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The Role of Adjuvants in Production of New Generation Vaccine

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Abstract: Inactivated vaccines require adjuvants to stimulate an immune response. The choice of adjuvant or immune enhancer determines whether the immune response is effective, ineffective, or damaging. Accordingly, there is a need for new vaccine that stimulate the appropriate immunity, for example, T cell immunity for intracellular pathogens and cancer vaccines. In several adjuvants, the identification of chemical groups that interact with specific cell toll-like receptors (Innate immunity) or receptors for costimulatory ligands (Adaptive immunity), have enabled the establishment of structure function relationships those are useful in the design of new adjuvants. Because of the crucial immunomodulating role of adjuvants, sub-unit vaccine development will remain dependent on new adjuvants. In the past decade, many receptors and signaling pathways in the innate immune system have been defined and these innate responses strongly influence the adaptive immune response. The focus of this review is to delineate the role of adjuvants and how adjuvants can be used to influence the magnitude and alter the quality of the adaptive response in order to provide maximum protection against specific pathogens. Despite the impressive success of currently approved adjuvants for generating immunity to viral and bacterial infections, there remains a need for improved adjuvants that enhance protective antibody responses, especially in populations that respond poorly to current vaccines. However, the larger challenge is to develop vaccines that generate strong T cell immunity with purified or recombinant vaccine antigens. Further, efforts are needed to develop and produce new generation of adjuvant vaccine with less adverse effect.

Key words: Adjuvants · Vaccine · Immune response

INTRODUCTION

Widespread vaccination in animals remains the most successful method to prevent losses in farm animals from infectious diseases [1]. Conventional veterinary vaccines have mainly consisted of live attenuated pathogens, completely inactivated organisms, or inactivated bacterial toxins [2]. Although attenuated forms of the pathogen are used as veterinary vaccines, however, concerns about these occasionally reverting to the virulent form still exist and employing killed organisms or parts thereof, is an alternative for these vaccines but they provide lesser degree of protection than attenuated forms. As a result of limitations of live attenuated vaccine and non-living vaccine several new approaches to veterinary vaccine development have emerged, which may have significant advantages over more traditional approaches [3].

The new vaccine approaches include recombinant protein subunits and plasmid DNA vaccine. While these new approaches may offer some advantages, a general problem is that these vaccines may not be cost effective for veterinary use and are often poorly immunogenic [4]. Moreover, require additional components to help stimulate protective immunity based on antibodies and effectors T cell functions. These additional components, termed adjuvants, provide the ‘‘Help’’ (From adjuvate, to help) needed to enhance the immunogenicity of vaccine antigens. Adjuvants currently in widespread use, either in

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man or in animals, have for the most part been developed empirically, without a clear understanding of their cellular and molecular mechanisms of action. However, recent data suggest that most, if not all, adjuvant enhance T and B cell responses by engaging components of the innate immune system, rather than by direct effects on the lymphocytes themselves [5].

Adjuvant can be broadly separated into two classes, based on their principal target to immune system; vaccine delivery systems and immune stimulatory adjuvants. Vaccine delivery systems are generally particulate e.g., oil emulsions, micro particles, immune stimulating complexes and liposomes and mainly function to target associated antigens into antigen presenting cells (APC). In contrast, immune stimulatory adjuvants are predominantly derived from pathogens and often represent pathogen associated molecular patterns (PAMP) e.g. lipopolysaccharide, monophosphoryl lipid A, cytosine phosphate guanine DNA vaccine, which activate cells of the innate immune system. The discovery of more potent adjuvants may allow the development of vaccines against infectious agents such as HIV, TB and other chronic diseases that do not naturally elicit protective immunity. New adjuants may also allow vaccines to be delivered through mucus membranes [6]. Therefore, the objective of this paper is to review the role of adjuvants in production of new generation vaccine.

