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Adjuvants for veterinary vaccines – types and modes of action

Adjuvantien für Veterinärimpfstoffe – Typen und Wirkweise

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Summary

Adjuvants are used to improve the immune response to vaccines. Formulation with adjuvants can result in an earlier onset of immunity, an overall stronger immune response, a specific type of immunity, or a longer duration of immunity to the vaccine. Adjuvants were discovered empirically, and for decades, have been used in both humans and animals without understanding the mechanisms of action. With an improved understanding of the immune system, and in particular the interplay between innate and adaptive immunity, we are now getting better insight into the function of adjuvants. As a result, new adjuvants are being developed that are safe and highly effective for common use in humans and animals, as well as for use in high risk populations such as immunocompromised animals, neonates or very old animals. Furthermore, adjuvants can help to reduce the amount of antigen needed in the vaccine, increase the stability of the vaccine and enable alternative administration routes such as needle-free delivery of the vaccine. Here, I will provide an overview of the existing adjuvant technologies for veterinary vaccines and provide an outlook into some of the new technologies in preclinical and clinical development.

Keywords: adjuvants, veterinary vaccines

Zusammenfassung

Adjuvantien sind wichtige Bestandteile von Impfstoffen und werden zur Steigerung der Wirksamkeit verwendet. Adjuvantien können unter anderem zu einer schnelleren und länger anhaltenden Immunantwort führen, sowie einen bestimmten Typ der Immunantwort einleiten. Adjuvantien wurden in den letzten hundert Jahren zumeist empirisch eingesetzt; erst in den letzten Jahren hat sich durch ein besseres Verständnis des Zusammenspiels von angeborener und erworbener Immunität eine eigene Forschungsrichtung etabliert. So wurden in den vergangenen Jahren verschiedene neuartige Adjuvantien für human- und veterinärmedizinische Anwendungen entwickelt, welche zum Beispiel die Wirksamkeit des Impfstoffes verbessern, die Stabilität erhöhen und die Menge des benötigen Antigens reduzieren können. Dieser Artikel beschreibt die Wirkungsweise von Adjuvantien und stellt einige veterinärmedizinisch relevante Adjuvantien vor.

Schüsselwörter: Adjuvantien, Veterinärvakzinen

Introduction

Vaccination remains the most effective medical intervention in history. Aside from providing access to clean water, vaccination has saved more lives than any other medical mitigation strategy. Vaccines have been used for hundreds of years, and remain common practice in livestock, poultry, exotic and companion animals. Interestingly, the challenges associated with vaccinating animals, such as low cost requirements, enhanced stability, ease of administration etc., require innovative solutions in vaccine development. As a result, we have seen several vaccine technologies and adjuvants first developed for use in animals before making their way into human medicine.

Adjuvants are important components of vaccines, commonly used to elicit stronger, faster, and longer

