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Veterinary vaccines: alternatives to antibiotics?*

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Abstract

The prevention of infectious diseases of animals by vaccination has been routinely practiced for decades and has proved to be one of the most cost-effective methods of disease control. However, since the pioneering work of Pasteur in the 1880s, the composition of veterinary vaccines has changed very little from a conceptual perspective and this has, in turn, limited their application in areas such as the control of chronic infectious diseases. New technologies in the areas of vaccine formulation and delivery as well as our increased knowledge of disease pathogenesis and the host responses associated with protection from disease offer promising alternatives for vaccine formulation as well as targets for the prevention of bacterial disease. These new vaccines have the potential to lessen our reliance on antibiotics for disease control, but will only reach their full potential when used in combination with other intervention strategies.

Keywords: Vaccine, vetinary, adjuvant, immunomodulator, infectious disease, antigen, immunization, pathogenesis

Introduction

The practice of vaccination against infectious diseases has been practiced for centuries and has been shown to be the single most efficient means of preventing bacterial, viral and parasitic infection. Indeed, the eradication of smallpox in humans was a testament to the power of vaccination programs when used in conjunction with traditional disease management strategies. Despite the success stories, infectious diseases remain a significant threat to animal populations, including many persisting diseases as well as emerging ones. For example, vaccines for bovine respiratory disease (BRD) have been available for decades, yet the incidence of disease is still significant. There are numerous reasons for this, including the ways in which food-producing animals are housed and managed in intensive livestock operations and a failure to administer vaccines using recommended procedures. The growing trend toward the use of single-dose vaccines for bacterial diseases such as respiratory infections caused by *Mannheimia haemolytica* also does not often provide an optimal duration of immunity.

Despite phenomenal advances in the fields of molecular biology, immunology and genomics, most vaccines available today are conceptually similar to those developed 50–100 years ago. This is due largely to the imbalance between efforts in antigen identification versus formulation and delivery aspects of vaccine development. During the past decade, there has been an explosion in the generation of genomic sequences for most bacterial pathogens of humans and animals, as well as the use of *in silico* technologies to generate lists of potential protective antigens. Likewise, technologies such as combinatorial peptide chemistry have made it possible to reverse engineer vaccine candidates without the need to culture or characterize the infectious agent. However, the antigen is only one component of the final vaccine formulation, and the development and characterization of novel adjuvant formulations remain the rate-limiting step in the enhancement of vaccine efficacy. The present review will focus on three areas: bacterial vaccine technologies, vaccine formulation and delivery and, finally, viral vaccines. The latter area is of particular interest since many common bacterial diseases of animals are the result of a predisposing viral infection and, thus,

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Table 1. Overview of advantages and disadvantages of commonly used veterinary vaccine technologies

Vaccine type	Advantages	Disadvantages
Live attenuated	Strong immune response; induction of both cell-mediated and humoral immunity; easy to produce	Lower safety profile; risk of reverting to full virulence; cold chain required
Inactivated whole-cell	Safe to use; inexpensive; stable on storage; no cold chain required	Induces mainly humoral immune responses; requires adjuvants; killing may affect antigen structure
Subunit	Safe to use; excellent immune responses; easily combined with other antigens	Induces mainly humoral immunity; requires adjuvants; can be expensive to produce
Vectored	Induces both cell-mediated and humoral immunity; inexpensive to produce	Good safety profile; responses may be affected by pre-existing immunity to the vector; cold chain required
DNA	Induces both cell-mediated and humoral immunity; very high safety profile	Inefficient uptake of the vaccine; often very low immune responses

the control of the viral component can be a useful control method.

Bacterial vaccines

Vaccines for bacterial diseases have been routinely used for decades in a variety of animal species. Pasteur demonstrated the principle of protecting animals against bacterial disease in the late 1800s using avirulent cultures of *Pasteurella septica* grown at elevated temperatures and stationary phase *Bacillus anthracis* (Pasteur, 1880, 1881). Subsequent to Pasteur's pioneering work, *in vitro*-grown bacterial cultures were routinely killed using physical or chemical means and combined with oil-based adjuvants to enhance immune responses. These basic methods of vaccine development changed very little over the next century and did prove remarkably successful in the development of vaccines for a variety of infectious diseases. This type of killed vaccine had the advantage of being quite inexpensive to produce but also had numerous disadvantages. These include a lack of relevant protective antigens due to *in vitro* growth conditions, antigenic competition between non-protective and protective components, a lack of safety due to potentially harmful components such as lipopolysaccharide, and finally a lack of broad-spectrum protection. For diseases such as those caused by *Streptococcus suis* or *Actinobacillus pleuropneumoniae* in swine, vaccines must protect against multiple serotypes, which increases cost and compounds the disadvantages listed above. Thus, over the past 20 years, a number of new strategies for vaccine development have evolved, leading to novel subunit products as well as rational attenuation of live vaccines. These vaccine types are summarized in Table 1.

