

Novel vaccines from biotechnology

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Summary

Vaccination continues to be the main approach to protecting animals from infectious diseases. Until recently, all licensed vaccines were developed using conventional technologies. However, the introduction of modern molecular biological tools and genomics, combined with a better understanding of not only which antigens are critical in inducing protection, but an appreciation of host defences that must be stimulated, has opened a new opportunity to develop safer and more effective vaccines. The authors describe the current and future trends in vaccine development and stress that in addition to identifying and producing the protective antigens, it is critical to formulate and deliver these vaccines appropriately to maximise the potential of modern advances in pathogenesis and vaccinology.

Keywords

Deoxyribonucleic acid immunisation – Immunity – Live vaccine – Marker vaccine – Subunit vaccine – Vaccine delivery.

Introduction

Vaccination is one of the most important and cost-effective methods of preventing infectious diseases of animals. To date, no other method in human or veterinary medicine has had such an impact in reducing morbidity and mortality and increasing the overall well-being of humans and animals. The first scientifically based approach to controlling infectious diseases in humans was achieved by Edward Jenner in 1796 when he inoculated an eight-year-old boy with cowpox (vaccinia virus) which in turn provided protection when subsequently challenged with virulent smallpox. Although vaccine technology has made substantial progress in the 200 years following Jenner, with the development of numerous vaccines against a variety of animal and human diseases, the basic concept remains the same. It is the exploitation of these concepts that has led to the eradication of smallpox and that is responsible for keeping many other diseases under control.

In veterinary medicine, vaccines have played an enormous role in the development of the modern livestock industry through the efficacious and cost-effective control of viral diseases, against which antibiotics have had no therapeutic effect. In wildlife management the use of bait vaccination programmes against rabies has been instrumental in

controlling these diseases among wildlife populations. This in turn has led to a dramatic decline in the transmission of these diseases to humans and domesticated animals.

Currently, the majority of licensed bacterial and viral vaccines are either live attenuated or killed. Live attenuated vaccines are very efficient in inducing long-lasting immunity through cell-mediated and humoral immunological responses. However, live attenuated vaccines present a potential risk for pregnant and immunocompromised animals as their potential to revert back to virulence has been constantly questioned. Inactivated vaccines cannot replicate and are, therefore, not infectious. However, they lack the ability to induce a robust immunological response, especially cell-mediated responses, and are therefore generally inferior in comparison with live attenuated vaccines.

There has been increasing pressure applied by the regulatory authorities, both human and veterinary, to specifically define the protective antigens and produce vaccines free from pathogen-associated toxins and immunosuppressive components. Subunit vaccines based on recombinant protein immunogens, deoxyribonucleic

acid (DNA) immunogens, and non-pathogenic vectors are currently the most cost-effective methods of producing antigens free from exogenous material characteristic of conventional vaccines. The current review will try to highlight some recent advances in vaccine development including formulation and delivery of vaccines.

Subunit vaccines

Initially, subunit vaccines were produced by purifying the specific antigens from cultures of the pathogenic bacteria or viruses. However, this required large-scale production facilities and costly downstream processing procedures. While the subunit antigen is free from toxins and immunosuppressive components associated with the pathogen, there is a significant risk during production, especially with Level III organisms, due to the potential of accidental release or escape to the external environment and subsequent transmission. It is for these reasons that native-organism purified subunit vaccines are not often economically viable for use in veterinary vaccines except as crude preparations.

Molecular biology and genetic engineering have had an enormous impact on vaccine development by providing the tools and techniques to produce a single protein in a prokaryotic or eukaryotic system. Furthermore, if the protein is produced in prokaryotic systems, it can be tailored in such a way that the protein of interest is expressed on the surface of the bacteria, in the periplasm, as insoluble inclusion bodies or secreted in the media. The recombinant approach to subunit vaccines is to clone the gene that encodes the protective antigen into a secondary, preferably non-pathogenic, organism that is capable of expressing the immunogen in its native form or with minimal alteration. This protein can then be expressed and harvested using traditional bacterial antigen production methods, or delivered by a live non-pathogenic vector. Recombinant subunit vaccines eliminate the risks associated with handling a pathogenic organism, and the risks associated with live or killed products reverting to a pathogenic state due to incomplete inactivation or attenuation (34, 75, 106). However, the most significant advantage provided by recombinant DNA technology is in the complete characterisation of the immunogen and the resulting product, thereby allowing commercial manufacturers to comply with good-manufacturing procedures and licensing regulations in a cost-effective and timely manner.

