



Perspectives in vaccine adjuvants for allergen-specific immunotherapy



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ABSTRACT

The design of more powerful adjuvants is a tool of crucial interest to ameliorate vaccination strategies to reduce injections and/or dose of antigen, induce local immunity and obtain better protection. Effective anti-infectious vaccines should elicit protective T_H1 responses, cytotoxic CD8+ cells and antibody-forming cells. However, cytokine microenvironment is a key point also in targeted therapeutic vaccinations, such as allergen-specific immunotherapy, where the interference with an already-existing but inappropriate immunity is required. In this case, safe, appropriately conditioning and potentially orally available adjuvants together with delivery to appropriate subsets of dendritic cells would be highly appreciated to properly boost innate immune cells. In fact, aluminium hydroxide, although safe, has been classically associated with the induction of a $TH2$ response to co-formulated antigens. Thus, detoxified lipopolysaccharide (MPL-A), CpG oligonucleotides, imidazoquinolines and adenine derivatives acting via innate sensors may represent improvements in therapeutic vaccinations for allergy as able to interfere with pathogenic T_H2 cells with eventual induction of T_H1 differentiation.

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1. Introduction

Allergic diseases are characterized by the expansion of $TH2$ cells specific for otherwise innocuous antigens (allergens) accumulating at the sites of inflammation (respiratory and/or gastro-intestinal tract, skin) and being responsible of IgE production, eosinophil recruitment and $Fc\epsilon RI+$ cell activation [1]. Allergen-specific immunotherapy (SIT) is a therapeutic vaccination based on the administration of gradually increasing amounts of an allergen and it is the only treatment able to prevent disease progression and further sensitizations. Decreased activity of pathogenic $TH2$ cells, redirection of allergen-specific cells towards a more protective phenotype or both, likely represent the mechanisms of action [2]. Different approaches are under investigation to overcome the use of aluminium hydroxide which is able to induce, at least in the mouse, $TH2$ responses such as CCL11-driven eosinophil recruitment and isotype switching to IgG1 and IgE [3]. Recently, several low molecular weight synthetic compounds triggering endosomal Toll-like receptors (TLRs) have been proposed as adjuvants in novel SIT approaches as able to provide the optimal cytokine milieu to interfere with pathogenic $TH2$ cells and induce T_H1 differentiation.

2. Monophosphoryl lipid A in allergy vaccines

Lipopolysaccharides (LPS) are structural components of the outer membrane of Gram-negative bacteria exerting strong induction of innate immunity via TLR4 trigger. The hydrophobic domain lipid A contains the functional moiety of the molecule but is useless due to toxicity. The lipid A analogue monophosphoryl lipid A (MPLA) obtained by controlled hydrolysis of LPS of *Salmonella minnesota* R595 is approximately 10^{-3} less toxic than the native molecule [4] but retains immunomodulatory properties through not completely known pathways. The prevalent activation of Toll-interleukin-1 receptor (TIR)-domain-containing adapter inducing IFN- β (TRAM/TRIF) without MAL/MyD88 activation was suggested to explain its activity together with low caspase-1, strong p38 MAPK and weak JNK activation [5,6]. In vitro increased levels of IP-10/CXCL10, TNF- α , IL-10 and IL-12 transcripts, reduced expression of costimulatory molecules on human oral Langerhans cells [6], induction of Foxp3 expression and T_H1 response, induction of IgG and IgA synthesis and generation of protective memory CD8 lymphocytes were all observed. In experimental models, MPLA provides interesting effects in difficult infections, still unsatisfactory vaccines, cancer immunotherapy, and abuse drugs [4].

Approved for human use in 2009, in allergy MPLA is included in subcutaneous and sublingual adjuvanted immunotherapy. MPL incorporated together with allergens was successful in reducing symptoms, medication scores and IgE rise after only four pre-seasonal injections (Pollinex Quattro) [7]. These results have been

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recently confirmed in a large cohort of patients exposed in the environmental exposure chamber and treated with short Ragweed pollen allergoid absorbed to L-tyrosine and mixed with MPL [8]. Similar preparations have been also customized with mites [9], recombinant Phleum [10], and Parietaria [11]. MPLA-adjuvanted allergen-specific vaccines exhibit long lasting effects as improvement in clinical scores and reduction in wheal diameters after skin tests were still observed 5 years after immunotherapy discontinuation [12]. In vitro, MPLA redirects allergen-induced T_H2 -cell responses towards 'protective' T_H1 in an IL-12 and monocyte-dependent fashion [13] whereas, in vivo, it reduces basophil activation and seasonally boosted immunoglobulin E production, increasing IgG4 blocking antibodies and IgG2 [10].

