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BRIEF REVIEW

Adjuvants and delivery systems in veterinary vaccinology: current state and future developments

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Abstract Modern adjuvants should induce strong and balanced immune responses, and it is often desirable to induce specific types of immunity. As an example, efficient Th1-immunity-inducing adjuvants are highly in demand. Such adjuvants promote good cell-mediated immunity against subunit vaccines that have low immunogenicity themselves. The development of such adjuvants may take advantage of the increased knowledge of the molecular mechanisms and factors controlling these responses. However, knowledge of such molecular details of immune mechanisms is relatively scarce for species other than humans and laboratory rodents, and in addition, there are special considerations pertaining to the use of adjuvants in veterinary animals, such as production and companion animals. With a focus on veterinary animals, this review highlights a number of approaches being pursued, including cytokines, CpG oligonucleotides, microparticles and liposomes.

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Introduction

Vaccines are formulations containing non-pathogenic, and sometimes non-infectious, variants of infectious or transmissible pathogenic agents. When administered to a host, they induce short-term (within weeks) as well as long-lasting (years, sometimes lifelong) specific immunity in the vaccinated host against the particular pathogen imitated by the vaccine. Immunity can be defined as preparedness and capacity to neutralize a given agent by appropriate, protective defence responses.

Since the late 18th century discovery by Jenner that vaccinia (cowpox virus) could be used as a reliable vaccine against the related smallpox virus [108], vaccine development has been largely empirical, using live attenuated or killed microorganisms and/or detoxified versions of their toxins [38, 71, 119, 121].

Still, mass vaccination programmes have eradicated smallpox, dramatically reduced polio in humans, and eradicated rinderpest in cattle [74, 116], and vaccination remains the most cost-effective way of controlling infectious diseases [71]. Closely linked to vaccine development,

vaccine-potentiating compounds (adjuvants) have been developed continuously. They include a broad variety of molecules, some of which have been used widely for many years, and they have many different mechanisms of action [34, 128, 135].

Although vaccines for use in livestock animals such as cattle, pigs, sheep, goat, poultry and farmed fish, as well as in companion animals such as dogs, cats, and horses, have the same general goal as those used for humans, there are a range of different demands to be met. Some of these are related to the use of the animal for human consumption or as companion animals, while others relate to how to design and use vaccines in the best way, considering animal production (rearing) practices, herd epidemiology and animal trade. With livestock animals, there is also a considerable focus on direct (vaccine) and indirect (effect on growth rates) costs of vaccines, which is not a primary concern for companion animals. Additional points are the challenges and opportunities presented by different species (e.g. fish and chickens) with respect to administration practices and routes, especially when attempting to vaccinate large numbers of animals in a short time.

Thus, the use of adjuvants and, especially, the rational development of new adjuvants and immunostimulators for veterinary animals demand special attention, and it is appropriate to consider some recent examples of adjuvant research with a specific focus on their use in animals of veterinary significance.

Background

A number of recent reviews giving excellent updates on the immune system and the mechanisms of adjuvants have been published [1, 48, 125]. For the sake of clarity and consistency, a few central concepts will be restated here.

The pivotal discrimination between threats (pathogens and their toxins) and non-threats (self antigens, innocuous environmental antigens) is exerted by the physiological barriers of the body together with the innate part of the immune system [29, 120]. The central innate cellular actors, the dendritic cells (DCs), when activated, secrete potent pleiotropic signal molecules that activate the adaptive immune system to respond against any foreign molecule. If the innate immune system is not activated, adaptive immune responses are very rarely induced. A limited number of highly conserved molecular structures specific to microbial pathogens, the so-called pathogen-associated molecular patterns (PAMPs) are well-described innate immune triggers [57]. When bound by pattern recognition receptors (PRRs) including the Toll-like receptors (TLR) on DCs, very fast innate responses are induced [120]. The TLR family was named after the Toll protein, which was originally found to be involved in drosophila development [5] and later found to have structural homologies with the human IL-1 receptor, implying immunological functions. This led to the identification of a growing family of vertebrate TLR, each associated with a particular ligand (agonist) type and cellular location (Table 1).

While the distinction between threats and non-threats takes place in the innate immune system self vs. non-self discrimination takes place in the adaptive immune system after activation by the innate immune system. The adaptive immune system, with its wide range of exquisitely fine-tuned antigen-specific responses, controlled by T and B cells in combination with antigen-presenting cells (APCs), brings antigenic specificity to immune responses and provides memory (long-lasting immunity). Memory is a central asset of immunity [20, 71] that relies upon the generation of long-lived optimally reactive antigen-specific T and B cells and long-lived plasma cells producing high-affinity antibodies [20, 71]. A subset of effector T cells, after performing their tasks at the site of infection survive and differentiate into long-lasting memory T cells that are able to respond rapidly to low doses of antigen [86, 125]. Memory B cells rapidly express high-affinity antibodies [125] upon antigenic restimulation. While the composition of the preferred immune response induced by a vaccine depends on the type of infectious agent targeted by the vaccine, a vaccine should always induce immunological memory.

The innate immune system also controls the type of adaptive response. This is mediated by soluble molecules secreted by activated DCs [120, 125, 140, 141] (see Figure 1, Suppl. info.). Some pathogens activate DCs to produce cytokines (including interleukin [IL]-12) that stimulate the development of T helper cells that produce interferon (IFN) γ and promote cell-mediated immunity (Th1 cells). Other types of stimuli [161] induce Th2 cells producing IL-4, IL-5 and IL-13, promoting antibody-based immunity that is efficient at neutralizing extracellular pathogens and toxins [86, 125] (see Figure 1, Suppl. info.).

Table 1 Summary of Toll-like receptor biology (agonists and cellular location)

Toll-like receptor	Structure recognised	TLR location
1	Bacterial lipoproteins	Cell surface
2	Bacterial cell wall components	Cell surface
3	Double-stranded RNA	Endosome
4	Bacterial cell wall components	Cell surface
5	Bacterial flagellin	Cell surface
6	Bacterial lipoproteins	Cell surface
7	Single-stranded RNA	Endosome
8	Single-stranded RNA	Endosome
9	Bacterial CpG DNA	Endosome

Also, generation of immunological memory is intimately dependent on innate immune reactions accompanying the initial encounter between host and vaccine [125].

Adjuvants primarily affect the innate, immunity-shaping pre-primary immune response, and innate immunity has been called "The Science of Adjuvants" [125].

Adjuvants must enhance both antigen specific immune responses as well as increase immunological memory and improve protection through stimulation of optimal types of immunity. Adjuvants also need to have low levels of adverse effects, including, in the case of veterinary animals, adverse effects that negatively influence the growth of the animal, the reproduction rate, the comfort and welfare of the animal or cause carcass blemish. Adjuvants must also be stable, easy to use, and convenient to inject.

Traditional adjuvants are mostly complex and not very well defined mixtures of surface-active compounds, microbial components and/or various polymers and lipids, and they can be classified as delivery systems/antigen modifiers, immune potentiators, or a combination of both (see Table 2) [42, 94]. Delivery systems/antigen modifiers function by presenting, aggregating and/or polymerising antigens [42, 48, 162]. Immunopotentiators include PAMP structures, endogenous immunoactive compounds (e.g., cytokines), and a range of surface active molecules that stimulate innate immune cells directly. Examples of PAMP structures used as immunopotentiators are monophosphoryl lipid A and unmethylated CpG oligonucleotides [42, 48]. After alum (aluminium salts), which is still the only adjuvant class generally approved for human use, emulsions of water-in-oil, or vice versa, stabilised by surfactants, are the most widely used adjuvant systems, which include Freund's adjuvant. Other well-known delivery systems include liposomes and microparticles, which are inert carriers of antigen unless immune potentiators have been added to them [48, 119].