In all subunit vaccine approaches, the identification of proteins or epitopes involved in eliciting a protective immune response is crucial to the development of a vaccine capable of inhibiting infection and disease in the body. Enormous advances in genomic and proteomic

bioinformatics has made the rapid identification of protective epitopes possible, including the cross-species identification of functionally similar proteins. For example, in recent years a large amount of information has been gathered on viral glycoproteins involved in virus attachment and entry, as in the case of glycoproteins (g)C, gB, and gD of bovine herpesvirus type 1 (BHV-1) (5, 96). The BHV-1 glycoproteins were purified by affinity chromatography and used to immunise animals where it was concluded that the individual glycoproteins elicited neutralising antibodies, which in turn had the capacity to block viral infectivity *in vitro*, significantly limiting replication of the virus (5). Similar approaches are being considered for other viruses, such as the paramyxovirus where the fusion (F) and haemagglutinin-neuraminidase (HN) glycoproteins are responsible for viral attachment and are therefore prospective candidates for a subunit vaccine (93).

The power of recombinant technology lies not only in single protein or epitope subunit vaccines, but also in generating fusion epitopes. In 1989, Brideau *et al.* (11) demonstrated that when paramyxovirus F and HN glycoproteins were expressed as a single viral fusion protein, a greater protective response was induced than from the individual proteins alone. In 1993, Whitton *et al.* (118) proposed that it should be possible to clone multiple protective epitopes from a variety of pathogens together as a single F protein. This 'string of beads' vaccine should be capable of inducing protective immunity to a wide range of viruses in a single subunit. The combination of genomics, bioinformatics and recombinant technology has even allowed for the development of vaccine candidates before the pathogen could even be cultured (90). Indeed, it is still not possible to culture hepatitis B, but a vaccine has been available for over a decade. Although not mutually exclusive, recombinant technologies and subunit vaccines are mutually compatible and together are revolutionising the veterinary vaccine industry.

Commercial production of a recombinant subunit vaccine requires the selection of an appropriate expression system based on the nature of the protein being expressed. Critical factors in selection of a biopharmaceutical expression system include: the production of an immunologically protective epitope, affordable protein production, affordable extraction and cleanup, minimal immunological interference from host proteins, and minimal pyrogen production. In this light we will present the various expression systems with examples of veterinary pharmaceuticals which they have been used to produce.

Bacterial expression

For the production of non-glycosylated proteins, bacterial expression systems are excellent candidates. Organisms

such as *Escherichia coli* and *Salmonella typhimurium* have been used extensively for the expression of a wide variety of foreign genes and as a result many production, stabilisation and optimisation strategies have been described. A large number of veterinary biologics have and are being produced in prokaryotic systems, including protein G of the respiratory syncytial virus expressed in *E. coli* (65, 79), antigens of feline leukaemia virus expressed in *E. coli* (64), and lipopolysaccharide (LPS) from *Shigella sonnei* expressed in *Vibrio cholerae* and *Bacillus brevis* (80). While prokaryotic expression is efficient and affordable for the production of a broad range of immunogens, including a few natively glycosylated proteins, production of many viral glycoproteins in prokaryotic systems does not result in immunologically protective proteins due to the lack of glycosylation, despite producing significant immune responses (3). Additionally, the presence of lipopolysaccharides and other pyrogens entails various complications including interference and possible injection-site reactions. Therefore, for the expression of glycoproteins and other modified proteins, eukaryotic expression systems are much more suitable. The eukaryotic systems like yeast, insect cells, plants and mammalian cells have been systems of choice for many natively expressed immunogens.

