CHAPTER 2.3.1.

THE APPLICATION OF BIOTECHNOLOGY TO THE DEVELOPMENT OF VETERINARY VACCINES

INTRODUCTION

The practice of vaccination for the prevention of animal disease has been used for centuries and has proven to be a powerful tool for the alleviation of animal suffering as well as the economic well being of producers of animal products. Up until 15–20 years ago, vaccines had changed little from those originally pioneered by Jenner and Pasteur. Since that time there have been significant changes in the types of vaccines available owing to a number of factors, including compatibility with eradication programmes and international trade policies as well as cost-effectiveness of production. The first recombinant vaccines were introduced in the late 1980s to control Aujeszky's disease and rabies in wild-life (Pastoret et al., 1988) and are the forerunners of similar products that will be available in the future.

The approaches used in the development of vaccines have expanded rapidly as the result of increased knowledge of the mechanisms by which protective immunity is induced, and the explosion of genomic data on both pathogens and their hosts. The associated evolution of new technology in the field of molecular biology and immunology has furthermore had a large impact on the development of new vaccine strategies and the quality of the products that are produced. It has enabled the design of vaccines targeted for the control and eradication of specific pathogens within the framework of regional, national and international requirements. Use of recombinant technologies bring with it the need for the application of a risk–benefit assessment that takes into account the specific aspects that need to be considered, particularly with respect to safety (see Appendix 1.1.8.1 Risk analysis for biologicals for veterinary use*, to Chapter 1.1.8* Principles of veterinary vaccine production *of this* Terrestrial Manual*).*

This chapter describes a range of technologies that are used to produce vaccines engineered for a specific purpose. The categorisation is aimed to assist the reader to understand the technologies employed, but it should be recognised that the categories are not mutually exclusive (i.e. reverse genetics may be used to produce a chimeric vaccine). In principle, the technologies can be used to change the target pathogen itself to alter its properties by deletion, insertion, other genetic modifications, or they can be used to modify the isolated genes or coding sequences of pathogens to produce specific immunogens associated with protective immunity.

A. REVERSE GENETICS

The development of a reverse genetics system for a range of different RNA and DNA viruses has revolutionised the field of virology by making it possible to introduce designed mutations, insertions and deletions into the viral genome of live viruses. It has by now been used in a range of applications that include the attenuation of viruses, the modification of host specificity and the generation of replication-deficient viruses. These strategies have also been applied to the development of new vaccine strategies and are widely used in the characterisation of the structure and function of individual viral genes and coding sequences.

The technology of reverse genetics involves the generation of a cloned copy of complementary DNA (cDNA) from RNA by reverse transcription *in vitro*, manipulating DNA *in vitro* followed by generating the modified live virus by transfection of permissive cells with the cloned DNA(s).The technology was first demonstrated using the bacteriophage Q-Beta, a positive-strand RNA virus (Taniguchi, 1978). Subsequently, a large number of positive-strand RNA viruses including severe acute respiratory syndrome (SARS) coronavirus, with large genomes have

been rescued, which has helped in the study of the biology of these viruses and the development of new live attenuated viral vaccines. For example, reverse genetics was used to develop an infectious clone of transmissible gastroenteritis virus (TGEV), which induced lactogenic immunity in immunised pigs (Sola *et al.,* 2003). This novel technique has also been used to develop a modified porcine respiratory and reproductive syndrome virus, which can be used as a DIVA (differentiating infected and vaccinated animals) vaccine to help differentiate between vaccinated and infected pigs (de Lima *et al*., 2008).

Owing to the inherent characteristics of negative-strand RNA viruses, it took years of work before this technique could be developed and used for generating engineered viruses containing negative-strand RNA genomes. Reverse genetics was first developed for influenza virus, a segmented negative-strand RNA virus. Since then, this technique has been successfully used for the generation of a number of RNA viruses containing either unsegmented or segmented negative-strand genomes. For example, the use of this technique has led to the development of a vaccine for avian influenza virus in which the engineered virus contained a haemagglutinin (HA) gene from an H5N1 virus and a neuraminidase (NA) gene from a H2N3 virus, using a H1N1 backbone (Meeusen *et al*., 2007). The resultant inactivated H5N3 virus vaccine induced complete protection in birds against highly pathogenic H5N1 challenge. A reverse genetics strategy has also been used in the development of Foot and mouth disease, Classical swine fever, and Newcastle disease vaccines (see chapters 3.1.8, 3.8.3 and 3.3.14, respectively). More recently, reverse genetics systems have been developed for segmented double-stranded RNA viruses including bluetongue virus (BTV) introducing the possibility of new vaccine development strategies for these viruses (Boyce *et al*., 2008).