Modern vaccines based on subunits of pathogens, e.g., purified proteins, are often unable to evoke strong immune responses. Therefore, adjuvants, in particular safe and efficient Th1-inducing adjuvants, are in increasing demand

Table 2 : Examples of conventional adjuvants with indication of dominant mode of action

Type	Common name or brand name	Ingredients	Mode of action	Type of immunity (if known)
Water-in-oil emulsion	Freund's complete	Mineral oil, surfactant, killed mycobacteria	D, I, Mix	Th1
	Freund's incomplete	Mineral oil, surfactant	D	Th1
	Montanide®	Mineral oil, surfactant, immunomodulator	D, I	
	Titermax®	Squalene (metab. oil), emulsifier, block copolymer, microparticulate silica	D, I	
Oil-in-water emulsion	Ribi®	Squalene (metab. oil), Tween 80 surfactant, immunostim.: TDM, MPL, CWS	I	
	SAF®	Squalane (metab. oil), Tween 80, block co-polymers, tMDP	I	
	MF59	Squalene (metab. oil), Tween 80, Span 85	I	
Particles	Alum, Alhydrogel	Aluminium hydroxide Aluminium phosphate	D, I	Th2
	ISCOMs	Cholesterol, phospholipid, saponin Quil A, detergent and antigen 40 nm particles	D, I	Th1
	PLG microparticles	Poly[lactide-coglycolide] polyester, bio-degradable	D	
		Micrometer scale[a]		
	Alginate microparticles	Polysaccharide biodegradable	D	
		Micrometer scale[a]		
	Liposomes	Phospholipid vesicles	D	
		Micrometer scale[a]		
Active compounds[b]	Saponin (Quil A, QS21)	Amphipathic, haemolytic triterpenoid saponin	M	Th1
	Monophosphoryl lipid A (MPL)	Chemically modified lipo-polysaccharide core	M, I	
	DDA	Amphiphile dioctadecylammonium chloride	M	
	CpG	Oligonucleotide w. unmethylated CpG motif(s)	I	Th1

The table is based on references [43, 46, 49, 120, 135, 141]

D delivery system (aggregates, particles), *I* immunostimulatory, *M* membrane-active (detergent-like), *Mix* mixed mode, *TDM* trehalose 6,6′-dimycolate, *MPL* monophosphoryl lipid A, *CWS* cell wall skeleton of mycobacteria, *tMDP* threonyl muramyl dipeptide

[a] <10 μm: particles are efficiently taken up by APCs; >20 μm: particles ensure long-term antigen release

[b] Often used together with other substances, e.g., microparticles

[134]. Freund's complete adjuvant [1, 48] is an efficient Th1 inducer, but it has a high and generally unacceptable level of adverse local effects.

Adjuvant safety: efficiently inducing immunity without causing harm

Adverse effects and potential hazards

Mild local and systemic reactions to vaccines and their adjuvants are to be expected as a natural consequence of vigorously stimulating the immune system [129, 137]. These adverse effects are influenced by the interactions of the specific adjuvant and antigen, and the type of adverse reaction will vary according to the vaccine used. For example, a temperature rise, with associated reduction or cessation of feeding, dullness and reduced milk production (if lactating), is frequently associated with live vaccines. Bacterial crude extracts often induce strong local reactions when administered in emulsion, as they may contain strongly immunostimulating compounds like lipopolysaccharide or peptidoglycan fragments, which are responsible for the induction of secondary reactions.

Many vaccine side effects are trivial and of short duration, and they are usually associated with *live vaccines*. Sometimes, adjuvants in a vaccine can cause an adverse reaction, sometimes latent infections can be reactivated, and sometimes an animal may fail to respond [107]. Other well-known side effects include transient swelling at the site of injection and a reaction that may change coat colour in the area, transient fever, respiratory distress, salivation, vomiting, diarrhoea, urticaria, arthritis, uveitis, anorexia, soreness, lethargy, reduced fertility, foetal deformities and abortion. Considering the many millions of doses of vaccine sold annually and used in farm animals, such adverse side effects are very rare [131] when vaccines are used as intended by the manufacturers, and safety testing of vaccines helps prevent their occurrence. With *killed vaccines,* the most common side effects are local, presenting themselves around the injection site, especially if using an adjuvant that delays antigen release. However, such reactions can also occur in vaccines that do not contain an adjuvant, and therefore, it is usually advised that the vaccine, particularly if injected subcutaneously, should be introduced into an area of the animal that is not used for human consumption, such as behind the animal's ear or in the area of the chest wall behind the elbow. Then, if there is any residual vaccine left, or any reaction to it, it will not involve an edible part of the carcass or cause losses in food animals [131].

Injection site reactions

Injection site reactions are of great concern in food-producing animals, as reviewed by Roth [129] and Spickler and Roth [137] for cattle, swine, sheep and chickens. These reactions may lead to unacceptable blemishes in, or decreased quality of, meat intended for human consumption. There are many possible causes of injection-site lesions, including introduction of organisms with a contaminated needle, contamination of the vaccine with live organisms, adjuvant-induced reactions, cytokine release, hypersensitivity reactions (type I, II, III, or IV), trauma, and haemorrhage. Injection of vaccines into the subcutis can result in the development of palpable granulomatous nodules due to adjuvants and other highly immunostimulatory vaccine components that induce persistent local immune responses [50]. Histological changes consist of a localized area of deep dermal or subcutaneous necrosis containing foreign material thought to be adjuvant or vaccine components. The central zone of foreign and necrotic material is bordered by macrophages and multinucleated giant cells with a peripheral zone of lymphocytes and variable numbers of plasma cells and eosinophils (foreign body granuloma) (Figure 2, Suppl. info.). Macrophages usually contain amphiphilic granular foreign material. Lymphoid follicular development at the margins of these lesions can be extensive. Vaccine-associated granulomatous inflammation has also been reported in the peritoneum [123] and muscle [79] of fish, with consequent retarded growth and downgrading during processing.

Vaccine-induced neoplastic disease

Although injection-site lesions often heal without serious consequences, in cats, there is a causal relationship between post-vaccination inflammation and development of fibrosarcomas, osteosarcomas, rhabdomyosarcomas, malignant fibrous histiocytomas, and chondrosarcomas [53]. The antigen load and degree of persistent inflammation and eventual fibroblastic proliferation caused by subcutaneous vaccine administration are thought to be important factors predisposing to tumour development in cats. It is speculated that during tissue repair, fibroblasts or myofibroblasts are stimulated by the immunostimulating substances in the vaccine reaction site, and this, in combination with other factors such as oncogene alterations or unidentified carcinogens, leads to malignant transformation of cells. Tumour development can take months to years, with eventual neoplastic transformation of mesenchymal cells.

The incidence of such fibrosarcomas occurring at sites commonly used for vaccination in cats has increased in recent years [53], and although still regarded as rare

(approx. 1 to 2 cases per 10,000 vaccinated cats), vaccine-associated fibrosarcomas are arguably the most serious vaccine-related adverse events reported in cats (Figure 3. Suppl. info.). More recently, a similar pathological entity has also been reported in ferrets [109] and dogs [154]. Histologically, feline postinjection fibrosarcomas are characterized by inflammatory peritumoral infiltration, multinucleated giant cells, and myofibroblastic cells.

Vaccine-associated sarcomas were recognized in 1991 following the introduction of an alum-adjuvanted FeLV vaccine and the transition from modified live rabies virus vaccines to adjuvanted killed rabies virus vaccines in the mid-1980s. Epidemiological evidence of a causal association between vaccination with aluminium-salt-adjuvanted rabies virus and FeLV vaccines has also been established [69], and vaccine-adjuvant-induced inflammation at the injection site has been implicated as the cause [61]. However, results of a multicenter case-control study [70] of risk factors did not support the hypothesis that the risk of sarcoma formation was associated with specific brands or types of vaccines. A direct association between the presence or severity of postvaccinal inflammation and tumour risk has not specifically been established, but after taking all currently available evidence into consideration, the 2006 American Association of Feline Practitioners Feline Vaccine Advisory Panel Report suggested that veterinarians should use less inflammatory products whenever possible [127]. Adjuvanted rabies vaccines appear to induce greater inflammation than do non-adjuvanted rabies vaccines, and the same appears true for FeLV vaccines. Administering injectable vaccines in specific recommended sites on the body facilitates monitoring vaccine site reactions and managing sarcomas, should they develop [127].

Safety of oil-based adjuvants

Oil-based adjuvants are among the most efficient adjuvants known, and they are in wide use for veterinary vaccines; however, they may induce local and general reactions, like granuloma, abscesses or fever [8]. The mineral oils in use for these purposes are a mix of several hydrocarbon chains of different lengths. Small chains are efficient but induce local reactions, whereas longer chains (>C14) are safer but less efficient. Medium-length chains are preferred (C16-C20). The solubilising and detergent properties of small chains are probably responsible for these local reactions. Highly purified non-mineral oils are well tolerated, as they are rapidly metabolised and eliminated from the injection site, inducing only a weak and transient local inflammatory reaction. In contrast, mineral oils tend to remain at the injection site and are progressively eliminated by competent cells such as macrophages as well as being partially metabolised to fatty acids, triglycerides, phospholipids or sterols. Bollinger et al. [15] demonstrated that, in rats and squirrel monkeys, 30% of the mineral oil disappears during the first month, and the majority of the oil found outside the injection site is in the liver and fat tissues in the form of phospholipids and fatty acids.