History of Adjuvants: Adjuvants for toxoid vaccines developed from the 1920s to the 1940s. The broadened use of oils and aluminum adjuvants is recorded from the 1940s to the 1970s. Synthetic adjuvants and second-generation delivery depot systems were developed from the 1970s to the 1990s. The development of rational receptor associated adjuvants that activate the innate immune system recorded from the 1990s until the present day [7]. The suspension of killed Salmonella Typhimurium with mineral oil emulsion elicited increased immune response. Later, water in oil type of emulsion adjuvant was prepared using paraffin oil mixed with killed Mycobacteria called Freund's complete adjuvant (FCA) and without *Mycobacterium* referred as Freund's in complete adjuvant (FIA). The FIA forms depot at the site of injection and slow release of antigen with the stimulation of antibody producing cells led to poor immuno modulatory effect (Freund, 1956). Earlier, FIA was used in human vaccine formulations such as influenza and killed poliomyelitis vaccines [8, 9].

Importance of Adjuvants: Adjuvants have been traditionally used to increase the magnitude of an adaptive response to a vaccine, based on antibody titer or ability to prevent infection and a second role for adjutants has become increasingly important: guiding the type of adaptive response to produce the most effective forms of immunity for each specific pathogen. Thus, there are four distinct reasons to incorporate an adjuvant into a vaccine. Adjuvants are currently used clinically to: (1) increase the response to a vaccine in the general population, increasing mean antibody titers and/or the fraction of subjects that become protectively immunized; (2) increase seroconversion rates in populations with reduced responsiveness because of age (Both infants and the elderly), disease, or therapeutic interventions, as in the use on the microfluid59 adjuvant to enhance the response of older subjects to influenza vaccine [10] (3) facilitate the use of smaller doses of antigen [11] because the ability of an adjuvant to permit comparable responses with substantially lower amounts of antigen could be important in circumstances in which large-scale vaccination is urgent and production facilities limiting, as in the emergence of a pandemic influenza strain and (4) permit immunization with fewer doses of vaccine. The requirement of many vaccines for multiple injections presents compliance issues and, in much of the world, significant logistic challenges. Adjuvants can reduce the number of doses required to achieve protection [12].

The second reason for incorporating an adjuvant into a vaccine is to achieve qualitative alteration of the immune response. For vaccines currently under development, adjuvants are increasingly used to promote types of immunity not effectively generated by the non-adjuvanted antigens. For example, adjuvants have been used in preclinical and clinical studies to: (1) provide functionally appropriate types of immune response (e.g., T helper 1 cell versus Th2 cell, CD8+ versus CD4+ T cells, specific antibody isotopes) (2) increase the generation of memory especially T cell memory [13] (3) increase speed of initial response, which may be critical in a pandemic outbreak of infection [14] and (4) alter the breadth, specificity, or affinity of the response [15].

Potent adjuvants can improve the effectiveness of vaccines by accelerating the generation of robust immune responses, sustaining responses for a longer duration, inducing local mucosal immune responses, generating antibodies with increased avidity or affinity and neutralization capacity, eliciting CTLs, enhancing immune

responses in individuals with weakened immune systems (*e.g.* children, elderly or immunocompromised adults), increasing the response rate in low-responder individuals and reducing the amount of antigen needed, thus reducing the cost of vaccination programmes. Immunopotentiators activate innate immunity directly (*e.g.* cytokines) or through pattern recognition receptors (PRRs) (Such as bacterial components), whereas delivery systems (*e.g.* microparticles and nanoparticles) concentrate the antigen and display antigens in repetitive patterns, target vaccine antigens to APCs and help co-localize antigens and immunopotentiators. Thus, both immunopotentiators and delivery systems can serve to augment antigen-specific immune response *in vivo*. For subunit vaccines, it is highly desirable that the combination of delivery systems and immune potentiators be required to elicit optimal immune responses [16].