Antigen identification and subunit vaccines

During the late 1970s and early 1980s, there was growing recognition that bacterial cells grown *in vitro* were quite distinct from the same organism grown in the host or under conditions that partially mimicked those found in the host. Many organisms have adapted to survival in environments with different nutrient sources and physical conditions and they have evolved to include mechanisms to sense their environment and produce components appropriate to the specific conditions. A classic example of this is growth under conditions where free iron is limited. Gram-negative organisms adapt to grow in the absence of free iron by producing a number of novel proteins on their surface which are able to sequester iron from host iron-binding proteins such as transferrin, lactoferrin, heme compounds and others (Bullen *et al.*, 1972, 1978; Griffiths *et al.*, 1980, 1985). Members of the Pasteurellaceae are excellent examples of this phenomenon (Deneer and Potter, 1989a, b; Ogunnariwo and Schryvers, 1990; Ogunnariwo *et al.*, 1991, 1997) and a number of groups have demonstrated that vaccines enriched in these novel proteins were capable of inducing enhanced protection following immunization (Gilmour *et al.*, 1991; Gerlach *et al.*, 1992; Potter *et al.*, 1999). Recombinant subunit vaccines based on the transferrin-binding protein TfbB are available for *A. pleuropneumoniae* and conventional vaccines have been developed for both bovine and ovine isolates of *M. haemolytica*. A number of laboratory techniques have since been developed to identify *in vivo*-expressed antigens, including *in vivo* expression technology (IVET) and conceptually related technologies (Slauch *et al.*, 1994; Slauch and Camilli, 2000) and this has become a common methodology for antigen identification. Both IVET and signature-tagged mutagenesis have been successfully used in veterinary pathogens (Fuller *et al.*, 1999, 2000). In conjunction with traditional techniques for protein characterization, a number of virulence determinants with a role in infection have been targeted for veterinary vaccine development, including those based on adhesins, toxins, outer membrane proteins, capsular polysaccharide and others.

The field of genomics has revolutionized how bacterial antigens are identified since the first bacterial genome

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Table 2. Examples of attenuating mutations used in live bacterial vaccines

Gene	Cellular function
aroA, aroC, aroD	Synthesis of aromatic amino acids
cya, crp	Adenylate cyclase
carAB	Arginine and pyrimidine biosynthesis
phoP, phoQ	Phosphatase, regulatory
galE	Galactose epimerase
ompR	Porin regulator
emm	M protein (*Streptococcus*)
stx	Shiga toxin
lktA, lktC	Leukotoxin (Pasteurellaceae)
toxA	*S. aureus* alpha-toxin
fur	Ferric uptake regulator

was sequenced over a decade ago (Fleischmann *et al.*, 1995). Researchers now have access to sequences covering virtually all pathogens of animals and the bioinformatics tools to identify surface proteins, secreted products, B- and T-cell epitopes, etc. are commonly used in the vaccine development field (Scarselli *et al.*, 2005). The first example of the use of a genome sequence to produce vaccine antigens was described by Pizza *et al.* (2000) for *Neisseria meningitidis* and several other examples with veterinary application followed. The use of these techniques is only useful in the identification of putative protective antigens and they must still be screened for their ability to induce protective immunity in animal models. However, for organisms that are fastidious in nature or non-cultivatable, genomic approaches can cut vaccine development times by several years.

Live vaccines

Live attenuated bacterial vaccines have several potential advantages over killed or subunit products, but their use in veterinary medicine has been limited to date. These advantages include the presence of the full repertoire of protective antigens, including those produced *in vivo*, the ability to induce balanced Th1/Th2 responses, the potential for single dose administration and lower production costs than subunit vaccines. Historically, attenuation has been carried out by using altered growth conditions, chemical mutagenesis or passage through alternative hosts. The use of *Brucella abortis* strain 19 is an excellent example of a traditional live vaccine that has proved effective in the control of brucellosis (Nicoletti and Milward, 1983). There are two areas that have limited the use of live vaccines in animals. First, the routine use of antibiotics in animal husbandry has precluded their use for obvious reasons, since there is a need for limited replication and transient colonization to occur. The second issue has been one of safety; since the attenuating mutation(s) were not known in many prototype vaccines, there have been concerns of reversion to virulence. Advances in molecular genetics in the 1970s and 1980s made it possible to rapidly construct defined insertions and deletions in specific genes, making rational attenuation possible and the first live attenuated bacterial vectors included transposon insertions of *Salmonella enterica* serovars (Stocker, 2000). These defined mutations resulted in safer vaccine strains, which could also be used for delivering a variety of foreign antigens to animals (Stocker, 2000; Thole *et al.*, 2000; Garmory *et al.*, 2003). Furthermore, by selecting the appropriate target genes for deletion, it is possible to tailor the degree of attenuation in a similar fashion to that routinely carried out in the development of modified live viral vaccines. For example, classical *aroA* mutants of *S. enterica* serovar Typhimurium (*Salmonella* Typhimurium) are able to persist in the host to a greater degree than the same strain carrying defects in purine metabolism (Charles and Dougan, 1990). Target genes for rational attenuation include those coding for proteins involved in physiological processes as well as those coding for virulence determinants. Numerous examples of each have been described and representative examples are listed in Table 2. The use of defined mutations is not without risk since vaccine strains are still capable of interacting with other microbes in the host. For example, the administration of *Salmonella* Typhimurium *aroA* mutants was observed to exacerbate infection with wild-type *Salmonella* Typhimurium in a mouse model of infection via a Toll-like receptor-4 (TLR-4)-dependent mechanism (Foster *et al.*, 2008).