Generally, water-in-oil emulsions (W/O) are recommended for bovines, small ruminants, poultry and fish when long-term immunity is required. In the case of foot-and-mouth disease, mineral-oil-based emulsions can protect bovines for one year with one vaccination, whereas formulations based on aluminium hydroxide required two boosts or more. Even if some local reactions occur, W/O emulsions can be used when the degree of protection against specific diseases is enough compared to other formulations or other routes of administration to justify some side effects. This is the case for fish vaccines against furunculosis, where the procedures can be limited to one injection, as the protection is maintained during the whole growing period. W/O emulsions also allow reduction of the vaccine dose or the antigen concentration. W/O formulations can also enhance cellular immune responses. Vaccination of sheep against heartwater with W/O formulations enhances protection against challenge, and these formulations are well tolerated. Miglyol-840-based (medium-chained triglyceride) vaccines containing Newcastle disease virus and infectious bronchitis (IB) virus produced no local reactions at all, nor any vaccine residues, except when the W/O type of emulsion was used, in which case traces of the inoculum were found. In contrast, equivalent vaccines containing mineral oil showed moderate local reactions at the injection site 12 weeks after inoculation when monitored by post-mortem macroscopic inspection [59]. Similarly, Fukanoki et al. [43] detected cyst formation, granulomatous reaction and abscesses in chickens at the injection site 8-16 weeks after administration of oil-adjuvanted vaccines prepared with various liquid paraffins. The vaccine with liquid paraffin mainly consisted of n-C16H34 ~ n-C20H42 and induced less severe problems due to adverse local reactions such as inflammatory responses and persistent residual oil.

Water-in-oil-in-water emulsions (W/O/W) are of interest for their low viscosity and their ability to enhance both short- and long-term immune responses. In the case of foot-and-mouth disease, such formulations are able to protect swine as well as bovines against the disease only four days after vaccination, which can be very useful in cases of outbreaks. However, multiphasic emulsions can also induce long-term immunity and protect bovines against haemorrhagic septicaemia for one year after only one vaccination. Those based on mineral oil are recommended for swine; however, with reactive antigens, it is preferable to avoid vaccination of fattening pigs, because this can

cause carcass blemish. Oil-in-water emulsions (O/W) are very fluid, well tolerated and induce strong short-term immune responses. The oil-phase content is very low, between 15 and 25%, which partly explains their safety. Emulsions based on mineral oil can be used safely for fattening pigs in order to enhance antibody responses against bacterial or viral antigens, and they also increase the potency of live vaccines such as the pseudorabies vaccine.

Vaccines for pets and horses must not induce any local reactions, and therefore, O/W emulsions based on non-mineral oil are used. Water-dispersed liquid nanoparticles (10 to 500 nm) combined with an immunostimulating compound is an interesting new adjuvant concept (so-called "immunosol" adjuvants) [8]. Trials in swine against atrophic rhinitis or pleuropneumonia demonstrated that such formulations could enhance the immune response without inducing local reactions [87, 88], and vaccination of bovines against anaplasmosis gave 100% protection. Moreover, fish trials have confirmed their efficacy and safety, and various trials in pets and horses are ongoing [8].

Conclusions and perspectives

There is no universal adjuvant, and there is always a tradeoff between safety and efficacy [8]. Also, the adjuvant must be adapted to the target species, the antigens, the desired type of immune response, the route of inoculation, and the desired duration of immunity.

Water-in-oil (W/O) emulsions induce strong, long-term immunity but can sometimes induce local reactions with reactive antigens. Non-mineral oils are well tolerated but less efficient with poor immunogens. Multiphasic (W/O/W) emulsions can induce short-and long-term immune responses, and oil-in-water (O/W) emulsions are well tolerated and induce a short-term immune response. Bovines and chickens can be vaccinated with W/O emulsions, whereas swine generally require a well-tolerated adjuvant such as an O/W emulsion. Also, the antigen type influences the selection. Mineral oils can be used when non-reactive antigens such as purified proteins or synthetic peptides are used. Non-mineral oils are preferable with antigens that are more reactive. W/O/W and O/W can be used with live or DNA vaccines. Adjuvants must be able to enhance humoral or cell-mediated immunity according to the mechanism of protection against the disease. W/O emulsions are able to induce cellular responses. W/O/W or O/W enhance humoral responses but have also been associated with enhancement of cellular responses. The duration of immunity also has to be considered, and the selection of the adjuvant is different if short- or long-term immunity is required. The route of inoculation is also important; e.g., subcutaneous and intramuscular administration of the same vaccine formulation can give different immune responses. New generations of oils and surfactants may allow the development of stable, safe and fluid emulsions [8, 26].

Molecularly defined adjuvants: Toll-like receptors and the use of PAMP agonists as adjuvants in farm animals

As described above, the innate immune response to pathogens provides a rapid early reaction to host invasion. This response initiates a range of inflammatory reactions through the expression of signalling proteins by infected and antigen-presenting cells. Proteins such as interferons, cytokines and chemokines mediate this activation as well as the chemo-attraction of a number of cell types including macrophages, lymphocytes, polymorphonuclear leukocytes and mast cells. This, in turn, paves the way for the development of the adaptive immune response and influences the development of antibody- and cell-mediated responses.

Whilst the innate immune response has evolved to enhance the survival of animals in the face of microbial invasion, it can itself result in damage to cells and structures within the host. Indeed, chemokines have been associated with autoimmune and cardiovascular disease in domestic animals (reviewed by Gangur et al. [44]), and many viruses encode genes that mimic chemokines or their receptors [111]. Unsurprisingly, the control of the innate immune system occurs early in the recognition process and is provided by groups of receptor proteins, both at the cell surface and intracellularly, that detect pathogens through engagement with integral pathogen structures known as pathogen-associated molecular patterns (PAMP). Examples of PAMPs are cell wall components of bacteria or particular nucleic acid structures unique to viruses, such as double-stranded RNA [33, 96, 142].

A key component of this pathogen detection system is the toll-like receptor (TLR) family, whose members have been identified throughout the animal kingdom. A principal benefit for the evolution of this system is economy. A relatively small number of TLRs and other receptors, including NOD-like and RIG-I receptors (reviewed by Creagh and O'Neill [24]), are able to detect a large range of pathogens at locations where pathogen-cell interactions can take place, i.e., at the cell surface, within endosomes and within the cell cytoplasm. The identification of such receptors and their agonists provides an opportunity to develop vaccine strategies that both enhance and focus the immune response in ways that are beneficial to the animal. In contrast to the TLR family, homologues for NOD-like and RIG-I-like receptors have not been identified in large farm animals.

A key feature of the TLRs is their conserved structure. Each mature TLR can be divided into three functional domains [12]: an ectodomain that is dominated by a continuous series of leucine-rich repeats (LRR) that forms the pattern-recognition structure, a transmembrane domain that anchors the glycoprotein in the cell membranes and a Toll IL-receptor (TIR) domain, which forms the cytoplasmic signalling domain. Specific PAMP recognition occurs through the LRRs, of which there are between 16 and 28 within the ectodomain. Individual LRRs typically have a structure LxxLxLxxNxL, in which "L" is Leu, Ile, Val or Phe and "N" is Asn, Thr, Ser or Cys (extensively reviewed by Matsushima et al. [101]).

TLR3 provides a useful example of the features of this family of receptors. It is associated with endosomal membranes with the ectodomain directed to the lumen of the endosome itself. TLR3 binds double-stranded RNA [3] and is a key receptor for the detection of RNA viruses [100, 102]. The crystal structure of the human TLR3 ectodomain has been solved at 2.1-angstrom resolution and reveals a horseshoe-shaped molecule. The structure is heavily glycosylated, with a single glycosylation-free surface that could bind ligands, and the conserved segments provide a likely site for homodimer formation [23]. Complete TLR3 coding sequences have been identified in a range of large veterinary animals including cattle (NM_001008664; [21]), pig (DQ647698) and horses (DQ266434). A partial sequence for sheep has also been obtained (AY957614; [104]). Alignment of human and bovine TLR3 (Figure 4, Suppl. info.) demonstrates a high degree of sequence homology, suggesting a conservation of function among higher mammals. This also implies that TLR agonists (discussed below) that have been developed in small animal models should engage TLRs in larger animals, although this must be tested empirically for both toxicity and efficacy. Certainly, bovine macrophages and dendritic cells produce nitric oxide (NO) and cytokines in response to some common TLR agonists including lipopolysaccharide, poly (I:C) RNA and CpG-DNA [158].