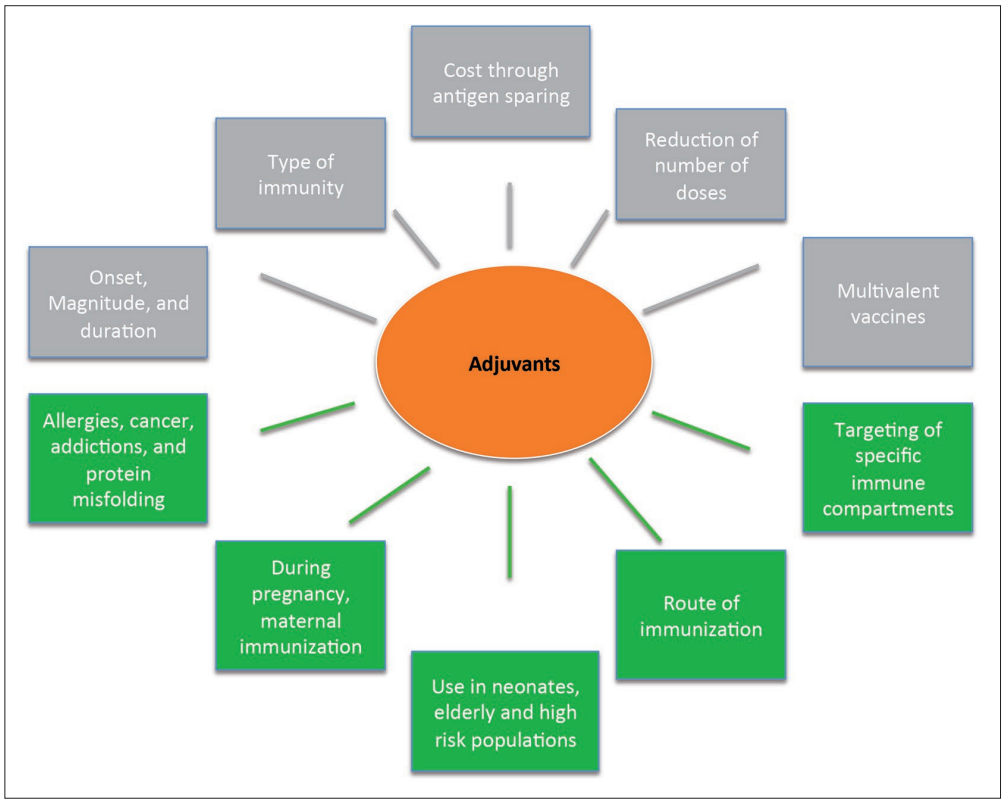

FIGURE 1: *Adjuvants can have a variety of effects on the outcome of vaccination. Some of these effects are listed in the figure.*

lasting immune responses to vaccines. Named after the latin word "adjuvare", which translates into "to help", adjuvants are used to enhance or shape the immune response to vaccines. For example, adjuvants can specifically enhance either antibody- or cell-mediated immune responses (type of immunity), they can be used to provide an earlier onset of immunity as needed for vaccination in the face of an outbreak for example, they can reduce the number of immunizations needed, or they can be used to induce immunity in the very young or the elderly (reviewed in Coffman et al., 2010; Fig. 1). Furthermore, adjuvants can help to reduce the amount of vaccine material (antigen sparing) needed and sometimes facilitate administration of the vaccine via an alternative route, such as the mucosal surfaces (reviewed in Coffman et al., 2010; Cox and Coulter, 1997).

The use of adjuvants was first described almost one hundred years ago. As one of the first, the French veterinarian and biologist Ramon reported in the 1920s that after adding foreign substances to a diphtheria vaccine candidate in horses, the antibody response to the vaccine was enhanced, and that large abscesses at the injection site seemed to correlate with an augmented antibody response (G.R. 1925; Ramon, 1926). Glenny et al. (1926) described the use of mineral salts for the enhancement of immunity through formation of precipitates (Glenny et al., 1926). In the 1930s, Freund introduced a combination of mineral oils and bacterial cell components for the enhancement of vaccine immune responses, a combination that is still available as "Freund's complete adjuvant" (Freund et al., 1937). However, due to its reactivity and side effects, many jurisdictions are now banning the use of Freund's adjuvant in animals and recommend use of less reactive adjuvants. Mineral salts, such as alum-based vaccines, and oil-in water emulsions have been successfully used since the early 40s in both human and animal vaccines, and are still being used today. For example, aluminum-based adjuvants are the most commonly used adjuvant in humans (Marrack et al., 2009). However, with an improved understanding of the immune system and the interactions between innate and adaptive immunity, many new adjuvants have recently been developed, both for veterinary and human applications. Indeed, adjuvant research has now become a field of its own, which over the past decade transitioned from empirical testing to sophisticated design and screening procedures (O'Hagan and Fox, 2015).

Most adjuvants are used with inactivated or subunit vaccines. These vaccine types have the disadvantage of being less immunogenic; however, they have an excellent safety profile due to the non-replicating nature of the vaccine itself, and thus are commonly used in humans and animals. Live-attenuated vaccines, on the other hand, rarely require adjuvants due to a different mode of action resulting in higher immunogenicity. However, live-attenuated vaccines have the disadvantage of a lower safety profile due to improper attenuation and the risk of reverting back to virulence. Thus, by using adjuvants one can overcome the challenges of reduced immunogenicity of inactivated and subunit vaccines while maintaining the high safety profile of the vaccine. Moreover, several adjuvants are under development to specifically tailor the immune response to live-attenuated vaccines.