Other vaccine types

While live and killed vaccines account for the majority of licensed products currently available, a number of alternative technologies are also being employed for the development of new vaccines. These include DNA vaccines, bacterial spores, bacterial ghosts and peptide-based technologies. Immunization with plasmid DNA-containing genes coding for protective antigens from bacteria, viruses and parasites has been common practice in research laboratories for the past decade and a half. There has been significant interest in developing the technology for use in animals since it has a number of potential advantages over existing production methods. These include low production cost, correct post-translational modification for viral antigens, ability to induce balanced Th1/Th2 responses without an adjuvant and finally the ability to induce responses in the face of maternal antibodies. Most of the literature dealing with DNA vaccines has described the use of viral antigens delivered in mouse models and the results have been promising. However, when large animals have been used, results have been disappointing. Cox *et al.* (1993) described the first example of vaccination of a non-rodent species with a DNA vaccine and they showed that immunization of cattle with nucleic acid encoding a

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bovine herpesvirus-1 glycoprotein was able to induce cellular and humoral immune responses. However, the dose of plasmid DNA was high, and repeated immunizations were required for the induction of immunity. Since that time, DNA vaccines have been shown to be effective in cattle, sheep, poultry, horses and fish and commercially available products have been produced for the latter two species. No vaccines for bacterial diseases are currently available, although experimental vaccines have been described for *Mycobacterium avium* subspecies *paratuberculosis*, *Campylobacter jejuni*, *B. anthracis*, *Pasteurella multocida*, *Salmonella* species and others. These are unlikely to see commercial development until the plasmid dose can be reduced and the method of delivery optimized (e.g. prime-boost strategies). Gerdts (Gerdts *et al.*, 2000) demonstrated that *in utero* immunization of sheep resulted in protective immunity, a finding which has implications for potential fetal delivery of animal vaccines.

The development of oral vaccines for animals has been a goal of many groups, with most work focused on the use of live attenuated or live vectored vaccines. Subunit antigens containing mucosal adjuvants such as the cholera toxin or heat-labile enterotoxin have also been successfully employed, as have plant-derived vaccines (Rice *et al.*, 2005). However, the use of oral subunit vaccines in large animals remains problematic due to degradation of the antigen and poor absorption in the gut. There has been recent interest in the use of bacterial spores as delivery vehicles for heterologous antigens, with *B. subtilis* and *B. anthracis* representing two of the hosts used (Aloni-Grinstein *et al.*, 2005; Zhou *et al.*, 2008a, b). Spores have a number of advantages over conventional live vaccines due to their stability and resistance to degradation by chemical and physical means. Foreign antigens, including a fragment of tetanus toxin and a *Clonorchis sinensis* tegument protein, have been fused to a major spore coat protein (CotC) and, following oral administration in mice, shown to induce protective immunity (Zhou *et al.*, 2008a, b). This represents an intriguing possibility for the production of low-cost, stable oral vaccines for animals.

Vaccine targets

Vaccines for bacterial pathogens of livestock and poultry have historically been targeted at organisms that were significant disease threats in the target species. In the case of zoonotic diseases, these included brucellosis, anthrax, clostridial disease and others. Vaccination was able to protect not only the immunized host, but it also reduced the risk of transmission to humans through a reduction in the pathogen reservoir. However, many newer zoonotic diseases of interest do not cause clinical signs of disease in the target species. For example, the 33 million cases of food-borne illness reported each year in North America are due to a variety of bacteria, viruses and parasites with over one-half belonging to six bacterial species including *C. jejuni*, Shiga toxin-producing *Escherichia coli*, non-typhoid *S. enterica* species, *Listeria monocytogenes*, *Staphylococcus aureus* and *Clostridium perfringens*. Most chickens are exposed to *C. jejuni* and *S. enteriditis* during their life with no clinical signs of infection and *E. coli* O157:H7 colonizes cattle with no impact on health or productivity. From a vaccine development perspective, protection against colonization rather than disease presents several issues not encountered with traditional disease targets ranging from the choice of antigens to be included in the vaccine to regulatory issues regarding the choice of animal models. The first vaccines for non-disease-causing zoonotic pathogens were developed in the United Kingdom for *Salmonella* in poultry (Gast *et al.*, 1993). These killed vaccines were shown to significantly reduce contamination of eggs and, since then, live attenuated vaccines have also been commercialized. No vaccines are commercially available for *C. jejuni* or *E. coli* O157 although proof of concept for immunization against both bacterial species has been demonstrated in chickens and cattle, respectively. In the case of *E. coli* O157, vaccination with lipopolysaccharide (Conlan *et al.*, 1999, 2000), intimin (Dean-Nystrom *et al.*, 2002) and type III-secreted proteins (Potter *et al.*, 2004) has been shown to reduce colonization, while immunization with bacterins has not shown any beneficial effect. We anticipate that this area will continue to see significant growth over the next decade.