3. CpG oligonucleotides

The discovery that DNA may exert anti-tumour and pro-inflammatory activity derived from the observation of Tokunaga et al. who attributed to DNA the immunostimulatory activity of portions of Calmette and Guérin mycobacteria. Prokaryotic but not eukaryotic DNA contains extremely high numbers of unmethylated CpG dinucleotides and several short (12–30 bases) DNA sequences or CpG oligonucleotides (ODNs) were then described as immune modulators [14]. Four different classes of CpG-ODNs are described on the basis of their structure and activities: (i) D- or A-type inducing IFN- α in plasmacytoid dendritic cell (pDCs); (ii) K- or B-type prevalently, but not uniquely, acting on B cells; (iii) C-type and (iv) P-type ODNs acting on both B lymphocytes and pDCs. Generally, the presence of the phosphorothioate (instead of the native phosphodiester) backbone confers stability and resistance to nucleases [15,16].

TLR9 localized in the endolysosomal compartment senses CpG-ODNs [17] but the amino acids within the ectodomain involved in recognition and binding are still unknown [18]. The interaction between TLR9 and CpG-ODNs induces the downstream activation of MyD-88-, IL-1 receptor-associated kinase (IRAK-) and TNF receptor-associated factor (TRAF)-6 followed by the recruitment of MAP-kinases, NF- κ B, interferon-regulatory factor (IRF) and AP-1 with a fast up-regulation of hundreds of genes [16]. In addition to B cells, ODN-stimulated pDCs increase the expression of MHC Class II and costimulatory antigens favouring antigen presentation, and produce a series of cytokines including type-I IFNs and IL-12, supporting maturation and differentiation of natural killer cells, influencing the functional phenotype of CD4+ T lymphocytes and monocytes/macrophages and promoting CD8-mediated responses [16]. Hundreds of publications concern experimental models and human trials using ODNs as adjuvants to target infectious diseases and cancers via systemic or mucosal routes of administration [15,16]. Conjugation with antigen proteins, addition to other adjuvants as alum, incorporation into lipid vesicles or emulsions, microparticle formulations and nanoparticle-based delivery are additional attempts to improve activity and safety of ODNs as adjuvants and spare doses and/or boosts [15].

The potential of oligonucleotides as adjuvants in immunotherapy of allergy was also quickly recognized. First, certain phosphorothioate sequences (but not its phosphodiester homologue) stimulate dendritic cells, crucially involved in T cell priming and functional education, to produce IL-12p70 [19]. CpG ODNs inhibit the in vitro spontaneous IgE production from allergic individuals and induce PBMCs to produce IL-12, IL-18 and IFN- γ [20]. Phosphorothioate ODNs promote the shift of human allergen-specific T_H2 cells into a T_H1/T_H0 phenotype and increase IFN- γ production in NK cells. The addition of neutralizing anti-IL-12 and anti-IFN- α mAbs could revert this activity indicating that the immunostimulatory sequences activate innate cells

[21]. In mouse models of acute asthma after intraperitoneal ovalbumin (OVA) or major birch allergen Bet V 1 followed by intranasal challenge, administration of phosphorothioate oligonucleotides at the time of sensitization down-regulated T_H2 -oriented responses ameliorating inflammatory airway infiltration and hyperresponsiveness but with conflicting results about the up-regulation of IFN- γ and/or T_H1 stimulation [22–25]. Recently, in subcutaneously sensitized mice with grass pollen Phl p 5, CpG-ODN 1826 reduced eosinophil lung infiltration and T_H2 cytokines, suppressed allergen-specific IgE and IgG1, increased IgG2a production with variable effects on IFN- γ [26].

To improve specificity and immunogenicity and reduce allergenicity, an innovative approach was used based on the conjugation of stimulatory sequences to allergenic proteins. N-ethylmaleimide-treated sulfo-succinimidyl-cyclohexane activated major allergen of Ragweed Amb a 1, mixed with a 5'-thio CpG-ODN, produces a conjugate containing an average number of 4 ODNs/one molecule of Amb a 1, inducing allergen-specific IgG2a production and IFN- γ release by murine splenocytes as well as IgG production in rabbits and monkeys. Amb a 1 conjugate prevented T_H2 responses in mice and exhibited a reduced ability to induce histamine release from human sensitized basophils [27]. Two independent studies showed that immunotherapy with ODN-Amb a 1 conjugate was safe and induced amelioration of symptoms in allergic patients [28,29]. In 2006, a Phase II randomized double-blind, placebo-controlled study of 25 rhinitic patients, 6 subcutaneous injections produced significant long lasting improvement of symptom scores, reduction in specific seasonal-induced IgE levels and suppression of immediate and late-phase responses after skin tests, without variation in the in vitro cytokine production by peripheral blood mononuclear cells (PBMCs) [30]. Despite these initial results, a prolonged study failed to confirm the promises and attempts are under study to improve allergy vaccines with the incorporation of CpG-ODNs into virus-like particles (VLPs) [31].