Mechanisms of action

Adjuvants can function in a variety of ways, from acting as specific delivery vehicle or targeting molecule to acting as a depot at the site of injection to representing a specific danger signal that induces a very specific type of

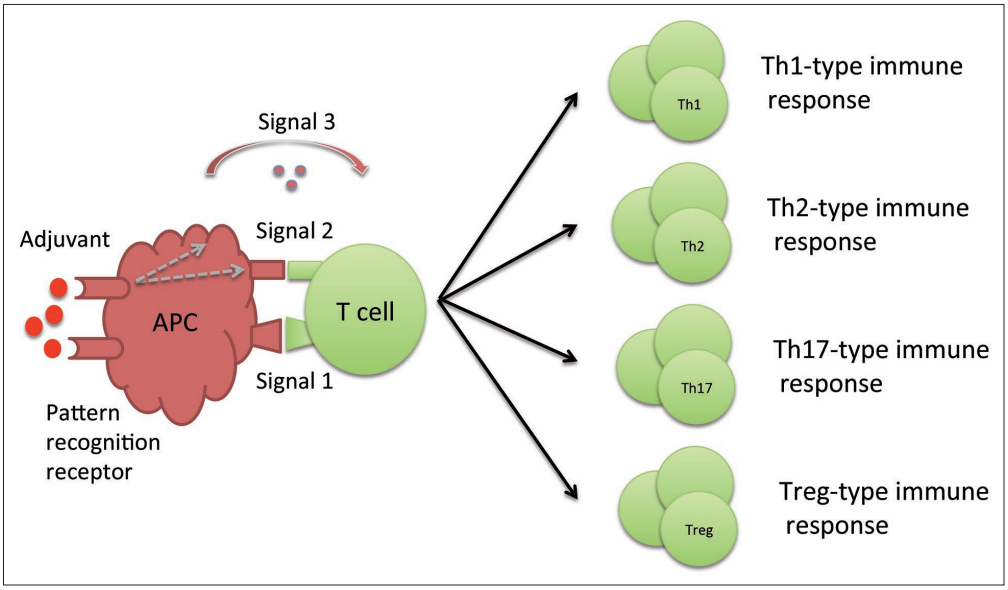

FIGURE 2: *Antigen presentation by an antigen presenting cell to a T cell. Antigen presenting cells (APC) are key cells of the immune system. They are presenting antigen to T cells via MHC molecules (signal 1). In addition they are providing co-stimulatory signals that determine the type of immune response (signal 2 and 3). Which co-stimulatory signals is provided depends on engagement of the pattern recognition receptors (PPR), which allows the APC to distinguish between various danger-associated molecular pattern. Adjuvants are recognized by these PPR and thus can drive the type of immune response as indicated by the arrows.*

immune response (reviewed in Awate et al., 2013 and Lambrecht et al., 2009; Fig. 2). In most cases, adjuvants cause some sort of tissue injury, which subsequently leads to recruitment of immune cells and recognition by the immune system and engagement of highly sophisticated mechanisms resulting in stimulation and activation of innate and adaptive immunity (Calabro et al., 2011). In most cases, adjuvants induce localized pro-inflammatory immune responses that result in recruitment and activation of immune cells at the site of injection (Awate et al., 2013; Goto and Akama, 1982; Mosca et al., 2008). Sometimes, such activation can result in apoptosis or necrosis at the site of injection (Mosca et al., 2008; Reed et al., 2013; Seubert et al., 2008), and is characterized by a recruitment of immune cells such as macrophages, neutrophils and dendritic cells. Local inflammation can result in redness, swelling and local pain, which may trigger comments such as"the vaccine is working"by some owners, whereas others may be concerned about adverse events to the vaccine. Especially for use in humans, modern adjuvants rarely induce macroscopically visible inflammatory signs.

Acting as danger signal

The immune system of our domestic animals is equipped with an innate and acquired immune system. Innate immunity provides early protection against many pathogens and is based on recognition of highly conserved molecular pattern that trigger a non-specific immune response (Medzhitov and Janeway, 1997; Medzhitov et al., 1997). Recognition of such danger-associated molecular pattern (DAMPs) allows differentiation into broad categories, such as infection versus traumatic injury, extracellular versus intracellular pathogens, or viral versus bacterial or parasitic pathogens. Engagement of these pattern recognition receptors (PRRs) informs the immune system of an ongoing event, a "danger" (Medzhitov et al., 1997). As such, the host up-regulates it's innate defense mechanisms, which are non-specific and typically last only for days, while activating the acquired immune system, also referred to as adaptive immunity. However, the danger signal determines the expression of co-stimulatory molecules, which are used by the antigen-presenting cell to drive a specific type of adaptive immunity (Fig. 2)(Hoebe et al., 2004).