Vaccine formulation and delivery

Key to the success of a vaccine is its formulation and delivery. Traditionally, most vaccines are administered systemically, either intramuscularly or subcutaneously. However, since over 90% of pathogens enter and initiate infection at mucosal surfaces, the best target for an effective vaccine is the mucosal surface in order to reduce the ability of the pathogen to become established. Thus, the induction of immune responses should ideally occur at the mucosal site. Vaccine effectiveness also greatly depends on the formulation of the antigen with adjuvants and carrier systems. These enhance and modulate the immune response and can be used to deliver the vaccine to either specific compartments or to facilitate uptake via the mucosal surfaces.

Live vectors

Live vectored vaccines facilitate the delivery of recombinant proteins expressed within the vector itself, or genetic information either integrated into the genome or as plasmid DNA. Both viral and bacterial vectors have been developed for vaccine delivery in humans and animals that are incapable of reverting to full virulence and are

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therefore safe to use. Various bacterial delivery systems have been optimized for the delivery of recombinant antigens as well as plasmid DNA (Garmory *et al.*, 2003). For example, *S. enterica* serovars or *Shigella* species can target mucosal tissues and deliver the foreign antigen specifically to antigen-presenting cells (APCs) via bacterial secretion systems (Gentschev *et al.*, 1996, 2002; Autenrieth and Schmidt, 2000). Other vectors include commensal microorganisms such as *Lactobacillus*, *Lactococcus*, *Staphylococcus*, *Streptococcus* species and attenuated pathogenic microorganisms such as *Salmonella*, *Shigella*, BCG, *Corynebacterium*, *Bacillus*, *Yersinia*, *Vibrio*, *Erysipelothrix* and *Bordetella* species (Sizemore *et al.*, 1995, 1997; Stocker, 2000; Darji *et al.*, 2000; Locht, 2000; Dietrich *et al.*, 2003; Roland *et al.*, 2005). Other bacterial vectors have been demonstrated to carry plasmid DNA across the mucosal surfaces (Sizemore *et al.*, 1996; Darji *et al.*, 2000; Dietrich *et al.*, 2003; Loessner and Weiss, 2004), and more recently, bacterial ghosts were demonstrated to represent effective means to deliver plasmid DNA (Ebensen *et al.*, 2004).

Several viral vectors including poxviruses, herpesviruses and adenoviruses have been used as vectors for veterinary vaccines (Yokoyama *et al.*, 1997). The usefulness of viral vectors for livestock species and poultry greatly depends on their host range and tropism. Ideally, a vector has a broad host range, infects and replicates within a wide range of tissues and ensures optimal post-translational modifications of the recombinant antigen including proper folding and processing (Yokoyama *et al.*, 1997; Sheppard, 1999). Canarypox-based vaccines are already licensed for the use in both large and small animals, and other viral vectors are currently being evaluated for vaccine delivery including DNA viruses such as adenoviruses, herpesviruses and RNA viruses such as Newcastle disease virus (NDV), Venezuelan equine encephalitis (VEE), Semliki forest virus (SFV) and a few retroviruses. For example, a recombinant alphaherpesvirus, pseudorabies virus (PRV), carrying the E1 glycoprotein of the classical swine fever virus (CSFV), was used by van Zijl *et al.* (1991) to protect pigs against challenge infection with CSFV. Kit *et al.* (1991) used the bovine herpesvirus type 1 (BHV-1) to deliver the viral protein (VP) 1 of foot-and-mouth disease virus (FMDV) to young calves, and Kweon *et al.* (1999) used BHV-1 as a vector for delivering the glycoprotein E2 of the bovine virus diarrhea virus (BVDV) to calves. These and numerous other examples demonstrate the potency of live viral vectors for vaccine delivery.