In particular, the historical emphasis on humoral immune responses has led to the development of adjuvants with the ability to enhance antibody responses. Therefore, most commonly used adjuvants are effective at elevating serum antibody titres, but do not elicit significant Th1 responses or CTLs. The ability of an adjuvant to qualitatively affect the outcome of the immune response is an important consideration, because the need for vaccines against chronic infections [*e.g.*, HIV, hepatitis C virus (HCV), tuberculosis and herpes simplex virus (HSV)] and cancer has shifted the focus to generation of cellular immune responses and adjuvants specifically geared towards eliciting this effect [11]. An expanded understanding of the immunobiology of toll like receptors (TLR) and other pathogen recognition receptor (PRRs), immunoregulatory cells, dendritic cells DCs and the importance of specific T helper cell responses (Th1 versus Th2) in resolving particular diseases provides a framework for their continued optimization [17].

Types of Adjuvants: Adjuvants can be broadly separated into two classes, based on their principal mechanisms of target function to immune system: vaccine delivery systems and immunostimulatory adjuvants. Vaccine delivery systems are generally particulate e.g. emulsions, microparticles and liposomes, immune stimulating complexes and mainly function to target associated antigens into antigen presenting cells (APC). In contrast, immunostimulatory adjuvants are predominantly derived from pathogens and often represent pathogen associated molecular patterns (PAMP) e.g. lipopolysacchride, monophosphoryl lipid A, cytosine phosphate guanine DNAvaccine, which activate cells of the innate immune system [18].

Delivery Systems Adjuvants: Several groups have evaluated the use of particulate adjuvants or antigen delivery systems, as alternatives to immunostimulatory adjuvants. Particulate adjuvants (E.g. emulsions, microparticles, immune stimulating complexes, liposomes and mineral salts) have comparable dimensions to the pathogens, which the immune system evolved to combat [19].

Oil Emulsion Adjuvants: Several water-in-oil (w/o) and oil-in water (o/w) emulsions with or without mineral oils have found mass applications in vaccination against different infectious diseases such as foot-and mouth disease and Newcastle diseases in farm animals.Vaccination for the foot-and-mouth disease in animals has been extensively carried out in two mineral oil emulsions from Seppice Montanide immune stimulating antigen (ISA 206 and ISA 25). Emulsified vaccines based on mineral oils like Drakeol and Marcol also induce high levels of immunity in cattle and pigs [20].The Freud's complete adjuvant can induce strong Th1 and Th2 immune response because of the combination of *Mycobacterium tuberculosis* with water in oil type of emulsion However, inducing toxic effect is the major disadvantage of this adjuvant [21].

Oil-in-water emulsions Microfluid59 and AS03MF59 (Novartis) and AS03 (GlaxoSmithKline) are both oil-in water emulsions based on squalene, an oil that is more readily metabolized than the paraffin oil used in Freund's adjuvants.MF59 is licensed in most of Europe for use with seasonal flu vaccines in the elderly and both are used in approved pandemic flu vaccines. As a result, there are considerable human data comparing flu vaccination with these adjuvants to the same vaccine without adjuvant or with alum [22]. These emulsions stimulate stronger antibody responses, permit fewer doses and antigen dose sparing and generate marked memory responses, with a mixed Th1-Th2 cell phenotype [23]. MF59 induces substantial local stimulation, recruitment of DCs, granulocytes and differentiation of monocytes into DCs. Intramuscular injection of MF59leads to a pattern of induced genes that is both larger and distinct from that induced by either alum or a TLR9 agonist [24].

Microparticles as Adjuvants: Antigen uptake by APC is enhanced by association of antigen with polymeric microparticles or by the use of polymers or proteins that self-assemble into particles. The biodegradable and biocompatible polyesters, the polylactide-co-glycolides are the primary candidates for the development of microparticles as adjuvants, since they have been used in humans and animals for many years as suture material and as controlled release drug delivery systems [25].

Microparticles also appear to have significant potential as an adjuvant for DNA vaccine. Importantly, the cationic, microparticle enhanced responses in a range of animal models including non-human primates. The microparticles appeared to be effective as a consequence of efficient delivery of the adsorbed plasmids into dendritic cells, the most important APC for presentation of antigen to naive T cells [26]. In addition, cationic microparticles can be used as delivery systems for adjuvant active molecules, including polylactide-coglycolide microparticle [27]. Similar anionic microparticle scan also be used for delivery of adsorbed proteins and are effective for cytotoxic T lymphocyte induction in mice [28].