Concerning TLRs in farm animals, there is growing evidence that they are actively engaged in response to infection [159]. In cattle, specific increases in TLR2 have been observed during mastitis [46], and porcine macrophages up-regulate TLR2 and 6 in response to infection with *Mycoplasma hyopneumoniae* [110], a major contributor to endemic pneumonia in swine. Many innate response genes, including TLR3, are activated in newborn calves after bovine rotavirus infection [2]. However, there is further evidence of pathogen subversion of this system. TLR2 detects peptidoglycan (Table 1), a major component of bacterial cell walls, including those of *Mycobacterium* spp. However, *Mycobacterium bovis* may use this to enhance their uptake into phagocytes. Bovine viral diarrhoea virus (BVDV) infection has been shown to modulate TLR expression in bovine macrophages [41] and in bovine peripheral blood monocytes [90], influencing the production of NO, TNFa production and type 1 interferon gene expression.

Can the TLR system be used to enhance veterinary vaccines? Engagement of TLRs activates at least two signalling pathways [11] that lead to the induction of antimicrobial genes and inflammatory cytokines [58]. Among these are the type 1 interferons, tumour necrosis factor-alpha and interleukin-6. This suggests that inclusion of a TLR agonist in a vaccine preparation induces a local inflammatory reaction and therefore fulfils the primary role of an adjuvant. TLR agonists also have the capacity to influence the development of the adaptive immune response, probably through recruitment and engagement of dendritic cells [58, 160]. Currently, such agonists are being used as topical antiviral agents in humans [7, 14] and are being included in vaccine vehicles for in vivo use [164]. Indeed, one company (InvivoGen) is offering a mouse TLR agonist kit containing an agonist for each class of TLR. Despite a number of setbacks in the field of human vaccine development, approval has now been gained for the first alum-based vaccine prepared in combination with a TLR agonist (MPL®, Glaxo-SmithKline), named AS04 and developed for use with a hepatitis B vaccine.

The ability of TLR agonists to influence the adaptive immune system has obvious attractions for the development of vaccines against a number of infectious diseases of livestock and man. Addition of such agonists would be used primarily to increase the magnitude of the immunological response, whether antibody- or cell-mediated. This might also enhance the development of immunological memory. Secondly, the formulation could sway the immune response towards a particular Th response and would be of specific interest to those developing animal vaccines against intracellular pathogens, e.g., bovine tuberculosis (*Mycobacterium bovis*), that need a Th1-like response to be counteracted efficiently. Thirdly, inclusion of a TLR agonist might reduce the response time between vaccination and protection. This would be particularly useful for reactive vaccination in response to outbreaks such as foot-and-mouth disease and bluetongue virus or where post-exposure prophylaxis is required, i.e., rabies virus. Currently, most of what we know of the TLR system has been derived from the mouse model and its practical applications, unsurprisingly, are in the field of human medicine. However, the inclusion of TLR agonists in human vaccines heralds the way for use of such compounds in the veterinary field.

Molecularly defined adjuvants: CpG oligonucleotides as PAMP adjuvants in pigs

Certain short, synthetic unmethylated oligodeoxynucleotides (ODNs) containing CpG (5'cytosine-guanine 3') dinucleotide motifs show a strong immunostimulatory activity towards murine B cells [81], emulating the immune stimulatory activity of bacterial DNA preparations [145] (see reviews by Krieg [82] and Klinman et al. [78]). Unmethylated CpG motifs occur much more frequently in bacterial and protozoan DNA than in vertebrate DNA and therefore represent a class of PAMPs.

The immunostimulatory activity depends on the intracellular CpG-specific Toll-like receptor 9 (TLR9) [47], with CpG being taken up by the cell by endocytosis [113]. In humans, B cells and plasmacytoid dendritic cells (pDCs) express TLR9 and are the primary targets for CpG ODNs. The responsiveness of immature plasmacytoid dendritic cells, also called natural interferon-producing cells, to CpG ODNs has been confirmed with pig cells [49].

As originally shown by Krieg et al. [81], CpG ODNs have immediate (8 hours), highly sequence-specific stimulatory effects on mouse spleen cell cytokine and IgM secretion. Methylation of cytosine as well as replacement of either C or G completely abolished activity. Also, two purine bases should flank the CpG motif at its 5' end and two pyrimidine bases should be present at the 3' end for optimal activity, i.e., PuPuCGPyPy. GACGTT is a preferred motif in mice [81], and multiple CpG motifs increase activity. These initial studies in mice have been followed up by studies in cattle, sheep, pigs, horses, dogs, cats and fish (see Mutwiri et al. [113]. CpG motifs with optimal stimulatory activity differ between species, possibly reflecting differences in the specific pathogens that preferentially infect these species. In cattle, Brown et al. [19] showed that *Babesia bovis* DNA stimulates bovine B cells to proliferate and to produce IgG, which is related to the presence of unmethylated CpG sequences in the DNA, which could be mimicked by synthetic ODNs, with one immunostimulating sequence being identified as AACGTT. In humans, the optimal sequences were ATCGAT (type D, phosphodiester, conforming to the PuPyCGPuPy rule) and GTCGTT (type K, phosphorothioate) [156].[1] In pigs, the palindromic type D sequence ATCGAT in a 20-mer ODN with a phosphodiester core flanked by phosphorothioates at both ends was one of the most active motifs [65, 155]. The high activity of ODNs with phosphodiester cores and phosphorothioate 5' and 3' ends (chimeric ODNs) is a general finding with type D ODNs, which are further enhanced by poly G stretches at both ends of the ODN [84]. Type K is optimally active as an all-phosphorothioate molecule, often containing multiple CpG motifs and with no requirement for poly G motifs [82]. In contrast to what has been demonstrated in sheep [16], the cytokine response of pig lymph-node-derived cells was found to be lower than that of pig PBMCs [27], and this is possibly linked to lower constitutive TLR9 expression and a lower frequency in pigs of IFN-alpha-producing cells in lymph node cells compared to PBMCs.

Immunostimulating CpG ODNs generally induce Th1-like responses characterized by IL-1, IFN-α, TNF-α, and IL-12 production, and in some cases, IFN-γ and IL-6, generating cytotoxic T cells [82, 156]. CpG ODNs can even shift an ongoing Th2 response to a Th1 response [113]. As Th1-type adjuvants, CpG ODNs are potentially useful in vaccines against intracellular pathogens, including viruses. It is also of great interest that CpG can work through mucosal routes of administration, enhancing mucosal responses [95, 115].

In vivo, a short half-life and adsorption by non-relevant tissue are limiting factors for the use of CpG ODNs. Natural backbone (phosphodiester) ODNs are degraded within minutes; however, inclusion of phosphorothioate backbones increases plasma half-lives to up to 60 minutes and tissue half-lives to around 48 hours, as shown by studies in mice [113]. Relevant delivery may be enhanced with targeting/delivery vehicles, e.g., transfection reagents, and/or by increasing concentration in the relevant tissue by increasing the dose and/or prolonging release. Furthermore, as with DNA vaccines [153], the efficiency of CpG ODNs depends on efficient translocation of DNA into cells. This can be achieved in a number of ways using traditional adjuvants, with liposomes and polymer-based particles also potentially useful. In a recent study in cattle [80], combinations of CpG ODN, indolicidin and polyphosphazene were tested for their ability to increase immunogenicity of hen egg white lysozyme, and the results indicated an enhancement of humoral and cellular immunity, both by complex formation between adjuvant and antigen and by the ability of polyphosphazene to increase the cytokine-inducing abilities of CpG ODN and indolicidin. Humoral and cellular immunity were increased to the same level as by the oil-in-water adjuvant Emulsigen®. It has generally been found that antigen-specific immunogenicity can be augmented by binding the CpG ODN to the antigen or maintaining close contact between the antigen and the CpG ODN in other ways [78].

Inter-individual variability in responses to CpG ODN has been a frequent finding in outbred populations, which may indicate that CpG sensitivity is partly genetically controlled [49, 65, 103, 113, 157].