Disabled infectious single-cycle (DISC) vaccine involve the deletion of an open reading frame coding for a key protein involved in the viral replication or viral capsid formation (Widman *et al*., 2008). The DISC virus is isolated in cells expressing the key protein, thus providing the missing protein in *trans*. Such virus, when injected in animals, can complete only one round of replication without producing a progeny virus. Vaccines based on DISC viruses are more stimulatory than a killed virus vaccine and are devoid of problems associated with live vaccines.

B. RECOMBINANT VECTOR TECHNOLOGY

Advances in reverse genetics, genomics, and proteomics have facilitated the identification of mechanisms of virulence, host-pathogen interactions, and protective antigens from many pathogenic microorganisms and also the development of suitable vehicles/vectors for delivery of these antigens to the host. The availability of bacterial and viral genome sequences has facilitated the rapid construction of defined deletions in the genomes of a wide variety of pathogens, which not only results in attenuation, but also creates space for the insertion of foreign genes coding for antigens from heterologous microbes. In general, live bacterial or viral vectors share several characteristics including ease and economy of production, non-integration into the host genome, stability and a reasonable capacity to insert genes coding for heterologous antigens. In addition, as with any live vaccine, the vector should be avirulent and the impact of immunity to the vector should be evaluated.

1. Bacterial vectors

In general bacterial vectors are attenuated by deletion of genes required for key metabolic processes or genes associated for virulence. Although they are not used routinely in animals, rapid progress is being made in developing and evaluating different bacteria as vectors. For several years, BCG (Bacillus Calmette–Guerin) and *Salmonella* have been developed as vectors for delivering vaccine antigens to animals and the latter has been used for the generation of live vaccine strains for poultry. There are currently a number of other bacterial vectors being developed based on commensal microorganisms (*Lactococcus, Streptococcus, Lactobacillus* and *Staphylococcus*) or attenuated pathogenic organisms (*Shigella, Bacillus, Yersinia, Vibrio, Cornebacteria,* and *Bordetella*), all of which are being evaluated for their ability to induce protective immunity.

2. Viral vectors

Most viral vectors are developed using viruses that are associated with mild or no disease or using viruses that are pathogenic but attenuated by deletion of virulence genes. Replication competent virus vectors, which can produce progeny virus, as well as replication-defective virus vectors, which do not produce progeny virus, have been developed and evaluated as vaccine delivery vehicles. A number of commercial vaccines based on DNA virus vectors, including poxviruses and herpesviruses, have been successfully licensed for use in veterinary medicine (reviewed in Gerdts *et al*., 2006). These include vectors based on vaccinia virus, canarypox virus, fowlpox

virus and turkey herpesvirus. A number of viral vectors have been developed or are in the process of being developed, improved and evaluated. These include RNA viruses such as Venezuelan equine encephalitis virus, Newcastle disease virus and feline foamy virus as well as DNA viruses such as adenoviruses, herpesviruses and pox viruses, Fowl pox and canary pox vectors have been used in a wide range of applications (MacLachlan *et al.,* 2007; Swayne, 2009) whereas replication-deficient human adenovirus vectors have been used very successfully in the development of FMDV vaccines (Rodriguez & Grubman, 2009). Licensed canary pox vaccines include vaccines against equine influenza and feline leukaemia. Other licensed vector vaccines include the herpesvirus of turkeys vectored with an infectious bursal disease insert.