The adaptive or acquired immune system provides a much more specific immune response, which is characterized by antibodies and T cells that recognize specific antigens through antigen-specific receptors. The development of an adaptive immune response can take days to weeks, during which the innate defense provides protection. However, as shown in Figure 1, the type of response is already determined by the danger signal itself. Thus, some adjuvants that act as danger signal can shape the immune response resulting in long-lasting immune responses of a specific type, such as a Th1-type responses (cellular immunity, needed for intracellular pathogens), Th2-type responses (humoral immunity, needed for parasites and some extracellular pathogens), Th17-type responses (extracellular pathogens, mucosal surfaces) and Treg-type responses (regulatory responses, suppression of immunity) (Fig. 2). Depending on the type of antigen, the local microenvironment and the extend of immune stimulation, adaptive immunity can last a very long time. Most importantly, adaptive immunity allows for the establishment of immune memory, which ensures that when encountering the pathogen for a second time, a more effective anamnestic or secondary immune response is mounted. Immune memory, therefore, forms the basis for vaccination. By vaccinating an animal, we are mimicking the first encounter without disease, which then ensures that an effective and fast immune response is in place when the animal encounters the real pathogen.

Acting as delivery vehicle

Forming a depot at the site of injection is another important function of some adjuvants. It is believed that such depots allow for slow release of the antigen and more effective antigen uptake by antigen-presenting cells (Fig. 3). Most salt-based adjuvants function this way, including alum ($ALK(SO_4)_2$; aluminum potassium sulphate), and most micro- and nanoparticle formulations. Antigen is absorbed or precipitated to the adjuvant and slowly released over days to weeks following injection (Iyer et al., 2003). Similarly, water-in oil adjuvants also depend on direct interaction between the antigen and the adjuvant, promoting enhanced antigen uptake by antigen presenting cells (Herbert, 1968). However, a depot effect