CpG ODN—as the only adjuvant tested so far—was found to significantly increase both cell-mediated

[1] Type D and K ODNs [156] are also called type A and B, respectively [84].

immunity and humoral responses against PRRSV in pigs as well as protective efficacy in challenge models, as reviewed by Charerntantanakul et al. [22, 95]. Subcutaneous injection of a K-type ODN with multiple phosphorothioate CpG motifs resulted in significant dose-dependent increases in PRRSV-specific antibody titres, MHC-II expression by PBMCs, IFN-gamma secretion upon antigen-specific stimulation of PBMCs in culture, and protection against disease and death [95]. The "reverse" GpC analogue was much less active.

Our own data (Sorensen et al., unpublished, and Figure 5, Suppl. info.) indicate that type D CpG, but not its "reverse" control ODN (GpC), is able to induce several cytokines in pig PBMCs, while a more type-K-like ODN is active in both its CpG and its GpC form and with increased induction of IL-6 by the GpC form. van der Stede et al. [152] also found the activity of a CpG and its GpC analogue to be similar upon immunization of pigs with ovalbumin when using a high intramuscular dose of 500 μg ODN.

CpG ODNs were also found to increase protection against experimental infection in pigs with the parasite *Toxoplasma gondii* when injected in combination with tachyzoites [83]. CpG ODNs provided better protection and led to higher serum anti-parasite antibody levels in comparison with Freund's incomplete adjuvant.

The highly active ATCGAT palindrome defined by Kamstrup et al. [65] was found to increase the efficiency of a DNA vaccine against pseudorabies virus infection in pigs, increasing protection and humoral as well as cell-mediated immunity [30]. The same vaccine was investigated by the oral route [95], where ODNs were found to increase systemic and mucosal antigen-specific antibody responses. Mixed phosphorothioate/phosphodiester backbones induced stronger IFN-gamma and proliferative responses than phosphorothioate-only ODNs, while antibody responses were similar. In another study, cell-mediated, Th1-type cytotoxic immunity against hepatitis C virus was achieved by immunization with a plasmid coding for the NS3 protein of hepatitis C virus, followed by boosting with recombinant NS3 mixed with CpG and Quil A (both in mice and pigs) [163].

As shown by Kekarainen et al. [73], CpG motifs in viral genomes (in this case, porcine circovirus type 2, PCV2) can also modulate host immune responses in a context-dependent way. For example, CpG motifs originating from the PCV2 Rep gene were superior for inhibiting IFN-alpha production induced in recall responses with pseudorabies virus compared to other viral CpG motifs.

Another use of CpG-based immunostimulators is for immediate and transient protection against infection, which has been demonstrated for a range of bacteria, viruses and protozoa (reviewed by Klinman et al. [77] and Mutwiri et al. [113]). Irrespective of the inoculation route, CpG ODNs protected mice against lethal doses of the intracellular bacteria *Listeria monocytogenes* and *Francisella tularensis*, provided that the ODNs were administered prior to infection (optimally 3-14 days before, and weaning off at 21 days before). If ODNs were repeatedly injected, administration of the specific pathogen within the protected time frame led to the creation of prolonged pathogen-specific immunity [77]. For "slow" pathogens, such as *Leishmania major*, protection could be achieved post-infection [77]. The effect of mucosal delivery (intrapulmonary) of CpG ODNs was studied in sheep, and it was found that a transient (lasting 2-5 days) systemic acute-phase and antiviral response took place, with the effect of the ODN greatly increased by including an oil-in-water adjuvant (Emulsigen®) [115]. These effects were also seen upon sc as well as intratracheal administration. In rainbow trout immunized with a combination of different salmonid rhabdovirus DNAs, protection against infection lasted for 4 days after vaccination and was apparently related to induction of type I interferon [35]. However, these effects are apparently not due to CpG, as the involvement of TLR9 could not be demonstrated [118]. Instead, the effect could be caused by the expressed rhabdovirus glycoproteins [76].

Such immediate innate responses mediated by CpG ODN do not rely on specific immunity and are not antigen dependent. The protection is short-lived (days), is non-specific, and has no memory component. This has relevance for peri-exposure prophylaxis of herds of production animals, e.g., against foot-and-mouth disease outbreaks in neighbouring herds, as has been investigated in mice, in which protection against challenge with 5 out of 6 different serotypes of foot-and-mouth disease virus (FMDV) was achieved [66]. The protection lasted for 14 days and, rather surprisingly, was also seen when ODN was administered simultaneously with or up to 12 hours after inoculation with the virus. Post-virus administration, however, had little effect on viraemia. For practical use as a means of FMDV control, both protection against disease and inhibition of virus secretion are pivotal. This protection strategy does not interfere with monitoring by analysis of antibodies in blood samples (serodiagnosis). However, more recent data indicate that CpG has no effect on innate-mediated early protection against FMDV-promoted disease in pigs [4], in contrast to the findings in mice. CpG ODN mixed with Emulsigen® and injected intramuscularly did induce IFN-α for at least 4 days after injection, but it did not protect pigs against disease at FMDV challenge 2 days after a combined injection of FMDV vaccine and CpG. Furthermore, with this specific vaccine, CpG did not increase the protective effect of the vaccine, as seen when challenge was performed more than 7 days after vaccination. This demonstrates the difficulties in comparing effects of this type of adjuvant between species.

As small, stable, and easily synthesizable molecules, CpG ODNs hold promise as molecular adjuvants. Their strongly Th1-biased immunostimulatory activity, without the adverse effects frequently seen with traditional Th1-inducing adjuvants (mineral oil adjuvants, see above) complements the Th2-skewed activity of generally approved aluminium-salt-based adjuvants. They have the potential to stimulate mucosal immunity, and they show immediate activity against intracellular infections. The challenge is to define CpG ODNs with optimal activity in the species of interest and broad activity between individual animals. More knowledge is needed to define the optimal combination of antigens and ODNs, to establish practical administration routes, and to elucidate long-term adverse effects related to the possible generation of anti-DNA autoimmunity.

Molecularly defined adjuvants: endogenous mediators for targeting lymphocytes to mucosal surfaces

Retinoic acid

Efficient induction of mucosal immunity most often employs vaccination at mucosal sites, as parenteral immunization is generally ineffective at generating mucosal immune responses. This relates to the compartmentalization of mucosal and systemic immune responses, which is mainly based on the selective expression of homing receptors on lymphocytes. These receptors target effector and memory cells to specific ligands expressed in the extralymphoid site of the original antigen encounter [106]. Thus, lymphocytes primed in the gut-associated immune system, such as Peyer's patches or mesenteric lymph nodes express the integrin $\alpha4\beta7$ and the chemokine receptor CCR9 and subsequently migrate to the mucosal tissues of the small intestine, where their ligands, mucosal addressin cell adhesion molecules 1 and CCL25, respectively, are expressed by postcapillary venules and intestinal epithelial cells, respectively. In contrast, lymphocytes primed in peripheral lymph nodes express ligands for vascular P- and E-selectins and the chemokine receptors CCR4 and/or CCR10. Local resident dendritic cells (DC) play an important role in instructing naïve lymphocytes to express the appropriate homing receptor profile [63, 105, 138]. For example, DCs isolated from Peyer's patches or mesenteric lymph nodes induced $\alpha4\beta7$ and CCR9 expression in co-cultured T or B cells, whereas DCs isolated from peripheral lymph nodes promoted the expression of P- and E-selectin ligands. The ability of intestinal DC to confer gut tropism to T cells may not necessarily be an attribute of a distinct, tissue-restricted DC subset [63, 105]. Rather, the local tissue (cytokine) environment and microbial signals play a dominant role in shaping the mucosal imprinting capacity of DCs [31, 32, 68].

In addition to their ability to promote gut tropism of T cells, mucosal DCs are characterized by their capacity to induce IgA responses and by their preferential secretion of the cytokines IL-10, TGF-β and IL-6 [55].

Interestingly, the ability of intestinal DC to promote lymphocyte targeting to the gut has been linked to their unique expression of retinoid hydrogenase enzymes, which convert dietary vitamin A to retinoic acid. Retinoic acid is a natural bioactive metabolite of vitamin A that regulates a broad range of biological processes including inflammation and cell differentiation and proliferation through binding to specific nuclear retinoid receptors that are present in many cell types, including T and B cells [10]. With regard to lymphocyte differentiation, retinoic acid has been shown to directly up-regulate $\alpha4\beta7$ and CCR9 expression on T cells [56]. In addition to this modulation of the homing properties of T lymphocytes to mucosal sites, gut DC-derived retinoic acid was also identified among other factors as an important inducer of IgA secretion in B cells [106].