4. Imidazoquinolines

Around mid-eighties, the new class of low-molecular-weight compounds Imidazoquinolines (IMQs), including Imiquimod (R-837), were described as potent anti-tumoral and anti-viral agents in animal models as inducing type I interferons, IL-6, IL-1 and TNF- α , among the other [32]. This activity was the basis for the approval of topic Imiquimod (Aldara) for the treatment of human genital warts caused by papillomavirus. The progenitor molecule and other more potent analogues including Resiquimod (R848), stimulated human PBMCs to produce cytokines, supported IFN- γ production in CD4+ and CD8+ lymphocytes and inhibited IL-4 and IL-5 production [33]. These results were further supported by the ability of R-837 and R-848 to in vitro shift pathogenic T_H2 allergen-specific human T cells into T_H0/T_H1 and to activate NK cells to produce IFN- γ [34]. Further, Imiquimod inhibited IgE production in CD40-stimulated IL-4 treated B cells [35]. Thus, a possible use as adjuvants in the immunotherapy of T_H2 -mediated disorders was envisaged. The receptors triggered by imidazoquinolines remained elusive till the beginning of 2000 and were finally disclosed as being TLR7 (Imiquimod) together with TLR8 (Resiquimod) [35]. The induction of IFN- α from pDCs [38] and IL-12 from monocytes and monocyte-derived DCs [36], respectively, explain the modulation of T_H2 responses but the interest in these compounds for immunotherapy of allergy and asthma overcomes the simple T_H1 -inducing effects. Allergen-induced airway hyperactivity, eosinophilic inflammation, goblet cell hyperplasia and airway remodelling are prevented by TLR7 triggering [37] which also relaxes airway smooth muscle, rapidly dilates the airways, inhibits viral replication in the lungs and prevents virus-induced

airway hyperreactivity [38,39]. Intranasally driven R848 dampens OVA-induced airway hyperresponsiveness, reduces eosinophil influx, IL-5 and leukotriene B₄ release in the bronchoalveolar lavage increasing IL-12, CXCL1 and CXCL9 or, alternatively, type-I IFN [40]. Other receptors besides TLRs may be finally implied such as stimulation of Opioid Growth Factor Receptor (OGF-R), caspase-1 mediated pathways and inhibition of microsomal PGES-1 [35,41]. These activities are globally relevant to include imidazoquinolines into the design of vaccines in cancer, atopic diseases or in early life. However, although as interesting as immune response modifiers, in allergy IMQs have been mainly limited to experimental models of asthma or in vitro cultured human cells. As decorating protein antigens by conjugation with properly efficient immunoenhancers (instead of a simple mix) can reduce the amount of adjuvant and/or the number of boosts, production of soluble imidazoquinolines for the so-called self-adjuvanted vaccines is currently under study [42].

5. Modified adenines as vaccine adjuvants

The use of imidazoquinolines, inducing systemically serious side effects (vomiting, hepatotoxicity, alterations in blood cell counts), had to be necessarily limited to topic application [15]. Thus, other safer heterocycles of simple chemical synthesis and retaining TLR-triggering activity were urgently needed. In addition to natural agonists of TLR7/8 and 9 such as guanosine- and uridine-rich ssRNA and bacterial and viral DNA, several synthetic purine and pyrimidine analogues exert immunostimulation and induce IFNs [43]. Selected guanosine analogues including loxoribine, enhanced the expression of MHC and costimulatory receptors in murine macrophages and DCs and activated human PBMCs to produce IFN- α , but not IL-12, via TLR7. The addition of a hydroxy or a mercapto-group in position 8- to 9-benzyl adenines strongly increased IFN levels in vitro but the 8-SH-derivatives exhibited a low in vivo activity. Thus, position 8 was crucial for activity whereas absence of acidic protons on the 8-substituents resulted into inactivity. Additional substitutes in clue positions of the adenine molecule remarkably improved (or reduced) the immunomodulatory activities. Introduction of alkyl-chain groups in position 2 – enhanced activity whereas replacement of the 6-amino group with other substituents resulted in inactivity [44]. Thus, optimal IFN-inducing activity resides into 9-benzyl-8-hydroxy-6-amino-2-alkyl adenines. Finally, introduction of an alkoxy group in 2 – increased oral availability of 8-OH adenines [45].