C. GENE-DELETED VACCINES

The knowledge of specific virulence factor(s) of a pathogen and the availability of recombinant DNA technology has facilitated the creation of specific gene-deleted pathogens for use as live vaccines. The approach of creating and testing defined gene deletions ultimately aids in reducing the pathogenicity/virulence of the organism without affecting the immunogenicity. Such gene-deleted organisms can be used as vaccines as they retain the immunogenic features of the wild-type organism but cannot cause disease. However, to be effective as viable vaccine(s), these organisms should be genetically stable, easy to grow and easy to administer. So far, genes involved either in determining virulence or regulating key metabolic pathways of the organism(s) have been targeted for such deletions.

This approach has been successfully used to create several live attenuated vaccine strains of bacterial pathogens that are genetically stable, safe to use and induce better protection than killed vaccines. Gene-deleted *Salmonella enterica* serovar *typhimurium* and serovar *enteritidis* vaccines have been licensed for use in poultry (Babu *et al*., 2004; Meesun *et al*., 2007) and similarly, an *aroA* gene-deleted *Streptococcus equi* vaccine has been licensed for use in horses (Jakobs *et al*., 2000; Meesun *et al*., 2007).

This technology has also been successfully used to create live attenuated vaccine strains of viral pathogens that are genetically stable and can be used as marker vaccines to differentiate between vaccinated and infected animals. A double gene (gE and TK) deleted pseudorabies virus marker vaccine has been licensed for use in pigs (Ferrari *et al*., 2000; Meesun *et al*., 2007) and similarly, gE deleted a bovine herpesvirus-1 marker vaccine has been licensed for use in cattle (Meesun *et al*., 2007; Van Oirschot *et al*., 1996).

D. CHIMERIC VIRUSES

Chimeric viruses are defined as recombinant viruses that may contain parts of two closely related viral genomes. For example, a chimeric virus could be one that contains structural genes of one viral serotype and nonstructural genes of another serotype of the same virus. Alternatively, a chimeric virus would be one that contains part of the genome from different members belonging to the same virus family. In principle, chimeric viruses display the biological characteristics of both the parent viruses. One of the main advantages of this approach is that a single dose of chimeric virus delivers the complete repertoire of antigens closely resembling the pathogen(s), which can induce protective immune response against multiple viral pathogens belonging to or different serotypes of the same viral pathogen.

The availability of infectious full-length complementary DNA (cDNA) clones of different RNA viruses using reverse genetics technologies has led to novel vaccine development strategies. Chimeric pestiviruses have been constructed using an infectious cDNA clone containing the classical swine fever virus (CSFV) genome or the bovine viral diarrhoea virus (BVDV) genome backbones. In one instance, a chimeric pestivirus was constructed by replacing the BVDV E2 coding sequence in the infectious DNA copy of BVDV strain CP7 with the corresponding E2 coding sequence of CSFV strain Alfort 187 (Reimann *et al.,* 2004). Another chimeric virus was constructed by replacing the CSFV E2 coding sequence in the infectious DNA copy of CSFV vaccine strain C with the corresponding E2 coding sequence from BVDV (van Gennip *et al.,* 2000). These chimeric viruses appeared to be attenuated in pigs, induced complete protection against CSFV challenge and helped to discriminate between vaccinated and infected pigs (Reimann *et al.,* 2004; van Gennip *et al.,* 2000).

In another application, chimeric porcine circoviruses (PCVs) have been isolated using infectious cDNA clones of porcine circovirus PCV1 in which the capsid protein from pathogenic PCV2 was used to replace the corresponding gene in the nonpathogenic PCV1 strain (PCV1-2). Likewise, the capsid gene in PCV2 has been replaced with the

gene from PCV1 (PCV2-1). The chimeric PCV1-2 virus appeared to be attenuated in pigs and induced protective immunity against wildtype PCV2 challenge in pigs (Fenaux *et al.,* 2004).

This platform technology has also been used to generate chimeric flaviviruses. In one example, a chimeric virus was generated by replacing the coding sequences for structural proteins of yellow fever YF-17D virus with those of West Nile virus (WNV). A single dose of this chimeric flavivirus vaccine induced both cell-mediated and humoral immune responses in horses, and provided protection against WNV challenge without causing any clinical illness (Meeusen *et al*., 2007). The same platform technology has also been used to develop human vaccines for Japanese encephalitis virus, WNV and Dengue virus. Although chimeric flavivirus vaccines have shown satisfactory safety profiles and protective efficacies, caution should be used in evaluating chimeric viruses for the change in virulence.