Particulate delivery systems

Particulate delivery systems have been developed as effective delivery vehicles for whole cells, subunit antigens and DNA. These systems can significantly improve the immunogenicity of both mucosally and systemically administered antigens by protecting the antigen from degradation, especially at the mucosal surfaces. Antigen uptake into specialized mucosa-associated lymphoid tissues (O'Hagan, 1996) as well as by APCs is increased, resulting in a better and more balanced immune response. Most particulate delivery systems are based on liposomes, polymers or a combination of the above. For example, a variety of polymers can be formed into microparticles including poly-lactide-co-glycolide (PLG), alginate, chitosan, polyphosphazenes and starch (Bowersock *et al.*, 1999; Illum *et al.*, 2001; Mutwiri *et al.*, 2005). The former have been extensively investigated for the delivery of micro-encapsulated vaccines with successful induction of protective immune responses against a variety of pathogens (Jones *et al.*, 1996; Allaoui-Attarki *et al.*, 1997; Chen *et al.*, 1998; O'Hagan, 1998; Herrmann *et al.*, 1999; Vajdy and O'Hagan, 2001; Fattal *et al.*, 2002) and have also been used to encapsulate plasmid DNA for delivery (Tacket *et al.*, 1994; Felder *et al.*, 2000; Singh *et al.*, 2001; Carcaboso *et al.*, 2004). However, its use may be limited by the fact that PLG is soluble only in organic solvents, which may denature critical epitopes required for inducing protection. Derivatives of PLG, including biodegradable polyesters, circumvent this problem and might represent better alternatives for future vaccine delivery.

Polyphosphazenes are synthetic biodegradable and water-soluble polymers that are easy to produce and that are stable at room temperature eliminating the need for refrigeration. Poly[di(sodium carboxylatophenoxy) phosphazene] (PCPP) has previously been shown to have adjuvant activity with a variety of viral and bacterial antigens in mice (Payne *et al.*, 1998; McNeal *et al.*, 1999; Wu *et al.*, 2001) and sheep (Mutwiri *et al.*, 2007a, b). In a direct comparison using the hepatitis B surface antigen (HBsAG), PCEP was more potent than the conventional adjuvant alum (Mutwiri *et al.*, 2007a). Polyphosphazenes not only enhance the magnitude of the immune response but also modulate the quality of the responses, resulting in a more balanced type of response (Mutwiri *et al.*, 2007b). Other examples of particulate delivery systems include immune stimulating complexes (ISCOMs), which are small 40 nm nanoparticles composed of saponin (adjuvant), lipids and antigen (Morein *et al.*, 1984). ISCOMs have adjuvant activity and also the ability to target APCs (Morein *et al.*, 2004). Numerous studies indicate that ISCOMs enhance immune responses to a variety of vaccine antigens in laboratory and domestic animals, and ISCOMs are now included in injectable commercial veterinary vaccines (Bowersock *et al.*, 1999; Morein *et al.*, 2004).

Similarly, liposomes are phospholipid vesicles which enhance immune responses primarily by increasing antigen uptake and presentation of the antigen (Gregoriadis, 1990). Liposomes have been used extensively in experimental vaccines for both human and veterinary vaccines using a variety of viral and bacterial pathogens. Liposomes have also been used to incorporate additional

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immune modulators, such as cholera toxin or recombinant cytokines such as interleukin-2 (IL-2) (Zhou *et al.*, 1995; Harokopakis *et al.*, 1998; Baca-Estrada *et al.*, 1999, 2000; Fukutome *et al.*, 2001; Tana *et al.*, 2003; Wang *et al.*, 2004). An alternative to co-delivering cytokines is to use liposomes containing cytokine-inducing immune modulators such as CpG oligodeoxyribonucleotides (CpG ODN). This would reduce the potential toxicity often associated with large doses of cytokines. For example, the adjuvant activity of CpG ODN was augmented by liposomal delivery in mice immunized intranasally against influenza and hepatitis B viruses (Joseph *et al.*, 2002).

Other particulate delivery systems include alginate and chitosan, naturally occurring carbohydrates that polymerize into particles upon contact with divalent cations (Bowersock *et al.*, 1999; Illum *et al.*, 2001; van der Lubben *et al.*, 2001; Mutwiri *et al.*, 2005; Douglas *et al.*, 2006; Greimel *et al.*, 2007). Oral immunization of cattle with the model antigen ovalbumin formulated in alginate microparticles enhanced IgA- and IgG-immune responses in the respiratory tract, providing evidence that they can stimulate the common mucosal immune system in a large animal (Bowersock *et al.*, 1998). Calves immunized intranasally with porcine serum albumin (PSA) in alginate microparticles developed high levels of anti-PSA IgG1 antibodies in serum, nasal secretions and saliva (Rebelatto *et al.*, 2001). In contrast, oral immunization did not induce any significant responses, suggesting that IN was more efficient for induction of nasal and serum responses. Rabbits orally immunized with *P. multocida* antigens encapsulated in alginate microparticles developed significantly higher nasal IgA responses (Bowersock *et al.*, 1999). Increased protection against *M. haemolytica* and *Streptococcus pneumoniae* challenge in mice was demonstrated by encapsulating antigens in alginate microparticles following oral immunization (Kidane *et al.*, 2001). These studies clearly show that alginate microparticles are effective for mucosal delivery of vaccines in small and large animals.