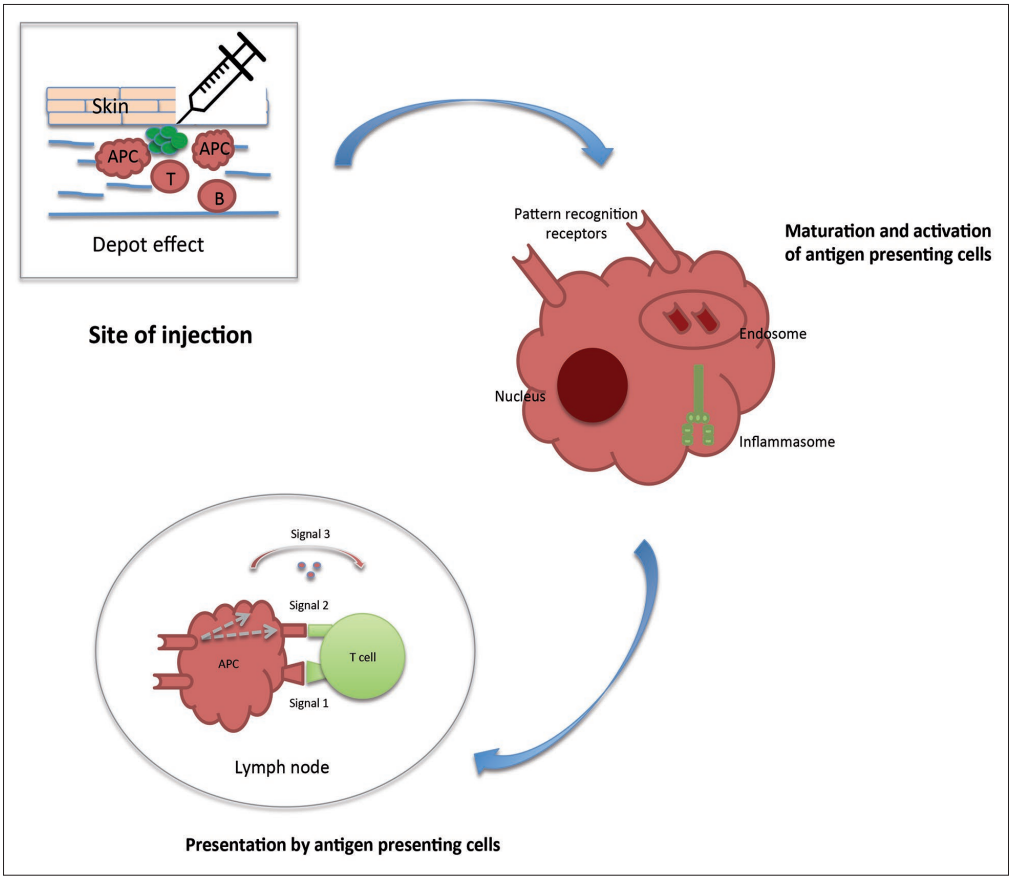

FIGURE 3: *Adjuvants have a variety of mechanisms of action including forming a depot at the injection site, activation and maturation of antigen-presenting cells including activation of the inflammasome as well as shaping the type if immunity during antigen presentation in the lymph node.*

is not necessarily beneficial for all adjuvants, and in some cases rapid transport to the draining lymph node seems to promote antigen-presentation more effectively (Morein and Bengtsson, 1999). Interestingly, some studies also demonstrated that surgical removal of the injection site had no impact on the immune response following injection, suggesting that a depot effect was not necessary for inducing effective immunity (Hutchison et al., 2012). However, it is not clear at the moment if this generally applies to all adjuvants or just to certain types. More research is required to fully answer these questions.

Activation of the inflammasome

Pattern recognition receptors (PRR) enable the immune system to recognize and respond to a variety of danger signals (Fig. 2). Several families of PRRs have been described over the past few years, including the family of nucleotide oligomerization domain (NOD) like receptors (NLRs). These receptors can be found on a variety of immune cells and tissues and ensure recognition of a wide variety of danger signals, including environmental and microbial stimuli (Latz et al., 2013). The inflammasome belongs to the family of NLRs and is comprised of a group of proteins involved in recognition of these danger signals and subsequent release of proinflammatory cytokines such as interleukin-1b, -18 and -33 (Latz et al., 2013). Interestingly, it remains unclear whether aluminum-based adjuvants signal through the inflammasome. Thus, while the inflammasome may account for activation of the innate immune response following stimulation with aluminium-based adjuvants, this may not be the case for the adaptive immune response.

Activation and maturation of antigen presenting cells

Amongst the many cells arriving at the injection site, antigen-presenting cells are of particular importance. These phagocytic cells take up and process the vaccine antigen and subsequently present short versions of these antigens to either T-helper or cytotoxic T lymphocytes in lymph nodes and spleen (Fig. 1). Upon recognition of their specific antigens (signal 1) activated T cells undergo clonal expansion and become antigen-specific effector cells that either lyse infected body cells (cytotoxic T cells) or that secrete cytokines to promote differentiation and maturation of other immune cells (T-helper cells). Importantly, in addition to the antigen itself, the antigen-presenting cell also provides information regarding the type of immune response needed to control the danger (signal 2 and 3), i. e. cell-mediated versus humoral for intracellular versus extracellular pathogens. Which co-stimulatory molecule (signal 2) and which cytokine (signal 3) is being expressed very much depends on the danger signal itself, and to this end, antigen-presenting cells are equipped with a broad range of pattern recognition receptors to recognize such danger signals. Thus, adjuvants can influence type of immune response being induced, e.g. a Th1, Th2, Th17 or regulatory immune response, respectively.