E. SUBUNIT VACCINES

Subunit vaccines composed of semi-pure or purified proteins have been commercially available since the early 1980s, with subunit components produced by recombinant DNA technology available since the 1990s (Cohen, 1993; Rhodes *et al.,* 1994; Ulmer *et al*., 1993; 1995). The latter have attracted growing interest and activity since that time. Subunit vaccines do not include live recombinant vector technologies, which provide the delivery of recombinant proteins *in vivo.* The field of genomics and related areas has revolutionised the manner in which microbial antigens are identified. Since the first bacterial genome was sequenced in 1995, there has been a huge increase in the number of bacterial, viral, and parasite genomes for which genome sequences are available. Indeed, virtually all pathogens of animals are represented and those pathogens that are not can readily be obtained in less than a day. More importantly, the development of the bioinformatics resources and tools that are required to analyse these genomes has proceeded in parallel and it is now relatively easy to identify surface-exposed antigens, specific B- and T-cell epitopes, etc. There is no requirement to have the ability to grow the organism in culture: for example subunit vaccines for *Piscirickettsia salmonis,* a salmonid pathogen, have been developed even though the organism could not be readily grown (Kuzyk *et al.,* 2001).

The production of subunit antigens can be achieved by both conventional biochemical or recombinant DNA technologies. The latter involves a range of prokaryotic and eukaryotic expression systems including yeast, insect cell and plants (Chichester & Yusibov, 2007) by means of a variety of integrated or transient expression strategies. Biochemical techniques remain useful in some cases where recombinant expression is not appropriate, such as antigens requiring complex assembly (e.g. fimbriae), or when post-translational modification is necessary. For example, *Campylobacter jejuni* is one bacterial species that glycosylates many surface proteins and, as such, they are best produced in *C. jejuni* rather than heterologous expression systems, although *Escherichia coli* strains have been engineered to carry out the same function (Wacker *et al.,* 2002). An excellent example of a subunit vaccine composed of an authentic antigen that retained three dimensional structure was the original *E. coli* K99 vaccine for calf scours, which was tested three decades ago (Acres *et al.,* 1979). This product was based on the K99 fimbrial antigen, which could readily be extracted from cells by heat treatment, thus retaining the three dimensional fimbrial structure. Another example includes a baculovirus expressed vaccine against porcine circovirus type 2 (Fachinger *et al.,* 2008). In a number of cases the expressed subunit vaccine protein spontaneously assembles into well defined particles that may resemble virus particles. These virus-like particles (VLPs) are a sub-class of subunit vaccines (Roy & Noad, 2008) and their application in vaccine development is reviewed in section F. Vaccines containing ORF 2 protein of PCV-2 expressed in baculovirus has been commercialised.

Subunit vaccines could have some advantages over live attenuated and inactivated vaccines, including the ability to induce strong humoral and cell-mediated immune response. The vaccines furthermore have an excellent safety profile, and can be used in combination with other subunit vaccines. However, efficacy is dependent on the protective immunity being induced by inoculation of a single or set of defined recombinant proteins. Experience has shown this may be affected by the gene expression system used. In addition, subunits vaccines may be expensive to produce for some glycoproteins and may require the use of adjuvants to enhance immune responses.

One of the biggest advantages of subunit vaccines is that they are generally compatible with DIVA strategies as long as the antigen is not being used as a marker. In the case of bovine herpesvirus, glycoprotein gD has been successfully used in subunit vaccine formulations. Although immunisation with gD has proven to be protective at an individual animal level (Harland *et al.,* 1992; van Drunen Littel-van den Hurk *et al*., 1994), it has not reduced the prevalence of the virus in the field, thus limiting its use. Subunit vaccines against a variety of other respiratory and