Liposomes Adjuvants: Liposomes have been commonly used in complex formulations, often including monophosphoryl lipid A, which makes it difficult to determine the contribution of the liposome to the overall adjuvant effect. Immunopotentiating reconstituted influenza virosomes are unilamellar liposomes comprising mainly phosphatidylcholine, with influenza hemagglutinin intercalated into the membrane. The use of viral membrane proteins in the formation of virosomes offers the opportunity to exploit the targeting and fusogenic properties of the native viral membrane proteins, perhaps resulting in effective delivery of entrapped antigens into the cytosol for CTL induction [29]. An alternative approach to vaccine delivery which may have some advantages over traditional liposomes has been described using 'Archaeosomes', which are vesicles prepared from the polar lipids of Archaeobacteria. In some studies, archaeosomes have been shown to be more potent than liposomes [30]. Cationic lipid vesicles have also been described, which comprise cationic cholesterol derivatives with or without neutral phospholipids [31].

Mineral Salts Adjuvants: Mineral salts such as alum and calcium phosphate have been used as adjuvant in vaccine formulations. The addition of potassium alum to diphtheria toxin resulted in a precipitate. The precipitated diphtheria toxin when injected into guinea pigs resulted in a higher number of antibody production when compared to normal non-precipitated diphtheria toxoid Alum is chemically potassium aluminum sulfate was initially used for purifying the protein antigen such as tetanus toxoid and diphtheria toxoid by precipitating them. However, aluminum compounds used as vaccine adjuvants include aluminum phosphate and aluminum hydroxide. In practice, the aluminum phosphate and aluminum hydroxide imperfectly referred as alum but they have different physical characteristics and differ in their adjuvant property. Among this, aluminum hydroxide showed higher adsorption property and found to be more potent than aluminum phosphate [32].

Aluminum salts are effective forming a short-term depot at the site of injection, slowly releasing antigen to the body's immune response system. Since last 90 years, various substances have been tried and used as vaccine adjuvant, most of these substances were never accepted for human vaccines due to their high level of toxicity and the alum salts remain the safest adjuvant approved [33]. However, the use of aluminum adjuvant in manufacturing vaccines is a difficult task because the adsorption of antigen on aluminum type adjuvant is based on physico-chemical characteristics of antigen. Although, aluminum salts remain the only adjuvant approved for human use it has some limitation such as lacking in inducing cytotoxic T lymphocyte (CTL) responses especially to protect from viral infection [34]. Besides, there are well documented problems of aluminum adjuvant that induce inflammation and stimulate local production of erythema, granuloma, subcutaneous nodules and contact hypersensitivity [35] in addition to that, alum cannot be frozen or lyophilized [36, 37].

Reported that aluminum hydroxide has attraction towards eosinophil that leads IgE mediated allergic reaction at the site of injection. On the other hand, Gupta *et al.* [33] stated that aluminum adjuvants have been used for many years for hypo sensitization of allergic patients without adverse effects. Clements and Griffth [38] reported that the alum has been established as a safety adjuvant for vaccine delivery since last 90 years. Furthermore, aluminum consumption from vaccines is far less than that received from the diet or medications such as antacids [35]. Nonetheless, the adjuvants received much attention because of the development of protein subunits made by rDNA technology [39]. The sub unit vaccines are weakly immunogenic when compared to whole-cell vaccines and therefore, need suitable adjuvants for delivering the antigens. Even though alum

has good properties it is not much suitable for small proteins because the alum adsorbed vaccines elicit a short term immuneresponse requiring many boosters for attaining minimum optimal thres hold immune response. Consequently, it is necessary to find a new adjuvant that can replace the alum type adjuvant [40].