Adjuvants

Adjuvants were originally described by Ramon (Ramon, 1924) as 'helper' substances that can enhance the immune responses to the antigen alone. Adjuvants have been developed mainly by trial and error, screening a wide variety of natural and synthetic molecules. Adjuvants can be classified into delivery systems and immunostimulators (Singh and O'Hagan, 2003) and include a wide variety of molecules and carrier systems. Several of these adjuvants have been developed for veterinary applications, including liposomes, oil-in-water emulsions (squalenes), saponins, carbomers, monophosphoryl lipid A, Quil A, ISCOMS, heat-labile enterotoxins, cholera toxin and QS21 to name a few and the advances in this field were recently summarized in an excellent review by Singh and O'Hagan (Singh and O'Hagan, 2003). Here, we will only focus on three adjuvant systems, which currently show great promise for adjuvanting vaccine responses.

Bacterial DNA as well as synthetic DNA containing CpG motifs (CpG ODN) represent very potent danger signals resulting in the induction of innate and adaptive immune responses. Signaling is mediated by TLR-9, which so far is the only identified receptor for synthetic double-stranded DNA. Numerous investigators have shown that treatment of animals with CpG ODN can protect against a variety of experimental infectious and non-infectious diseases (Wilson *et al.*, 2006). CpG ODNs promote predominantly Th1-type immune responses (Chu *et al.*, 1997; Krieg, 2006) and clinical studies are in various stages in humans to evaluate therapy against infectious disease, cancer, asthma and allergy (Krieg, 2006). Successful use of synthetic CpG DNA as a vaccine adjuvant has been demonstrated in various animal species including large animals such as cattle and pigs. For example, CpG ODN was shown to be an excellent adjuvant for stimulating immune responses against an experimental vaccine based on a subunit protein (gD antigen) of the BHV-1 in mice, sheep and cattle by producing enhanced serum Ig2a levels and interferon-γ (IFN-γ) in splenocytes or peripheral blood lymphocytes, indicating a more balanced or Th1-type response (Ioannou *et al.*, 2000b, 2002a). Interestingly, the use of CpG ODNs in combination with low levels of mineral oil enhanced the immune response and reduced the amount of tissue damage associated with conventional vaccine adjuvants in sheep (Ioannou *et al.*, 2002a) and cattle (Ioannou *et al.*, 2002b). Similarly, incorporation of CpG ODN in a commercial equine influenza virus vaccine resulted in significant enhancement of antibodies against influenza virus (Lopez *et al.*, 2006).

Cationic host defense peptides (HDPs) are endogenous effectors found in virtually every life form. They are short peptides that can be grouped as defensins and cathelicidins (Oppenheim *et al.*, 2003; Finlay and Hancock, 2004). These molecules are fundamental components of the innate immune response with a broad spectrum of functions including direct antimicrobial activities, immunostimulatory functions of both innate and acquired immunity, as well as involvement in wound healing, cell trafficking and vascular growth (Bowdish *et al.*, 2006; Brown and Hancock, 2006; Mookherjee *et al.*, 2006). While the antimicrobial activities of HDPs have been known for a long time, more recent evidence suggests that HDPs are immunomodulatory resulting in recruitment of immature DCs and T-cells, glucocorticoid production, phagocytosis, mast cell degranulation, complement activation, and IL-8 production by epithelial cells (Yang *et al.*, 1999, 2001, 2004). The immunoenhancing activity of HDPs has been demonstrated in several studies. For example, ovalbumin-specific immune responses were enhanced in mice when human neutrophil peptides 1, 2 and 3 (HNP1–3) were co-administered intranasally (Lillard *et al.*, 1999). It was also demonstrated that HIV

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gp120-specific mucosal, systemic and CTL immune responses could be achieved after immunization with a fusion DNA vaccine encoding the murine β-defensin 2 and the HIV gp120 gene (Biragyn *et al.*, 2001, 2002). These examples provide evidence that HDPs have been successfully used as adjuvants to enhance vaccine-specific immunity.

Synthetic single-stranded RNA (ssRNA) and small antiviral compounds (imidazoquinolines) have been shown to also promote Th1 rather than Th2 immune responses (Vasilakos *et al.*, 2000). Studies in our own laboratories have indicated that similar to bacterial DNA, ssRNA and imidazoquinolones signal through specific TLRs resulting in maturation and activation of effector cells such as porcine dendritic cells (G. Auray, 2008). Evidence from studies in mice and humans has confirmed that these molecules have adjuvant activity *in vivo*; however, extensive studies in livestock species have not been conducted.

Viral–bacterial synergism

Viral infections are known to predispose animals and humans to secondary bacterial infections and, as such, prevention of the primary viral disease can have a significant impact on bacterial disease. Overall, the most common synergisms have been observed for respiratory infections. In particular, BRD is a serious problem involving four viruses, BHV-1, BVDV, bovine parainfluenzavirus-3 (PI3) and bovine respiratory syncytial virus (BRSV), as well as the bacterial pathogens *M. haemolytica*, *Histophilus somni* (formerly *Haemophilus somnus*) and sometimes *P. multocida* (Bowland and Shewen, 2000) (Srikumaran *et al.*, 2007). Other viruses that have been implicated in BRD include coronavirus, adenovirus, parvovirus and rhinovirus (Storz *et al.*, 2000). Indeed, in Canada, more than 80% of the biologics licensed for use in cattle act against agents associated with BRD (Bowland and Shewen, 2000), which emphasizes the importance of this disease and the need for vaccination or treatment.