enteric viruses, including BVDV, BRSV, PI3, rotavirus, and coronavirus have been successfully tested, although none of these is used on a commercial basis. Bacterial subunits have arguably proven more successful than their viral counterparts. This is because of the cost-effectiveness of growth of both conventional and recombinant organisms and a general requirement for a Th_2-biased immune response in many cases. Recombinant vaccines are commercially available for respiratory pathogens such as *Mannheimia haemolytica* and *Actinobacillus pleuropneumoniae* based upon the leukotoxins produced by these organisms, as well as transferrin-binding proteins. *Actinobacillus pleuropneumoniae* is an excellent example of a vaccine composed of subunits selected on cross-serotype reactivity, thus providing broad-spectrum protection against disease. Likewise a vaccine against atrophic rhinitis containing a non-toxic derivative of *Pasteurella multocida* dermonecrotic toxin produced by a genetically modified *Escherichia coli* strain together with a conventional *B. bronchiseptica* bacterin has been commercialised.

Vaccines against CSF demonstrate well the need to target recombinant technology to a particular purpose. Conventional, live attenuated CSF vaccines have a rapid onset of immunity and are effective at preventing transmission of infection (Van Oirschot, 2003) but have the disadvantage that it is not possible to differentiate infected pigs from those that have merely been vaccinated. Commercial E_2 subunit vaccines have a slower onset of immunity and reduce but do not prevent viral shedding. However, they enable a DIVA strategy to be followed thereby facilitating a 'vaccination to live' strategy. Their use is therefore likely to be of particular benefit in high value breeder pigs where the vaccine can be used to limit the clinical impact of the disease whilst allowing individual infected pigs to be identified and eliminated.

F. VIRUS-LIKE PARTICLES

Virus-like particles (VLPs) are supra-molecular structures composed of one or more recombinant proteins. The particles form through self-assembly and typically range from 20 to 100 nm in size. Depending on the origin they can be icosahedral or rod-like in structure (reviewed in Jennings & Bachmann, 2008). VLPs offer the advantage of formulating the vaccine antigen in a particulate structure, thereby increasing the immunogenicity of the vaccine. VLPs can be used as either vaccine itself or as carrier for genetically fused (chimeric), incorporated or covalently linked antigens. VLPs have been extensively studied for the past 20 years, with human vaccines against hepatitis B virus (Zuckerman, 2006) and human papillomavirus (Stanley, 2008) commercially available and several vaccines for veterinary application in development. These include vaccine for bluetongue virus, rota- and parvovirus.

VLPs offer several advantages for the use as vaccine including a high safety profile, the similarity to viral and bacterial structures, the ability for large-scale production and the possibility of combining the VLPs with other adjuvants. Typically, immunisation with VLP induces rapid and strong antibody responses. Similar to viruses and bacteria multiple copies of the vaccine antigens are displayed in a highly repetitive and ordered, quasi crystalline-structure (Bachmann & Zinkernagel, 1996), which can cross-link the B cell receptor resulting in activation of the B cell and subsequent induction of T-independent IgM responses (Thyagarajan *et al*., 2003).). Furthermore, this enables interaction with the complement system resulting in increased phagocytosis. Moreover, the particulate structure of VLPs also enhances uptake by dendritic cells and subsequent cross-presentation of the antigen. It was demonstrated by Lenz *et al.* (2001) that cross-presentation of particulate antigens was more effective than presentations of soluble antigens. However, the induction of T cell responses overall is still not as effective as those induced by live vaccines. To overcome this, VLPs have been successfully combined with molecular adjuvants such as CpG ODN and single-stranded RNA. Other VLPs have been demonstrated to directly stimulate dendritic cells (DC.) For example, L1 protein-VLPs of papillomavirus have been shown to directly activate DC.

VLPs can either be used as vaccine itself or be used as carrier for recombinant antigens, either incorporated, directly, genetically fused or covalently linked. For example, the bovine rotavirus virus protein 6 (VP6) forms VLPs that are highly immunogenic and already confer protection against challenge infection (Redmond *et al.,* 1993). However, using the VP4 and VP7, other antigens can be covalently linked to the VP6 particles and used for immunisation (Redmond *et al.,* 1993). Other prominent examples include the hepatitis B surface antigen VLPs (HBsAg-VLP), human immunodeficiency virus 1, dengue virus VLPs, norovirus VLPs, and influenza A VLPs. Examples of VLPs used as carriers include the well characterised hepatitis B core antigen VLPs (HBcAg VLPs; [Blanchet & Sureau, 2006; Pumpens & Grens, 2001]) as carrier for the influenza a M2 protein (M2-HBcAg [Jegerlehner *et al.,* 2002]), or malaria B- and T-cell epitopes (Nardin *et al.,* 2004). While mostly administered systemically, some VLPs-based vaccines already have been tested for mucosal administration.