Yeast expression

Yeast has a long history of human use and is extensively used in the production of food, alcohol and fuel. The most characterised yeast is *Saccharomyces cerevisiae* which was used to produce the first ever subunit vaccine for hepatitis B (108), was licensed and commercialised in 1986. From a commercial perspective, yeast expression systems such as *S. cerevisiae* or *Pichia pastoris* are attractive due to their production similarities to bacterial-based systems: manufacturing facilities, cost of production, scalability and ease of genetic modification are all very similar. However, yeast systems have several distinct advantages over prokaryotes, including the ability to express glycosylated protein, the absence of pyrogens and the fact that the yeast cell is not excessively immunogenic. All of these combine to make yeast expression systems particularly attractive in antigen production for veterinary vaccines. Of note are the expression of hantavirus nucleocapsid proteins (89), the expression of protein E from Japanese encephalitis virus at 50 mg/L in *Pichia* (120) and the expression of gD from equine herpesvirus 1 in *Saccharomyces* or *Pichia* (94).

Insect cell expression

The insect cell expression system is based on the infection of cultured lepidopteran cells with a recombinant baculovirus designed to express the gene product under the control of the strong polyhedron promoter, typically resulting in a high yield of immunologically active protein.

Like yeast, insect cells also produce glycosylated proteins except that insect cell glycosylation patterns more accurately resemble higher eukaryote glycosylation, which is therefore believed to have a greater potential as protective immunogens. In 1999, Hu *et al.* (43) described the expression of several structural proteins of infective bursal disease virus, an important poultry pathogen, with a baculovirus/insect cell system and the subsequent improvement in yields through the optimisation of media, dissolved oxygen and the use of protease inhibitors to protect against protein degradation. The major limitation of insect cell expression has been the inability to achieve high densities due to the requirement of high dissolved-oxygen levels which cannot be achieved with traditional fermentation techniques due to the cells' sensitivity to shear forces. Traditional oxygen-supplementation methods such as sparging and mechanical agitation induce significant shear. However, the recent development of synthetic serum-free medium and lipid surfactants are allowing for the high-density fermentation of insect cells on a large scale (45, 46, 85) leading to cost-effective production for veterinary vaccines.

Viral expression

Viral expression systems remain the preferred method of commercial production for native glycoproteins. Examples include the expression of the Aujeszky's disease virus gp50 and gp63 glycoproteins in swinepox (97, 109), and the expression of rabbit haemorrhagic disease virus in canarypox virus (26). This technology was originally demonstrated with vaccinia virus as the vector (77). However, there are other viral systems used extensively for foreign gene expression (89). In fact, almost any virus can be used as an expression system for producing either whole proteins or epitopes.

Mammalian cell expression

Although expression of proteins in mammalian cells is generally expensive, for some viral glycoproteins such expression systems are critical. This is especially true for those glycoproteins where post-translational modification such as glycosylation is important for proper folding and generation of specific epitopes. In selecting an expression system, it is important to choose one that is robust and economical. Thus, almost any cell line that is free of extraneous agents, is not tumorigenic and grows well, preferably in suspension, could be employed. Since there is at least partial correlation between aggressive growth *in vitro* and transforming characteristics, it is critical to ensure low tumorigenicity of the cell for production of products. Another critical consideration is to develop an expression system where the glycoproteins (products) are secreted into the media and not retained in the cell. This is important for two reasons. First, over-expression of many proteins may

lead to death of the cell due to the toxic nature of many viral proteins. Secondly, if cells need to be lysed to extract the product, this adds excessively to the cost of production: not only is it necessary to remove host cellular proteins from the product, but this approach requires the growth of large quantities of cells. Thus, if the product is secreted into the media, the cell mass can be maintained over an extended period and the product harvested repeatedly. Since most glycoproteins are associated with membranes, it requires the removal of the transmembrane anchor to allow secretion of the glycoprotein into the media. This can only be done with glycoprotein/proteins in which the transmembrane requirement does not dramatically alter the conformational properties of the product. Similarly, it is possible to secrete other non-anchored, but cellular proteins, by the incorporation of a secretion signal sequence.

The feasibility of this technique was recently demonstrated using gD from BHV-1, where the gD was expressed in MDBK cells under the control of a heat-shock promoter. Although this glycoprotein is toxic to cells, the rapid secretion into the media following induction of gD synthesis allows the continuous harvesting of the product over at least one month (53). Since the cells can be grown in serum-free media, downstream processing is extremely economical. This is critical in veterinary vaccines where costs per dose must be cents/dose.