Saponin Based Adjuvants (Immune Stimulating Complexes): Immune stimulating complexes (ISCOMs) are cage like nanoparticles composed of saponins purified from the bark of a South American tree, Quillaja saponaria, formulated with cholesterol, phospholipid and antigen. Vaccine antigens need not be incorporated into the particles and most current applications use a mixture of soluble antigens and the antigen-free particle. ISCOMs do not act through any identified PRR; however, they enhance antigen uptake and prolong retention by DCs in draining lymph nodes, induce activation of DCs and lead to strong antibody and T cell responses Although ISCOMs are potent enhancers of Th cells, they do not impose a bias to either a Th1 or Th2 cell response [41].

Unlike most other adjuvants, ISCOMs enable substantial MHCclass I presentation and induce both CD8+ and CD4+ T cell responses to a variety of soluble protein antigens in man and experimental animals [42]. ISCOMs appear to destabilize the endosomal membrane, allowing greater cytoplasmic access for codelivered antigens compared to other forms of antigen delivery [43]. A heterogenous fraction of saponins, Quil A, is widely used for veterinary vaccines. ISCOM formulations have also evaluated in various animal models and a licensed ISCOM based vaccine is used to protect horses from equine influenza in Sweden [44].

Immunostimulatory Adjuvants: The immune stimulatory adjuvants, tend to stimulate immunity with minimal or no tissue damage. These adjuvants are predominantly microbial components and as the name suggests their adjuvant activity is dependent on their ability to stimulate innate immunity. Current understanding of how the body senses infectious threats involves the use of a variety of receptors, which sets the stage for a 'danger' signal that triggers a cascade of innate immune responses, subsequently leading to the recruitment and expansion of cells involved in the development of adaptive immunity. Indeed, several pathogen-derived components such as bacterial endotoxin (lipopolysaccharide [LPS]) and bacterial deoxyribonucleic acid (DNA), including synthetic CpG DNA can generate 'danger' signals and thus have adjuvant activity. Therefore, molecules that activate innate immunity provide a novel class of adjuvants that not only enhance immune responses but also can be selectively used to 'tailor' the quality of the desired response [45].

Lipopolysaccharide Adjuvants: Lipopolysaccharide (LPS) is a major constituent of the outer membrane of Gram-negative bacteria. LPS consists of a poly saccharide termed the O-specific chain, a non-repeating core oligosaccharide and a hydrophobic lipid component termed lipid A LPS is also known as an endotoxin and lipidA is responsible for its toxicity [46]. LPS from Gram-negative bacteria, such as *Escherichia coli*, trigger various path physiological responses via TLR-4 [47] For example, LPS stimulates macrophages, resulting in the production of proinflammatory cytokines such as TNF-_, IL-_, IL-6, IL-10 and IFN-_ Excess production of these cytokines induced by LPS causes endo toxin shock, which is characterized by inflammation, abnormal coagulation, profound hypotension and organ failure. In contrast, the ability of LPS to induce these immune responses has been suggested to make it useful as a potent adjuvant however, the margin between its clinical benefit and unacceptable toxicity is exceedingly narrow [48].

Monophosphoryl Lipid a Adjuvants: In the 1980s, Ribi and co-worker found a LPS-mimetic compound that exhibits potent adjuvant activity but is 100 to 10,000 fold less toxic than LPS This compound is monophosphoryllipid A (MPL), a lipid A derivative that lacks the phosphate attached at the 1-position of the reducing-terminal glucose amine moiety. However, the molecular mechanism underlying the low toxicity of MPL remains to be elucidated is responsible for the endotoxin activity of LPS and induces many inflammatory responses in macrophages [49].

Monophosphoryl lipid A is derived from LPS of *Salmonella Minnesota*, a gram negative bacteria and therefore, is classified as a pathogen associated molecular pattern. Like lipo polysaccharide, monophosphoryl lipid A is thought to interact with TLR4 on APC, resulting in the release of pro-inflammatory cytokines. In a number of preclinical studies, monophosphoryl lipid A has been shown toinduce the synthesis and release of IL-2 and IFN-g, which promote the generation of Th1 responses. Monophosphoryllipid A has been formulated into emulsions to enhance its potency [50].