Examples of types of adjuvants for animal vaccines

A wide range of adjuvants has been successfully used in commercial vaccines for animals and several new technologies are currently in preclinical development (Tab. 1). Some of these are briefly described below:

Mineral salts
Aluminium-based adjuvants were already described in the early 20s by Glenny et al. (Glenny et al., 1926), and since then have been used in a wide variety of both human and animal vaccines. Billions of children and animals have been immunized with vaccines containing aluminum salts, mostly aluminum hydroxide or aluminum phosphate. Aluminium-based salts include alum (aluminum potassium sulphate), alhydrogel (aluminum hydroxide); Adju-Plus (aluminum phosphate) and Imject Alum (aluminum hydroxide and magnesium hydroxide). Aluminum-based adjuvants are safe, and known to induce a Th2 immune response, which is predominantly mediated by antibodies and thus beneficial for extracellular pathogens. However, the exact mechanisms of action are still not fully understood (de Gregorio et al., 2008; Marrack et al., 2009; McKee et al., 2009). While aluminium-based adjuvants can retain the antigen at the site of injection, they also cause cell injury resulting in the release of danger signals, which can stimulate the immune system as described above and possibly activate the inflammasome resulting in caspase-1 dependent release of pro-inflammatory mediators such as IL-1b and IL-18 (Marrack et al., 2009).

Oil-in-water/water-in-oil emulsions
Emulsions such as MF59, Incomplete Freund's adjuvant or Emulsigen-D (MVP Technologies) have been used for a long time in animals, predominantly in large livestock species (Galliher-Beckley et al., 2015; Lai et al., 2015). Oil-in water and water-in oil emulsions contain uniformly dispersed, micron sized oil or water droplets, that provide stability and decreased viscosity. Emulsions are believed to act through formation of a depot at the injection site and slow release of the antigen, a feature that is further pursued through the development of self-emulsifying oil-in water emulsions (Shah et al., 2015). Several oil-in water emulsion are commercially available for veterinary use, including Montanide adjuvants (Seppic), Emulsigen-D, Incomplete Freund's adjuvant and others. Oil-in water emulsions are also commonly used in aquaculture, however, concern has been raised regarding the induction of adhesions following immunization (Bowden et al., 2003).

Saponins
Saponins are a group of glycosides commonly found in plants, and which are often promoted as nutraceuticals and dietary supplements. Various saponins have been tested and commercialized for use in animals, including Quil-A (InvivoGen), ISCOMS, ISCOMATRIX (CSL), and QS-21 (Cambridge Biotech Corp.) (de Costa et al., 2011; Drane et al., 2007; Morein et al., 1984; Morelli et al., 2012; Sanders et al., 2005; Sun et al., 2009). Although the mechanism of action is not fully understood, these molecules have been shown to be powerful inducers of both T cell and humoral immune responses

Toll like receptor (TLR) ligands and small molecules
Toll like receptors are pattern recognition receptors that recognize a variety of microbial danger signals, including bacterial and viral DNA, RNA, lipopolysaccharide (LPS), and flagellin to name a few. Engagement of these receptors by the ligand results in activation a various signalling pathways that eventually lead to expression of proinflammatory cytokines as a consequence. Prominent examples of TLR ligands include LPS, PolyI:C, and CpG ODN to name a few. Most of these ligands have been tested in animal species and have been combined with various experimental vaccine candidates. Most notably, the use of CpG oligonucleotides has proven highly efficacious in large animals (Dar et al., 2010; Mutwiri et al., 2003; Nichani et al., 2004). Currently, TLR ligands are included in a number of combination adjuvants, as described below. Small molecules, such as host defense peptides have been widely used in experimental and commercial adjuvants. For example, synthetic host defense peptides proved highly efficacious when combined with PolyI:C or Cpg ODN and polyphosphazenes in a variety of species and in conjunction with a variety of antigens. Many of the specific receptors for these small molecules are not known, however, evidence suggest that for example host defense peptides directly target dendritic cells (Dybvig et al., 2011; Garlapati et al., 2011; Gracia et al., 2011).

Particles
Particle-based adjuvants have been extensively studied, both in the form of nanoparticles and microparticles. Microparticles offer the advantage of delivering the vaccine antigens directly to antigen presenting cells, since antigen presenting cells are phagocytic cells that preferably take up particulate antigen. Particulate vaccine formulations also offer the advantages of delivering the vaccine to the mucosal surfaces, including oral and nasal routes of delivery (Mutwiri et al., 2005). Various synthetic and natural polymers have been developed and tested in both preclinical and clinical studies. Sizes range from 50–100 nanometer to 2–5 micrometer in size (Shah et al., 2014). For example, poly-(DL-lactide-coglycide) particles have been used with a wide variety of antigens in experimental species. Other examples include polyphosphazenes, a group of synthetic polymers that was recently evaluated and further optimized as vaccine adjuvant for use in a combination adjuvant (Eng et al., 2010a; Eng et al., 2010b).