Also, in recent experiments performed in large animal models, the potential of peripheral DC to be modulated towards a mucosa-type DC was investigated. This would enable the induction of mucosal immune responses after parenteral administration of antigen, and some support for the feasibility of this has been obtained [132]. Porcine-monocyte-derived DCs pre-treated with the vitamin A derivative all-trans retinoic acid (RA) acquired several attributes characteristic of mucosal DC, including (i) secretion of the cytokines TGF-β and IL-6, (ii) the capacity to induce IgA responses and (iii) the ability to induce expression of mucosal homing receptors in co-cultured lymphocytes [132]. Transwell experiments in which the cell populations were separated in an in vitro porcine co-culture model revealed that RA-treated monocyte-derived DCs mediate their effects through soluble factors rather than through cognate receptor interactions with lymphocytes. Although no IL-10 was detectable in supernatants from RA-treated monocyte-derived DCs, RA was found to induce TGF-β. Addition of a pan-TGF-β neutralizing monoclonal antibody reduced the capacity of the RA-treated monocyte-derived DCs to induce integrin $\alpha4\beta7$ up-regulation significantly in co-cultured lymphocytes. Up-regulation of CCR9 mRNA induced by RA-treated porcine monocyte-derived DCs was not affected by the presence of the TGF-β neutralizing monoclonal antibody. Thus, $\beta7$ integrin and CCR9 expression in this large-animal in vitro model appear to be differentially regulated in a way that is similar to what has been described in the mouse system [63, 105].

The ability to induce specific IgG and IgA responses has also been investigated using foot-and-mouth disease virus

(FMDV) as an antigen [132]. It was shown that RA-treated monocyte-derived porcine DCs are potent inducers of specific IgG and IgA responses. Similar experiments in the presence of an RA receptor antagonist revealed that $\alpha 4\beta 7$ integrin and CCR9 mRNA expression were suppressed, but IgA production remained unchanged, suggesting that a different mechanism contributes to these effects on T and B lymphocytes.

A role of RA as a mucosal immune modulator would be consistent with the location of mucosal DCs in the vicinity of RA-producing intestinal epithelial cells [85] and the autocrine production of RA by intestinal DCs. Initial *in vivo* immunization experiments revealed that the application of RA in a vitamin A deficiency model in rats enhanced the antibody response to tetanus toxoid [28]. The transcutaneous application of RA with cholera toxin and whole inactivated influenza virus augmented the intestinal anti-influenza virus response compared with a combination of cholera toxin and virus [136]. Whether migration of peripheral DCs to mucosal inductive sites or the imprinting of mucosal homing receptor expression in local draining lymph nodes accounts for these effects needs to be investigated in future studies [13, 36, 132].

These findings suggest a novel role for RA as a mucosal immune modulator targeting DCs, which is of particular interest for adjuvant and also nutritional applications.

Interferons

Dendritic cells (DCs), and particularly the interaction between conventional 'myeloid' DCs (cDCs) and plasmacytoid DCs (pDCs), are important for efficient immune defence functions (pDC are also known as natural interferon-producing cells). The host immune system is often manipulated by viral pathogens that infect DCs. The manner in which different viruses interfere with DC function depends on both the virus and the subset of DCs involved. The recognition of viral nucleic acids by pattern recognition receptors (PRRs) is the first step in inducing the innate immune system. Type I interferons, central mediators in antiviral innate immunity, along with other cytokines and chemokines, disrupt virus replication. Recent studies have indicated at least two distinct pathways for type I interferon induction by viral infection: one mediated by retinoic-acid-inducible gene-I (RIG-I) and one mediated by melanoma differentiation-associated gene 5 (MDA5).

In addition to their direct antiviral activity, type I interferons also possess major immunomodulatory abilities. *In vivo* experiments have shown that type I interferons can potently enhance humoral immunity and promote isotype switching [89]. Type I interferons secreted by pDCs have been shown to induce B-lymphocytes to differentiate into antibody-producing plasma cells and to be necessary for the production of both specific and polyclonal humoral immune responses after influenza virus infection [124]. With regard to an adjuvant effect in vaccination experiments, type I interferons have been shown to have potent activity when co-administered with inactivated vaccine preparations administered by the parenteral route [60, 150]. Humoral IgG and IgA levels were significantly elevated in a dose-dependent manner when interferon-alpha was co-administered with an inactivated influenza vaccine. In contrast, intraperitoneal application of interferon-alpha at a site distant from that of the vaccination antigen instead decreased the humoral immune response [150]. Characterization of the cell population activated by co-administration of interferon-alpha revealed that populations corresponding to cDCs and pDCs were involved. Therefore, trafficking of antigen-presenting cells towards the site of vaccination may, at least in part, explain the mechanism underlying the adjuvant activity of interferon-alpha when co-administered with inactivated vaccine preparations [150].

To investigate the species specificity of commercially available interferon-alpha, human (HRT-18, Panc), simian (Vero), and bovine (MDBK) cells were treated with commercially available human recombinant interferon-alpha expressed in *Escherichia coli* (Sigma-Aldrich®). The reactivity of the cells was tested by the induction of the antiviral Mx protein using the anti-Mx antibody M143 [39]. As shown in Figure 6 (Suppl. info), the human recombinant interferon induced Mx protein synthesis in human, simian and bovine cells in a dose-dependent manner. Interestingly, the strongest induction was observed in the cells of bovine origin, and this should be investigated further.

Well-defined carrier/delivery systems: particulate antigen delivery systems for mucosal immunity

The delivery of purified antigens by encapsulation into particulate, non-living, systems offers a range of advantages, such as 1) enhancement of the immunogenicity of soluble antigens, 2) increase of the antigen uptake, 3) protection of the antigens, 4) reduction of the antigen dose, and 5) control of antigen release. Another major advantage is the capacity for mucosal antigen delivery. This route is the most relevant for triggering protection, since almost 90% of all pathogens, regardless of which species, enter and initiate infection at mucosal surfaces [45]. IgA, the main mucosal immunoglobulin, cannot be induced by parenteral inoculation, and thus systemic (parenteral) vaccination only triggers incomplete protection against mucosal infections, although partial transfer of IgG1 and

IgG2 from serum into the lung has been demonstrated in cattle [17]. However, soluble antigens are poor immunogens when delivered by the oral or nasal route, and adjuvants or delivery systems for these kinds of antigens are needed to induce mucosal immune responses [25]. Mucosal immunization elicits strong mucosal immune responses, even in remote mucosal sites (the existence of a common mucosal immune system has been confirmed in large animals [133]), and in addition, a systemic immune response, depending on the size of the particles.

The advantages of particulate antigen delivery systems for mucosal immunization include 1) protection of antigen against the gastrointestinal environment (acid and proteolytic enzymes), 2) enhancement of antigen translocation to the mucosa-associated lymphoid tissue, 3) ease of delivery via the oral or intranasal route. A variety of particulate systems has been developed. Here, we will focus on microparticles and liposomes.

Microparticles: uses in ruminants

Microparticles (MPs) can be derived from different polymers, including poly-lactide-coglycolide (PLG) (Figure 7, Suppl. info.), alginate, and starch or other carbohydrate polymers. Compared to PLG MPs, the manufacture of which requires the use of organic solvents that may alter antigenic epitopes, alginate MPs are produced under mild conditions [45, 126], using an inexpensive, non-toxic, naturally occurring polysaccharide that is also biodegradable. Alginate MPs are compatible with a variety of antigens, have proved useful for circumventing maternal antibodies in vaccination of young animals [75], and also improves capture of DNA by antigen-presenting cells (APCs) [153]. A direct adjuvant role for alginate has also been suggested [126]. Biodegradable MPs were originally developed for oral delivery [126], however, although demonstrating high efficacy in small animal models, few of them have been tested in large animals.