Immunostimulatory DNA sequence (Cytosine phosphate Guanine oligodeoxynucleotides): As early as the 1890s, a surgeon in New York observed that cancer patients injected with crude bacterial preparations had significantly longer remission periods. Subsequently, bacterial DNA was identified as the primary mediator of anti-tumour immunity in mice [51]. It has now become clear that bacterial DNA, as well as synthetic oligodeoxynucleotides (ODN) containing Cytosine phosphate Guanine (CpG) motifs (CpG ODN), provides a 'Danger' signal that induces vigorous immune responses. Numerous investigators have shown that treatment of animals with CpG DNA can protect against a variety of experimental infectious and non-infectious diseases. Based on encouraging results from mouse models, human clinical studies are now being under taken to evaluate the efficacy of CpG ODN therapy against infectious disease, cancer, asthma and allergy [52]. In this regard, addition of CpG ODN to a commercial hepatitis B virus (HBV) vaccine resulted in significant increases in HBV surface antigen specific antibody response in human volunteers [53]. Furthermore, immunization of human immunodeficiency virus (HIV)-infected individuals with an HBV vaccine in the presence of CpG DNA significantly increased the number of seropositive subjects and increased theHBV-specific lymphocyte proliferative response. Thus, CpG DNA is a promising adjuvant for human vaccines [54].

Synthetic CpG DNA has been evaluated as a vaccine adjuvant in large animals. Unlike conventional oil-based adjuvants, which typically promote Th2 type immune responses that may not be protective against some infections, in these studies, CpG ODN promoted predominantly Th1 type immune responses. For example, cytosine phosphate guanine oligo deoxyribonucleic acid was shown to be an excellent adjuvant for stimulating immune responses against an experimental vaccine based on a subunit protein (gD antigen) of bovine herpesvirus-1 (BHV-1) in mice, sheep and cattle by producing enhanced serum immunoglobin2a levels and IFN-_ in splenocytes or peripheral blood lymphocytes, indicating a more balanced, or Th1 type, response. Interestingly, the use of CpG ODN in combination with low levels of mineral oil enhanced the immune response and reduced the amount of tissue damage associated with conventional vaccine adjuvants in sheep [55].

Incorporation of CpG ODN in a commercial equine influenza virus vaccine resulted in significant enhancement of antibody production against influenza virus [4].Therefore, CpG ODN is compatible with commercially available vaccines and in some cases CpG synergises with conventional adjuvants present in these vaccines, resulting in even greater enhancement of immune responses. This should expedite the application of CpG in commercial vaccines because there should be less need to perform all the safety trials required for new vaccines as new adjuvantsare simply being added to currently licensed vaccines. Indeed, clinical trials are currently in progress to evaluate the benefits of incorporating CpG DNA in commercial livestock vaccines [43].

Mechanism of Action of Adjuvants: Adjuvants are used in many vaccines, but their mechanisms of action are not fully understood [56]. Studies from the past decade on adjuvant mechanisms are slowly revealing the secrets of adjuvant activity. That adjuvants are "the immunologists' dirty little secret". However, recent advances in immuno biological research have revealed several mechanisms by which adjuvants act. Adjuvants may act by a combination of various mechanisms including formation of depot, induction of cytokines and chemokines, recruitment of immune cells, enhancement of antigen uptake and presentation and promoting antigen transport to draining lymph nodes. It appears that adjuvants activate innate immune responses to create a local immunocompetent environment at the injection site. Depending on the type of innate responses activated, adjutants can alter the quality and quantity of adaptive immune responses. Understanding the mechanisms of action of adjuvants will provide critical information on how innate immunity influences the development of adaptive immunity, help in rational design of vaccines against various diseases and can inform on adjuvant safety [57]. The adjuvants employ one or more of the following mechanisms to elicit immune responses.