An additional viral–bacterial synergism in cattle occurs between BVDV and *Mycobacterium bovis* (Kao *et al.*, 2007; Srikumaran *et al.*, 2007). Furthermore, BHV-2, vaccinia virus, cowpox, pseudocowpox, vesicular stomatitis, FMDVs and bovine papillomaviruses can play an indirect role in the etiology of bovine mastitis due to their immunosuppressive properties. However, a direct cause and effect relationship remains to be established (Wellenberg *et al.*, 2002). A correlation between rotavirus and coronavirus infection and the occurrence of enterotoxigenic *E. coli* (ETEC) has also been proposed (Hess *et al.*, 1984).

Mechanisms of viral–bacterial synergism

One of the more extensively studied synergisms between a viral and bacterial infection is that between BHV-1 and *M. haemolytica*. BHV-1 infects the epithelium of the upper respiratory tract, which results in rhinotracheitis. Lesions include loss of cilia and goblet cells leading to epithelial erosions, which could progress to necrosis of epithelium and adjacent lymphoid tissue (Schuh *et al.*, 1992). This facilitates migration and colonization of the lower respiratory tract by bacterial pathogens such as *M. haemolytica*. Furthermore, alveolar macrophages and peripheral blood leukocytes from cattle with active BVH-1 infection are more susceptible to the lethal effects of leukotoxin *ex vivo* than leukocytes from uninfected cattle. Czuprynski *et al.* (2004) reported that BHV-1 infection affects the response of bovine lung and peripheral blood leukocytes to *M. haemolytica* leukotoxin *ex vivo*. Active viral infection increased the susceptibility of bovine alveolar macrophages and leukocytes to *M. haemolytica* leukotoxin by increasing the production of proinflammatory cytokines (i.e. IL-1β, tumor necrosis factor-α and IFN-γ). This then leads to expression or activation of the β2-integrin CD11a/CD18 (LFA-1) on the leukocyte surface, which correlates with increased binding and responsiveness to leukotoxin. In support of this observation, *ex vivo* exposure to proinflammatory cytokines (i.e. IL-1β, tumor necrosis factor-α and IFN-γ) increased LFA-1 expression on bovine leukocytes, and *in vitro* incubation of bovine leukocytes with BHV-1 potentiated LFA-1 expression making them more responsive to leukotoxin (Leite *et al.*, 2004).

BVDV is also known to contribute to shipping fever. Recently, evidence was presented by Al-Haddawi *et al.* (2007), who demonstrated that infection with a type 2 BVDV isolate inhibited LPS-induced up-regulation of tracheal antimicrobial peptide (TAP) mRNA expression and lactoferrin secretion by tracheal epithelial cells. This suggests that BVDV-2 abrogates respiratory innate immune responses and thus predisposes to bacterial pneumonia in cattle.

BRSV was found to synergistically enhance *H. somni* infection (Gershwin *et al.*, 2005). When calves were either dually infected or singly infected with *H. somni* or BRSV, disease was significantly more severe in dually infected calves and high IgE and IgG responses were detected to *H. somni* antigens. An increase in IgE antibodies specific for antigens of *H. somni* presents a possible mechanism for pathogenesis of the disease enhancement. A recent report by Corbeil (2008) showed that the pathogenic synergy of BRSV and *H. somni* in calves can be attributed, in part at least, to the higher IgE anti-major membrane protein (MOMP) antibody responses in dually infected calves.

A number of strategies are used by the viruses involved in BRD to compromise the immune response to pathogens. BVDV infects immune cells and inhibits cell proliferation, whereas BHV-1 and BRSV only inhibit proliferation. BHV-1 induces apoptosis of CD4+ Th cells, which impairs cytokine production and the development of both humoral and cell-mediated immune responses.

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The ability of BHV-1 to down-regulate MHC class I expression compromises the development of CTL response against BHV-1 as well as other pathogens involved in BRD (Srikumaran *et al.*, 2007).

Effect of viral vaccines on disease

Vaccination against viral infections is expected to limit the chance of bacterial infections and thus reduce the need for antibiotic use. Although few controlled studies have been performed to assess the ability of viral vaccination to prevent bacterial infections, there are several reports on vaccination against the viruses involved in BRD that support this contention. According to Jericho *et al.* (1991), prevention of viral infection by vaccination can reduce the severity of disease after subsequent *M. haemolytica* exposure. We showed that calves vaccinated with one of the three major glycoproteins of BHV-1, gB, gC or gD, or various combinations experienced a significantly lower number of sick days and reduced lung pathology when they were subsequently challenged with BHV-1 followed by *M. haemolytica*. All calves in the vaccinated groups survived, whereas 60% of the animals in the control group died. Among these glycoproteins, gD was the superior protective antigen (Babiuk *et al.*, 1987; van Drunen Littel-van den Hurk *et al.*, 1990). Since it usually is not clear which virus is involved in field situations, calves need to be ultimately vaccinated against all four common viruses associated with BRD in order to have an impact on secondary bacterial infection. Stilwell *et al.* (2008) reported that the use of Rispoval 4I, a quadrivalent vaccine against respiratory viruses, reduced morbidity and mortality due to BRD in beef calves after weaning. This observation provides support for the value of viral vaccines in preventing overall disease, thus reducing the need for antibiotics.