G. DNA VACCINES

Immunisation with DNA represents a relatively new vaccination strategy that is based on a simple concept. DNA vaccines can be defined as antigen-encoding bacterial plasmids that are capable of inducing specific immune responses upon inoculation into a suitable host. Immunisation is accomplished by the uptake of purified plasmid in the host cells, where it persists extrachromosomally in the nuclei. Subsequent expression of protein results in the presentation of normally processed or modified forms of the protein to the immune system. In the host, native forms of the proteins have access to presentation pathways by class I major histocompatibility (MHC) antigens in addition to class II MHC presentation, which results in a balanced immune response. The use of pure plasmid DNA offers many advantages over other vaccine delivery vehicles. One of the greatest advantages is the ability of DNA vaccines to induce both humoral and cell-mediated immune responses, which is critical for protection from many diseases. There is also evidence that DNA vaccines can induce long-term immunity, which is a further requirement for vaccine efficacy. As the vector itself does not induce immune responses, DNA vaccines can be repeatedly administered without the interference of antibodies. From a technical viewpoint, DNA vaccines are easy to engineer, produce and purify, so new DNA vaccines can be constructed and evaluated in animal models within months. DNA vaccines are very stable and therefore have a long shelf life and can be transported without a cold chain. The safety of DNA vaccines has been established in various trials in several species including humans (Bagarazzi *et al.,* 1998; Kim *et al.,* 2001).

As soon as the concept of DNA immunisation began to be explored, this technology was found to be very effective in rodents, but initially did not perform as well in larger species. However, recent progress has resulted in the development of DNA vaccines in a number of target species (Carvalho *et al*., 2009; Redding & Weiner, 2009). Currently, four veterinary DNA vaccines have been licensed, against growth hormone releasing hormone for swine in Australia, infectious haematopoietic necrosis virus for salmon in Canada, WNV for horses and melanoma for dogs in the USA (Kutzler & Weiner, 2008). To achieve higher efficacy in large animal species, optimisation at various levels has been required, including (i) vector modifications; (ii) protein engineering to modify subcellular localisation; (iii) improvements in DNA delivery routes and methods; (iv) inclusion of adjuvants, as a gene or co-administered agent, and (v) antigen targeting to antigen-presenting cells (APCs). It is likely that the often unsatisfactory efficacy of DNA vaccines in large animals was caused by inefficient transfection, as well as 'immunological blandness', of the administered plasmids. The use of a needle-free vaccine delivery device was shown to reduce the effective dose of an experimental polyvalent DNA vaccine for avian influenza, and to rapidly deliver repeated injections in poultry (Rao *et al.,* 2009).

H. ANTIGEN DELIVERY AND MOLECULAR ADJUVANTS

Adjuvants are substances that enhance immune responses when co-administered with antigens. They are a critical component of killed (recombinant and subunit) vaccines, which are often poorly immunogenic. Adjuvants can be classified into two broad categories based on their presumed mechanism of action: i) delivery systems, and ii) immunostimulatory adjuvants. Delivery systems include many conventional adjuvants and numerous particulate adjuvants and will be discussed separately below.