Formation of Depot at the Site of Injection: The formation of a depot at the injection site is perhaps the oldest and most widely recognized mechanism of action of adjuvants. Antigen trapping and slow release at the site of injection ensures constant stimulation of the immune system for production of high antibody titers [58]. Glenny *et al.* [59] were the first to propose the importance of depot formation in the adjuvant activity of alum. Antigen was detected for 2–3weeks in alumina gel-induced granulomas [60]. Antigens are simply adsorbed onto the alum but the binding is proposed to be due to strong electrostatic interaction between antigen and alum [61] which enhanced antigen uptake and presentation by APCs [62] Various other adjuvants such as water-in-oil emulsions

[Complete Freunds Adjuvant (CFA)] and biodegradable micro-and nanoparticles were shown to act by depot effect to generate prolonged and sustained high antibody titers [63, 64]. AS04, an adjuvant combination consisting of monophosphoryl lipid A (MPL) and alum was shown to induce optimal immune responses only when co-localized with antigen. The presence of alum in AS04 is important in stabilizing the MPL and antigen within the vaccine [65].

Up-regulation of Cytokines and Chemokines Leading to Cellular Recruitment at the Injection Site: Recent studies on the mechanisms of adjuvants have focused on recruitment of innate immune cells at the site of injection. Particulate adjuvants have been shown to create a local pro-inflammatory environment to recruit immune cells [66]. Using genome wide microarray analys is demonstrated that a cluster of genes encoding cytokines, chemokines, innate immune receptors, interferon- induced genes and gene encoding adhesion molecules defined as "adjuvant core response genes" were commonly modulated by alum, MF59 and CpG-ODN at the site of injection. Compared with alum and CpG-ODN (TLR9 agonist), MF59 was a strong modulator of adjuvant core response genes. Chemokines, which play a critical role in tissue specific migration of immune cells, were shown to be up regulated by adjuvants at the injection site [24].

Cellular Recruitment at Site of Injection: Secreted cytokines and chemokines are involved in recruitment of various immune cells to the injection site. These recruited cells secrete cytokines and chemokines, in turn attract other immune cells. All these events lead to formation of a local immuno-competent environment at the injection site. The recruited APCs express various pathogen recognizing receptors (PRRs) both on the surface (Toll-like receptors) and intracellularly, which are recognized and/or are activated by the adjuvants this leads to maturation and activation of recruited APCs [67].

Antigen Presentation: Efficient antigen presentation by major histocompatabilities (MHC) on antigen presenting cells (APC) is important for the induction of adaptive immune response. Many adjuvants including alum, oil-based emulsions and micro particles act by "Targeting" antigens to APCs resulting in enhanced antigen presentation by MHC [68]. Alum was shown to increase antigen uptake by dendritic cells and alter the magnitude and duration of antigen presentation. Antigen adsorption on alum led to an increase in internalization of antigen [65]. Recent studies that have shown that alum does not enter DCs directly, but rather delivers the antigen via abortive phagocytosis [69].

Similarly, MF59facilitatedinternalization of gD2 antigen from type2 herpes simplex virus (HSV) by recruited APCs at the site of injection and increased phagocytosis in human [70]. Antigen size seems to play an important role in modulating the antigen presentation efficiency. Large lipid vesicles end up in early endosome/phagosomes and increases antigen presentation whereas smaller vesicles rapidly localize to late lysosomes leading to reduced antigen presentation [71].

Activation and Maturation of Dendritic Cells: Activation of DCs is essential for induction of adaptive immune responses. Increased expression of major histocompatability class II, activation marker CD86 and maturation marker CD83 leads to enhanced ability of APCs to induce T lymphocyte activation and differentiation [72]. Freund's complete adjuvant lipopolysaccharide (LPS), liposomes, CpG-ODN, MF59, AS04 and *a*-galactosylceramide (*a*-GAL) have all been shown to induce dendritic cell maturation to enhance adaptive immunity [73].