For parenteral delivery, a single injection in cattle of a *Staphylococcus aureus* lysate encapsulated in biodegradable PLG microparticles (Sta-MP) was investigated using a 50/50 mixture of small (<10 μm) and large (>10 μm) particles emulsified in Freund's incomplete adjuvant (FIA) [117]. This resulted in an antibody response similar to the one obtained when emulsifying the antigen in FIA, although at a lower level, likely due to the slow release of the antigens. The Sta-MP-elicited antibodies supported phagocytosis at a higher level than unencapsulated antigens. It has been suggested that the small MPs (<10 μm) are taken up by APCs, while the larger ones (>10 μm) serve as an antigen depot, slowly releasing the antigen over an extended period of time. It can therefore be envisaged that a long-term functional antibody response can be elicited by a single injection. In another study, the delivery of naked DNA plasmids encoding protective foot-and-mouth disease (FMD) antigens and ovine GM-CSF was investigated in sheep after formulation in PLG MPs, and this was compared to administering plasmids alone, plasmids in alum or plasmids in lipofectin, using intradermal and intramuscular injection [114]. The formulation in MPs proved to be the only one able to trigger an FMD-specific cell-mediated immune response. The level of protection against FMDV challenge was similar to that obtained using a conventional vaccine. In contrast, no immunity was induced in sheep by the nasal route, even in the presence of the *E. coli* labile toxin. These findings confirm that PLG MP can be used for DNA vaccination by the parenteral route.

The efficacy of MPs in inducing mucosal immunity was evaluated in cattle, and it was demonstrated that after oral delivery, particles smaller than 5 μm were rapidly translocated to the lymphatics and disseminated to the systemic lymphoid organs, while MPs larger than 5 μm remained in the Peyer's patches with a very slow translocation to the efferent lymphatics [75, 165]. Therefore, small MPs may induce a systemic as well as a mucosal immune response, whereas larger MPs may only induce a local response. The efficacy of alginate or PLG-MP to elicit a mucosal immune response against model antigens such as ovalbumin (OVA) and porcine serum albumin (PSA) was also studied in cattle. In one study, OVA was encapsulated in alginate MPs of which 70% had a diameter of less than 10 μm and 30% had a diameter of less than 50 μm [17]. These OVA-MPs were then entrapped in larger alginate macrospheres (4-5 mm) and placed in gelatine boluses for oral delivery to cattle. This system has been shown to release the encapsulated material into the lower intestinal tract. Compared to control animals, cattle receiving two oral regimens (each including five daily doses of 5 mg OVA) with a 2-week interval, had a significantly larger number of anti-OVA IgA antibody-secreting cells (ASCs) in their bronchoalveolar lavages (BAL). This study also showed that priming by the subcutaneous route with OVA greatly enhanced the mucosal response, with larger numbers of anti-OVA IgA, IgG1 and IgG2 ASCs in the BAL as well as anti-OVA IgG1 ASCs in the blood [17]. This indicates that the antigen was well protected by alginate macrosphere encapsulation and able to trigger an IgA response even in the pulmonary mucosal system after oral administration. Also, oral boosting with an antigen encapsulated in MP enhanced the protection achieved by subcutaneous vaccination with the same antigen.

A comparison between oral and intranasal delivery in cattle of pig serum albumin (PSA) contained within alginate MP [126] did not lead to the same conclusions as in the above study, as it was found that only intranasal

delivery led to a PSA-specific humoral response, but with IgG1 being the only significant immunoglobulin isotype in serum, saliva and nasal secretions, and with no cellular response and no statistically significant anti-PSA IgA response. The lack of a clear mucosal IgA response is surprising; however, 64 % of the particles were smaller than 2 μm and only 5% were larger than 5 μm, which is different from the above study. It is possible that, in cattle, this range of sizes allows the particles to bypass the local lymphoid tissues and go directly to the draining lymph nodes. Intranasal delivery was also investigated in cattle by Kavanagh et al. [72], using OVA-containing PLG MPs smaller than 2.5 μm. The objective was to define the optimal dose of OVA and timing of the booster inoculation to elicit an IgA response. A significant, but moderate, mucosal IgA and serum IgG response was achieved with 1 ml of PLG-MP containing 1 mg of OVA administered into each nostril. The determination of the timing of the booster inoculation did not result in clear-cut differences, although a boost at 3 weeks showed higher overall OVA-specific IgA levels. This finding might be due to sustained release of the antigen, which may give unpredictable results. Nevertheless, this sustained antigen release allowed the generation of a prolonged IgA response up to 5 months following an intranasal boost given at week 5.

These studies illustrate that mucosal immunization is more challenging than parenteral delivery. Critical factors include the size and composition of the MP as well as the protocol and anatomical site for the mucosal delivery. In ruminants, the gut-associated lymphoid tissues (GALT) are located as patches in the jejunum, ileum, colon and rectum, and M cells have been identified at all of these sites [92]. However, internalization of latex beads (250 and 610 nm) was only demonstrated with M cells of the ileum and not with those of the jejunum [92]. The nasal-associated lymphoid tissues (NALT) in ruminants are located in the nasopharynx, posterior to the opening of the Eustachian tube [92]. Besides the NALT, the lymphoid tissues of the Waldeyer's ring are even more developed in farm animals. They guard the nasal (pharyngeal and tubal tonsils), oral (lingual, palatine and soft palatine tonsils) and auditory passage into the pharynx. M-like cells have been identified in the pharyngeal and palatine tonsils of sheep [92], and the function of the ovine NALT as a potent antigen-sampling site has been demonstrated [139]. Furthermore, in ruminants, other lymphoid tissues, such as bronchus-associated lymphoid tissue (BALT), can be induced by antigen exposure [92]. Thus all of these lymphoid tissues play a major role as active inductive mucosal sites. The anatomical localization of intranasal immunization, depending on the particle size and delivery device, is thus also a critical point in triggering optimal mucosal responses. It will determine if the antigen-loaded MP will reach and be retained in the relevant tissues for sustained antigen release or be entrapped by and expelled with the nasal mucus.

To further characterize the uptake of alginate MPs and their ability to trigger a mucosal response, in vitro experiments were performed in a sheep intestinal "loop" model [75, 112] using PSA as the encapsulated antigen. First, a comparative analysis was done with MPs smaller than 10 μm and larger than 10 μm. The results revealed that only MPs smaller than 10 μm attached to the follicle-associated epithelium overlying the Peyer's patches within which M cells are contained, confirming previous observations made in mice and pigs [75]. The uptake of PSA-MPs was confirmed by the presence of numerous PSA-specific antibody-secreting cells of IgG and IgA isotypes in the Peyer's patches [75]. Induction of a systemic humoral response was also observed, but no cellular response was observed [112]. Although no cell-mediated immune response was detected in these studies, there is evidence that MPs can induce cellular immunity in mice following mucosal immunization with proteins and may preferentially induce a Th1 response [112].

These findings demonstrate that MPs with a diameter less than 10 μm can be an efficient antigen delivery system for oral immunization if they can reach the relevant mucosal lymphoid tissues where cellular uptake takes place. Similarly, Rebelatto et al. [126] reported an old study demonstrating that, in calves, tonsils could absorb resin particles of 1-5 μm in diameter. Then, as described above, an optimal balance between differently sized MS should be defined to allow both mucosal and systemic immune responses and long-term immunity. Appropriate delivery to the mucosal inductive sites is a critical requirement for vaccine efficacy.

Microparticles: uses in pigs

Since 1992, when Weng et al. reported on the protective effects of an oral microencapsulated *M. hyopneumoniae* vaccine against experimental infection in pigs [157], only a few studies have been published on the usability of microparticles for vaccine delivery in pigs. Some of these delivery systems remain unsuccessful or are largely untested for oral vaccine delivery in large animals [93, 112]. Felder et al. [37] examined the feasibility of peroral immunisation with microencapsulated *Escherichia coli* and detached fimbriae to prevent enterotoxigenic *E. coli* infections in pigs. Various MP formulations designed to deliver priming and booster doses were fed to newborn and weaned pigs. No significant serum IgA antibodies were induced, and after peroral homologous challenge 19 days after the booster vaccination, *E. coli* colonisation was not reduced.

The apparent discrepancy between the results obtained using small and large animals suggests that additional

barriers may impede the local trafficking of MP throughout the GALT. Torche et al. [149] investigated the systemic immune response after administration to pigs of a model antigen (IgY), either in solution or encapsulated in PLG MP. A surgical experimental model ensured local delivery of IgY at different GALT locations, including the intestinal lumen, in mesenteric lymph nodes and within Peyer's patches. It was found that PLG MPs were able to elicit a combined serum IgG2/G1 response with a predominance of IgG1 when administered locally. PLG MP could be a potential oral delivery system for antigen; however, these results further illustrate the difficulty associated with immunizing large animals such as pigs.

