al. 2012; Magiri et al. 2016, 2018). In human phase I clinical trials for three influenza viral strains (A/H3N2, A/H1N1 and B strain) targeted towards both young and elderly adults, up to 500 μg PCPP was shown to be safe, showing sterile abscesses and non-ulcerative necrosis at the site of inoculation (Le Cam et al. 1998). Phase I and phase II clinical trials of a vaccine formulated with PCPP and HIV-1 antigens did not result in either abscess at injection site, immune dysfunction, anaphylaxis, or allergy, whereas a vaccine formulated with Freund's complete adjuvant and HIV-1 was associated with definable long-term adverse events (Gilbert et al. 2003). Together, the results suggest that polyphosphazenes are well tolerated in humans and animals but detailed safety and toxicity studies per vaccine are still required.

Conclusion

The trend in vaccine development away from the use of whole-cell, virus vaccines or inactivated vaccines to subunit vaccines requires addition of potent adjuvants to induce protective immune responses. Thus, the long-term goal of vaccine development should be identification of key innate immune targets for induction of potent but safe antigen-specific immune responses. Recent advances in understanding of innate immunity has led to increased understanding of the MOA for adjuvants and how they drive antigen-specific immunity and immunological memory (Guy 2007; Coffman et al. 2010; Mohan et al. 2013). This new appreciation of innate defense mechanisms provides a solid foundation for rational approaches to adjuvant discovery and vaccine optimization. PZ adjuvants exhibit species-specific differences, hence adjuvant selection may need to be tailored to the species as well. Given these considerations, it should be increasingly possible to

design and select adjuvants tailored to the specific needs of the antigen, species and situation.

Many new adjuvants in clinical or preclinical development are focused on enhancing specific types of T cell responses and generating the multifaceted immune responses that may be needed for challenging diseases. Understanding how adjuvants activate the innate immune system will make a significant impact on vaccine development in the future.

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