Liposomes and virosomes:
Liposomes were first described more than 40 years ago and promoted as potent vaccine adjuvants (Cardella et al., 1974) are synthetic spheres consisting of lipid layers that encapsulate antigens and release these by integration into various cell compartments through fusion of the membranes. The potency of liposomes largely depends on size, polarity, number of lipid layers, electric charge, and assembly procedures (Alving et al., 2012; Schwendener, 2014). Liposomes have been used with a variety of antigens in a variety of species, including experimental vaccines and clinical vaccine candidates (Korsholm et al., 2012). Virosomes are non-replicating delivery vehicles for vaccine antigens. Virosomes consist of viral particles that have the vaccine antigens incorporated or linked and that by being of particulate shape themselves enhance vaccine uptake by antigen-presenting cells (Gerdts et al., 2013).

Combination adjuvants

Over the past decade, we have seen both in human and animal health registration of several new combination adjuvants including MF59™ (Novartis Inc.), AS03™

(Glaxo Smith Kline Inc.), and IC31™ (Valneva Inc.) to name a few (Mutwiri et al., 2007; Mutwiri et al., 2011; Skibinski et al., 2011). These adjuvants typically contain between two and three individual adjuvant components, often at a lower dose or formulated into smaller particles. With a better understanding of the mechanisms of action for each individual adjuvant, combining them allows to benefit form a synergistic effect that in many cases by far exceeds the sum of the individual effects seen when giving each component individually. For example, we recently developed a novel combination adjuvant that is comprised of three components, namely polyphosphazenes (a synthetic polymer), host defense peptides, and a TLR ligand, either CpG ODN or PolyI:C (Kindrachuk et al., 2009). When vaccine antigens were co-formulated with this combination, we saw a more than 1,000 fold increase in antibody titers compared to the vaccine alone (Garlapati et al., 2011; Gracia et al., 2011). The onset of immunity was much faster and highly effective even after a single immunization. Immune responses lasted for a very long time, and provided protection against infections with a variety of pathogens including swine influenza, respiratory syncytial virus, and *Bordetella pertussis*, to name a few (Dar et al., 2012; Garg et al., 2015; Garlapati et al., 2010; Garlapati et al., 2012; Khan et al., 2014). The combination adjuvant was highly effective in a variety of species including pigs, sheep, cattle, koalas, fish, cotton rats and mice and was stable for more than six months under accelerated conditions at 37 C in the light (Garg et al., 2014; Gracia et al., 2011; Khan et al., 2014; Polewicz et al., 2013; van der Merwe et al., 2011). Moreover, the combination adjuvant was cost effective, especially when using a single immunization, and easy to formulate and also allowed both systemic as well as mucosal administration of the vaccine (Garlapati et al., 2011). Analysis of the immune response demonstrated that the combination adjuvant targeted dendritic cells in particular, which then released a combination of cytokines that resulted in promotion of a balanced/ Th1-type immune response (Auray et al. 2013; Dar et al. 2012).

Future perspectives

With an increased understanding of the immune system and its various interactions between innate and adaptive immunity, our understanding of the mechanisms of action of adjuvants has greatly improved. As a result, adjuvant research has transitioned from empirical screening to a more sophisticated specific design and synthesis process. Molecules are specifically designed that can act synergistically with other adjuvants, and that can shape the immune response in a very specific way as required by the respective pathogen. Already we are seeing new adjuvants in development and on the market that are safe for use in both humans and animals, and that are potent enhancers of a specific type of immune response, as required by the specific vaccine. Future research will address the use of those adjuvants in combination with each other and assess the potential of using the same platform of adjuvant for multiple vaccine antigens, so that one can save money on each individual vaccine by reducing the total number of injections each animal receives. Furthermore, large emphasis lies on improving the safety profile of our vaccine formulations. It is expected that future adjuvants while being highly potent, will not induce a local proinflammatory response to avoid any potential adverse events and improve the carcass quality. At the same time, by using such highly effective adjuvants one can further reduce the amount of antigen needed in the vaccine, which will make it more cost effective for the producer.

Conflict of interest

The author has no conflict of interest.

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