During the last ten years, work has been performed on the targeting of MPs to antigen-presenting cells to improve their potency. Thus, modified PLGA MP with specific ligands on their surface for increasing their cellular uptake have been investigated *in vitro* on pig alveolar macrophages [146, 148]. An *ex vivo* assay was also performed on a pig ileal Peyer's patch segment to confirm the traffic of PLG MP throughout M cells [147]. Brandhonneur et al. [18] studied cationic (poly-L-lysine-rafted) PLG MPs and ligands such as wheat germ agglutinin, mannose-PEG3-NH2, and arginine-glycine-aspartic acid grafted on PLG MPs in this system and found their uptake by macrophages to be increased. However, the relative contribution of specific and nonspecific uptake varied according to the ligands, and was dependent on the particle-to-cell ratio. Jiang et al. [62] describe the potential of mannosylated chitosan microparticles to target mouse macrophage mannose receptors. Non-grafted chitosan microparticles were shown previously to be effective in pigs in inducing specific immune responses against *Bordetella bronchiseptica* [67].

Liposomes: uses in ruminants

Due to their flexibility with regard to size, composition, charge and bilayer fluidity, as well as their ability to incorporate large amounts of antigens and a variety of hydrophilic or hydrophobic compounds, liposomes are interesting antigen delivery systems. In vaccine applications, their main functions are to protect the antigens from clearance in the body and to deliver the antigens to professional antigen-presenting cells (Fig. 1). They are classically composed of natural, biodegradable, nontoxic and nonimmunogenic phospholipids in which antigen is either enclosed within the core, corresponding to an aqueous phase, or intercalated into the lipid layer [9, 54]. Liposomes might be used as carriers for proteins, peptide-derived antigens and nucleic acids encoding antigens (DNA plasmids, mRNA) or targeting genes (siRNA) [9, 51, 64].

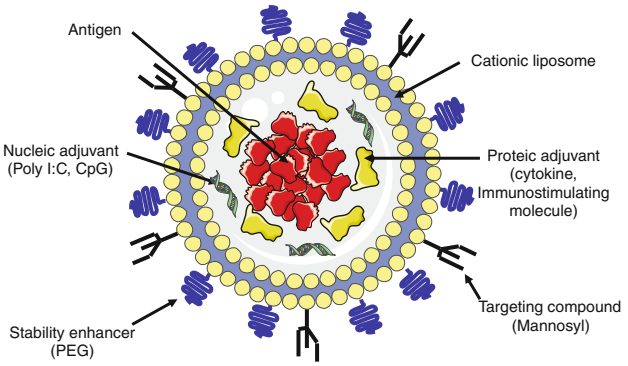

Fig. 1 Schematic diagram showing the possibility of incorporating various components with potential for improving vaccine delivery and efficacy into liposomes

Liposomes may have natural adjuvant properties, depending on their lipid composition. For example, an increase in IFN-γ secretion by murine spleen cells was observed *ex vivo* when phosphatidylserine was used as neutral lipid in liposome formulation [6]. Liposomes can also influence antigen presentation. It has been described that an antigenic protein delivered by conventional liposomes was processed via the class II molecules of the major histocompatibility complex (MHC II) pathway, while MHC I presentation could be achieved with pH-sensitive liposome carriers [91]. Also, liposomes can be made directly immunostimulatory by including microbial PAMPs. For example, antigen-presenting cell activation through interaction with TLR or CD14 was reported when lipopolysaccharide (LPS) derivatives were added to liposome formulations [54]. By optimizing the lipid composition and biophysical properties of liposomes, it is also possible to target specific tissues or cell types. Several groups report that cationic liposome-encapsulated antigens are phagocytosed by dendritic cells to a much greater extent than anionic particles, inducing their activation and leading to increased efficiency of presentation to the immune system [40, 130, 144] (Figure 8, Suppl. info.). The mechanism behind these differences in passive targeting of antigen presentation remains unknown. Active cell-targeting can be achieved by the use of engineered lipids. For example, it has been observed that addition of mannosylated phosphatidylethanolamine, which is specifically recognised by mannose receptors, leads to specific delivery to CD11c+ antigen-presenting spleen cells after systemic injection [52]. To target other cell types, liposomes coated with immunoglobulin or Fab fragments have been developed. In this case, specific attachment of "immunoliposomes" to cells is a function of the affinity and avidity of the Ig towards a cell-surface marker [99]. In addition, liposomes have been used widely for oral and intranasal delivery of antigens in mice. For this purpose, improved delivery was

achieved by conjugation of the liposomes with a recombinant B subunit of cholera toxin or IgA [45, 165].

Very few liposome formulations have been used in veterinary medicine; this includes uses as a novel antitumor drug delivery system or vaccine for dogs and cats [98, 122, 151]. Tana et al. showed that cationic liposomes were efficient in promoting diphtheria toxin A-chain gene delivery in cells infected with bovine leukemia virus after intratumoral injection [143]. Subunit vaccines based on liposome-encapsulated antigenic proteins have also been assessed. Subcutaneous immunisation against bovine herpesvirus type 1 with liposome-entrapped herpesvirus antigen and IL-12 has been reported to elicit induction of antigen-specific cellular and humoral immune responses [9]. More recently, liposomal delivery of recombinant antigen has been described to enhance the immune response against *Brucella abortus* in mice [97]. Assessment of intramuscular delivery in cattle of a liposome-based DNA vaccine against bovine viral diarrhoea virus was described by Harpin et al. [51]. These authors reported that lipoplexes, consisting of a plasmid encoding the major glycoprotein E2 and conventional cationic liposomes, were able to enhance the immune response compared to naked DNA.

A new generation of engineered liposomes holds promise for even more efficient immunostimulation. For example, immunization of cattle with recombinant major piroplasm surface protein from *Theileria sergenti* encapsulated by mannan-coated liposomes has been reported to be an efficient inducer of T-cell immune responses [64].

Conclusions and perspectives

There is considerable interest in mucosal routes of vaccination in livestock, since they obviate the problems associated with injection, stress and handling of the animals and do not require trained personnel, and as a large number of infections are of mucosal origin. Both microparticles and liposomes have proved efficient for mucosal vaccine delivery in small-animal models [45]. Their study in large animals is still at an early stage; however, the results achieved warrant further experiments. Microparticulate delivery represents an efficient and cost-effective vaccine strategy with particular relevance for developing countries. Oral delivery, the most attractive route for mucosal immunization of livestock, might be used to achieve mucosal immunity, not only in the digestive tract, but also in the lungs. Even so, nasal vaccination may provide a practical alternative to oral immunization because of its relative accessibility, high permeability of the local lymphoid tissues, less acidic pH and lower levels of enzymatic activity compared to the gut lymphoid tissues [139]. This may be of particular importance in ruminants, since orally administered antigens have to pass through the rumen before reaching the target GALT.

The studies reported above offer preliminary proof of concept that encapsulating antigens in a particulate system protects the antigen, facilitates and controls its uptake, either by antigen presenting cells or by the M cells of the nasal or intestinal mucosae, and triggers an immune response. Further enhancement of mucosal immune responses may be achieved by optimizing the antigen dose and the size and composition of the particles, by improving cell targeting, and by incorporating relevant adjuvants and immunomodulators. Particulate delivery systems can accommodate antigens, immunomodulators and targeting molecules at the same time, and the simultaneous presence of antigen, adjuvant and a targeted antigen-presenting cell (APC) will efficiently enhance APC activation.

Conclusions

The development of novel, safe, efficient, and yet cost-effective vaccine formulations is a great challenge, although a large number of innovative strategies are being investigated, and significant advances have been achieved. The majority of these studies are conducted in small-animal (rodent) models, and the translation of results from small to large animals is not a trivial task. Apart from a whole range of differences between species in the finer details of the molecular mechanisms and the physiology of the immune system, there are also major differences related to general physiology that invite the use of substantially different vaccine strategies and methods, especially when considering mucosal immunization. There are also interesting differences in reaction patterns towards different types of PAMPs, including CpG-containing DNA, possibly evolutionarily adapted to the spectrum of infections that commonly occur in a given species. Other variations in PAMP responsiveness are seen. For example, there are major differences between species in their responsiveness to lipopolysaccharides. On top of this, there are a number of practical considerations related to production/rearing practices, economic concerns and animal "use" considerations (meat quality, fur blemishes, etc.) that are not relevant for humans and for laboratory rodents.

Therefore, the potential of new vaccine formulation strategies for improving veterinary vaccines still remains largely unexploited, although there is a great need for needle-free, cost-effective, single-shot vaccines that trigger long-lasting immunity in large animals, including livestock.

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