Activation of Inflammasome: Innate immune cells express various pathogen recognition receptors (PRRs) to recognize infectious agents. In recent years, various new families of PRRs have been identified including TLRs, C-type lectin likereceptors (CLRs),nucleotide oligomerizationdomain (NOD) like receptors(NLRs),and Retinoic acid inducible gene1 (RIG-1) like receptors (RLRs).Many immunological adjuvants signal via PRRs or act as ligands for innate immune receptors. In contrast to TLR agonists, particulate adjuvants are not recognized by specific PRRs but they still induce adaptive immune responses proposed that apart from self/non self-discrimination against infection, danger signals from damaged cells can trigger activation of the immune system, first advanced the "danger" hypothesis [74]. Molecules associated with tissue damage such as uricacid, nucleotides, adenosine triphosphate(ATP),and reactive oxygen intermediates and cytokines are released at the injection site due to tissue damage [75].

Activated Antigen Presenting Cells Traffic to Drain Lymph Node: Mature APCs migrate to the draining lymph nodes to interact with antigen-specific B or T cell to activate potent antibody secreting B cells and/or effector

CD8 T cell responses. Some of delivery systems adjuvants may also be capable of moving away from the injection site in lymph and may deliver antigen directly to the lymph node. Nevertheless, the successful delivery of antigen to a lymph node will not necessarily result in the induction of an immune response, since the presence of antigen alone constitutes only 'Signal 1'. To successfully induce an immune response, it is necessary that 'signal 2' is also present. Signal 2 is represented by co-stimulatory molecules and cytokines, which are normally provided by APC and contribute to the priming of T helper cells. The role of the T helper cells is to provide antigen specific help for B cell proliferation and antibody induction and help for cytotoxic T lymphocyte (CTL) responses. In the absence of signal 2, non-responsiveness, or 'Immunological tolerance' will result. In addition to adjuvants which act predominantly as delivery systems, facilitating antigen uptake, transport or presentation by APC, there is a second broad classification of adjuvants which are thought to directly activate signal 2. This is achieved through stimulating the release of cytokines, or the expression of co-stimulatory molecules on APC [27].

Table 1: Functional elements of the immune response

Response/mediator	Function
APC	• Take up, process and present antigens as peptide through MHC I and II pathways
	• Provide co-stimulatory signals
	• DC has unique ability to present antigen to naý¨ve T cells
B-lymphocytes	• Antibody production and APC function
T lymphocytes CD8+ (MHC I)	• CTL Kill infected cells, secrete cytokines that suppresses viral replication
CD4+ (MHC II)	• Promote B cell and CTL differentiation and maturation
Th1	• Secrete IFN, mediates killing of intracellular pathogen
Th2	• Of intracellular pathogens Secrete IL-4
	• Provide help for antibody induction
Co-stimulatory signals e.g. B7, CD40, CD28	• Necessary for effective antigen presentation and T cell activation
Adjuvants	• Promote cytokine induction or co-stimulatory signals or enhance antigen uptake by APCs

Source: O'Hagan *et al.* [27]

CONCLUSION

Looking to the future, many new generation vaccines will consist of purified antigens and well-defined adjuvants and these vaccines will be expected to meet more stringent safety and efficacy requirements. The selection of adjuvants will be much more focused on stimulating specific immune responses and not just enhancing antibody responses. Thus, there will be more emphasis on the quality of the immune response with fewer adverse reactions. The use of better formulations adjuvants in vaccine should dramatically improve vaccine efficacy and reduce economic losses to the livestock industry. Furthermore, these more defined vaccine formulations, together with the understanding of their mode of action, should provide the regulatory agencies with a greater level of confidence in the new vaccines. Those vaccines currently being developed will be safer for use in livestock, which is particularly important for food-producing animals that will eventually be consumed by humans. Further efforts are needed to produce the safest vaccine adjuvant that improves animal health. Further studies to develop and produce new generation of adjuvant vaccine with less adverse effect are needed. The issue of toxicity should be considered while the formulation of adjuvants.

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