Despite the importance of adjuvants in vaccines, their mechanisms of action remains poorly understood. Recent advances in the understanding of innate immunity have provided important clues on the molecular mechanisms of action of immunostimulatory adjuvants. In this regard, immune cells express a variety of receptors, collectively termed pattern recognition receptors (PRR) that broadly detect conserved microbial components referred to as pathogen-associated molecular patterns (PAMPs). A number of PRR have been described including toll-like receptors (TLR); e.g. TLR 9 recognises bacterial CpG nucleic acid motifs, natural agonist of TLR7/8, single-stranded viral RNA (oligoribonucleotides, ORN) strongly activate innate immune responses in mice, humans and are particularly potent in large animals; TLR4 agonist such as lipolysaccharide (LPS) that is known for its powerful immunostimulatory and adjuvant properties, but unfortunately this molecule is highly toxic; nucleotide oligomerisation domain (NOD)-like receptor (NLL), retinoic acid inducible gene (RIG)-like receptors (RLL), and C-type lectin receptors (CLR), all of which detect microbial components. Engagement of these receptors by their agonists leads to a cascade of molecular and cellular events that result in activation of innate immunity, which directs antigen-specific adaptive immunity. Of these receptors, TLR agonists are most widely explored and have shown great promise as adjuvants. Interestingly, the live-attenuated yellow fever vaccine 17D (YF-17D), one of the most successful vaccines available, activates TLR2, 7, 8 and 9 (Querec *et al.,* 2006), suggesting that the success of at least some of the live vaccines may be the result of their ability to activate TLRs. This has generated a great deal of interest in TLR agonist as adjuvants.

The existing paradigm in the veterinary vaccine industry of "one adjuvant-one vaccine," is driven partly by costs associated with including more than one adjuvant in a vaccine; however, it may severely limit the efficacy of potentially safe vaccine candidates, and may explain, at least in part, why some vaccines or adjuvants have only achieved suboptimal efficacy. Evidence is slowly accumulating to indicate that multiple adjuvants may offer more than can be achieved with a single adjuvant. For example, although CpG ODNs are a good adjuvant, they can have even greater adjuvant activity if formulated or coadministered with other compounds, such as particulates, mineral salts, saponins, liposomes, cationic peptides, polysaccharides and bacterial toxins and the synthetic polymers, polyphosphazenes (Wack *et al.,* 2008).

The adjuvant effect of microparticles has been known for some time and has been previously reviewed (Mutwiri *et al.,* 2005). Particulate delivery systems are thought to promote trapping and retention of antigens in local lymph nodes. In addition, microparticles facilitate antigen presentation by APCs via both MHC class I and MHC class II restricted processing and presentation pathways. One of the main advantages of microparticles for targeted antigen delivery is that they can be a flexible delivery platform that can be used to deliver both antigens and immunostimulatory molecules.

Other potential antigen delivery systems include polyphosphazenes, a class of synthetic polymers consisting of a backbone with alternating phosphorus and nitrogen atoms and organic side groups attached to each phosphorus (Mutwiri *et al.,* 2007). Immune stimulating complex (ISCOM), which is a small 40 nm nanoparticle composed of saponin (adjuvant), lipids and antigen, and has been described as an antigen delivery system because it not only has adjuvant activity and but also the ability to target APC (Morein *et al.,* 2004). A commercial ISCOM-based vaccine against equine influenza has been licensed for many years (Heldens *et al*., 2009).

I. VACCINE DELIVERY

Vaccine delivery comprises a diverse range of approaches with the overall goal of providing vaccines for mass vaccination during disease outbreaks and delivery of vaccines to wildlife. Oral vaccines used in rabies vaccination of wildlife such as foxes were initially based on attenuated rabies vaccine viruses such as the ERA strain, but concerns that these vaccines could rarely cause rabies (Fehlner-Gardiner *et al*., 2008) have largely lead to their replacement (http://ec.europa.eu/food/animal/diseases/eradication/rabies_pres_19.pdf). In Canada, a live adenovirus-vectored rabies vaccine with a good safety profile (Knowles *et al.,* 2009) is currently being used in rabies vaccination campaigns directed at controlling skunk and raccoon rabies (Rosatte *et al*., 2009). The live oral vaccinia-rabies glycoprotein (V-RG) vaccine is widely used elsewhere, and attempts are being made to optimise the vaccine baits for efficacy for other species including dogs (Cliquet *et al.,* 2008). Rabies infection in stray dogs and wildlife represents a serious problem for humans globally, and research for safer, more stable and efficacious live oral rabies vaccines continue (Faber *et al.,* 2009). Other possibilities for mass vaccination using edible plant-made vaccines have been actively investigated, but in spite of biotechnological advances in plant expression of vaccine antigens, no commercial products for oral use have been identified to date (Rice *et al.,* 2005).

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