However, despite optimal chemical characterization and laborious synthetic procedures of numerous compounds, assessment of 8-OH adenines activity was limited for long time to murine unfractionated spleen cells neither binding receptor(s) were further investigated or effects on human system were detailed. We generated a 2-butoxy compound (SA-2) investigating the possible receptor(s) triggered by the molecule in comparison with a mock 9-benzyl adenine lacking the 8-OH. Following the hypothesis that SA-2 might target NF- κ B as IFN inducer, p50 and p65 subunits were found to be released by human monocytes. SA-2 and 8-OH adenines in general triggered human and mouse TLR7 without any ability to activate G-protein-coupled receptors, ion-channel nucleotide receptor, NOD receptors or MyD88-deficient cells [46,47]. The production of regulatory (IL-12p70, IFN- α , IL-10, CXCL10) and inflammatory (TNF- α , IL-6) cytokines and chemokines was induced by SA-2 in human monocytes and pDCs. In vitro, SA-2, when mixed together with allergens such as the group 2 major allergen of *Dermatophagoides pteronyssinus* (Der p 2) redirected allergen-specific T_H2 cells towards the T_H1/T_H0 phenotype downregulating IL-4 production and GATA-3 expression while favouring IFN- γ and T-Bet. These effects were abolished by the addition of neutralizing antibodies against IL-12 and IFN- α

[47]. In vivo, in both pre-priming and pre-challenge mouse model of OVA-induced respiratory allergy, SA-2 reduced lung inflammation, eosinophil infiltration in the airways and expression of mucus-encoding genes without any effect on the numbers of Foxp3+ Tregs. Increase in specific IgG2a with a parallel decrease in specific IgE was also observed. Despite the reported induction of autoimmunity after TLR7-triggering, titres of anti-nuclear antibodies were not different between SA-2 or mock-treated animals [48]. Finally, the production of potentially harmful cytokines such as IL-17A and F, involved in unregulated way in some immune-mediated disease, was inhibited by SA-2 as able to induce high levels of IL-10 [49]. Interestingly, as efficient techniques of stable conjugation between different compounds including proteins have been provided, a series of immunomodulatory substituted 8-OH adenines could be coupled to free amines on protein antigens (or allergens) via a spacer under undenaturing mild conditions (aqueous buffer, neutral pH) maintaining the intact protein structure. A SA-2 derivative [4-(6-amino-9-benzyl-8-hydroxy-9H-purin-2-ylsulfanyl)-butyric acid 2,5-dioxo-pyrrolidin-1-yl ester] coupled to Der p 2 maintained TLR7 triggering, induced the production of type I IFN, in vitro redirected human allergen-specific T_H2 responses and promoted a T_H1 profile in a murine model of allergic sensitization without induction of autoimmunity [47]. More recently, both OVA- and Der p 2-conjugates displayed the same effects also in therapeutic protocols of experimental allergy (Vultaggio et al., submitted for publication). In general, as this coupling procedure can be applied to different proteins provided that containing amine groups, the use of properly modified 8-OH adenines might be envisaged in all those prophylactic or therapeutic vaccines in which a protective T_H1 response is hopeful. Finally, as some modified 8-OH adenines are orally active, these compounds may also offer further opportunities for non-parentally administered vaccinations.

6. Conclusions

Encouraging results come from in vitro and in vivo studies, including some human clinical trials, showing the efficacy of several low molecular weight synthetic compounds exhibiting immunomodulatory activity as triggering endosomal TLR7, 8 and 9. In allergy, there is a general consensus about the need to ameliorate specific immunotherapy which materialized in the optimization of allergen sources and purification, of administration routes and improvement in immunostimulation and conditioned microenvironment. These attempts are relevant both to give opportunity to treat those more severe allergic diseases (atopic dermatitis, severe allergic asthma, food allergy) in which the interference with the allergen-specific response would be highly appreciable as well as the more ordinary allergic pathologies frequently trivially and hastily considered even if severely affecting the quality of life. Although the use of these compounds may raise concerns about safety especially for the wide expression of their receptors on both immune and extra-immune cells, this topic may be overcome by novel delivery systems or coupling procedures.

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