Vaccination has also been evaluated as an approach to reduce the severity of porcine respiratory disease (PRD) in pigs. In one study, pigs vaccinated with a PRRSV vaccine did not show decreased potentiation of PRRSV pneumonia by *Mycoplasma hyopneumoniae*, whereas vaccination with *M. hyopneumoniae* bacterin decreased the enhancement of PRRSV-induced pneumonia in the dually infected pigs (Thacker *et al.*, 2000). In contrast, in one field study, pigs vaccinated with a live PPRSV vaccine performed better with respect to feed conversion ratio and average daily gain as compared with non-vaccinated animals, and decreased incidence of respiratory bacterial infection was observed in vaccinated pigs compared with untreated ones, demonstrating that indeed a viral vaccine can protect from subsequent bacterial infection in pigs (Mavromatis *et al.*, 1999).

In chickens, live IBV vaccine can protect against intranasal challenge with a mixture of *E. coli* strains and IBV strains of the same serotype; this live IBV vaccine usually also protected against challenge with the *E. coli* pool and IBV strains of other serological types. Some protection, but less efficient, against challenge with IBV and the *E. coli* pool was also observed in chickens vaccinated with an inactivated IBV strain (Cook *et al.*, 1986). In another study, vaccination against IBV resulted in protection against predisposure for colibacillosis after a subsequent IBV infection. When 1-day-old commercial broilers were vaccinated ocularly with IBV vaccine and then challenged with the virulent IBV strain M41 followed by *E. coli* 506, it was apparent that IBV vaccination reduced both the number of broilers with *E. coli* airsacculitis and the severity of airsacculitis. In contrast, in spray-vaccinated groups, no significant reduction of the number of birds with systemic colibacillosis or the severity of this infection was obtained, whereas eye-drop vaccination resulted in conflicting results (Matthijs *et al.*, 2005).

An interesting result of vaccination of chickens with NDV vaccine was reported by Huang and Matsumoto (2000). When chickens at 5 weeks of age were vaccinated with a NDV vaccine at various days before challenge exposure with O1:K1 strain of *E. coli* via an intra-air sac route, viable numbers of *E. coli* in the spleen 24 h after the infection were reduced. This was effective for a period of 2–8 days post-vaccination. This suggests that innate immunity induced by NDV vaccination may suppress the multiplication of *E. coli* in chickens.

One may have to be careful with the use of live viral vaccines, as administration of a live vaccine containing IBV and NDV together with exposure to *Mycoplasma gallisepticum* and *E. coli* in chickens actually resulted in extensive multiplication of *E. coli* and severe and persistent histological lesions and mortality. This effect was less severe when the chickens were exposed to only the vaccine and *E. coli*, while chickens inoculated with only vaccine, or with *M. gallisepticum* and/or *E. coli*, did not show any lesions (Nakamura *et al.*, 1994).

Overall, however, the benefits of vaccination against the viral pathogens involved in mixed viral–bacterial infections have been clearly demonstrated and the results indicate that vaccination against viral components involved in diseases such as BRD can reduce the incidence of bacterial infection. In view of the numerous instances of viral–bacterial synergisms, additional controlled studies in this area would be beneficial in further reducing the use of antibiotics.

Conclusions

Vaccination of animals has proved to be a remarkably efficient means of preventing bacterial disease despite the fact that many of the vaccines currently in use are based on century-old technology. The diseases that have been successfully controlled by immunization have, by and large, represented acute disorders, or the 'low hanging fruit' of infectious diseases, whereas chronic diseases such

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as those caused by mycobacterial species remain elusive targets. The reason for this has been the lack of options for vaccine formulations that are capable of inducing strong cellular responses. Fortunately, this is changing and we are on the horizon of a new era of rational vaccine development in which a number of complementary technologies will be available to induce the appropriate type of immunity at the site(s) of infection. These include novel adjuvants, immunomodulators and delivery devices for mucosal administration of vaccines.

Are vaccines alternatives to antibiotics? The true power of vaccination programs is not likely to be realized in the absence of other intervention strategies as has been the case in the control of human diseases. Therefore, while vaccines may lessen our reliance on the use of antibiotics, they are complementary rather than a replacement. As further advances are made in the field of innate immunity, it is possible to envision a time in which traditional antimicrobial therapy will be rarely used in any animal species, and the development of therapeutic vaccines for chronic diseases will be another invaluable tool in our arsenal for the prevention and treatment of infectious diseases.

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