Plant-based expression

Recently plants have become the focus of a number of researchers in the development of biofactories for recombinant proteins and biological products (14, 40, 52). Of interest is the expression of biopharmaceuticals in plants as they possess the ability to produce glycosylated proteins similar to that of higher eukaryotes, and they have the potential to be administered as oral vaccines with minimal expense in downstream processing. The use of a cereal or oilseed crop also presents the possibility of the vaccine immunogen being stable at ambient temperatures, thereby allowing for long-term storage without the need of a cold chain. While the level of expression in plants varies greatly, it seems that how the expression product is regulated and where it is targeted has the greatest impact on yield; optimisation of these processes may allow for the development of predictable and reliable high yielding expression constructs in the near future. For example, subunit B of the cholera toxin (CT) has been expressed in potato tubers at 0.3% of the total soluble protein when targeted to the endoplasmic reticulum (2), yet yielded 4% of the total soluble protein when expressed in the chloroplast (20). Correspondingly, subunit B of the heat-labile enterotoxigenic *E. coli* toxin has been expressed in corn seed at 0.3% of the total soluble proteins and the modification of the regulatory sequences has resulted in yields up to 12% (17, 103).

The development of plant-based direct-fed vaccines offers considerable advantage when compared to the high cost of production, formulation and delivery of a conventional vaccine. Regardless of the delivery vehicle, oral immunisation typically requires 100-1,000 fold more antigen to be presented than is required for parenteral delivery (103). In the case of ruminants, this requires that the encapsulating cell remains intact during its passage through the rumen so as not to expose the immunogen to ruminal proteases, and subsequent inactivation; regardless of the species, this cell must lyse in the abomasum or proximal duodenum and adequately dissociate from the immunogen so as to present it effectively to the mucosal surface of the small intestine (102). Veterinary development projects include the expression of glycoprotein S of transmissible gastroenteritis virus in plants such as the arabidopsis leaf (32), the potato tuber (33), and maize seed (54); as well as the expression of rabies glycoproteins in alfalfa (70), and VP1 protein of foot and mouth disease in tobacco (119). Currently, the greatest concern regarding the use of direct-fed vaccines is the risk to the human food chain due to inadvertent cross-contamination and the potential issues of immunogenicity and tolerance to oral vaccines. Despite the demonstration of efficacy, no plant-based vaccine has been licensed to date.

Formulation and delivery

Subunit or killed vaccine specific antigens require specific adjuvants in order to present an immunological response tailored to mimic responses induced by natural infection. It has been generally accepted that the optimal protective response is achieved when the vaccine is administered via the same route by which the infection enters the body (21). Therefore, formulation is essential in the development of an effective vaccine as the adjuvant must be compatible with the route of administration and complementary to the antigen. Today's highly efficacious and safe vaccines, be they recombinant or conventional inactivated vaccines, would not be available if it were not for the adjuvants and delivery systems developed over the past three decades.

Any component that is added to the antigen in order to enhance the immunological and protective response to the antigen, and hence the efficacy of the vaccine, is called an adjuvant. The term is derived from the Latin verb 'adjuvare', meaning 'to help'. A variety of chemical and biologically derived compounds have been added to vaccines in order to increase the elicited immunological response, including aluminum salts, mineral oil, CT and *E. coli* labile toxin. More recently, several classes of compounds including immunostimulatory complexes (ISCOMs), liposomes, virosomes and microparticles have been employed to act as antigen-delivery vehicles and they are proving to be potent adjuvants, greatly enhancing the

magnitude and the duration of the immunological response to the formulation.

An efficacious response to non-replicating vaccines (subunit and inactivated) is entirely dependent on the adjuvant, which acts via a variety of pathways, but predominantly through one of three methods. The first mechanism known as the 'depot effect' presents the antigens to the immune system by physically associating with immunological cells so that the formulation is exposed for sufficient time or in sufficient levels to induce a significant immune response. The second method consists of targeting innate immune pathways, which when activated, will quantitatively and qualitatively direct the immunological response towards the specific antigen. The last method is to alter the properties of the antigen so that it increases its ability to effect either or both of the first two mechanisms (10).

Aluminum-based adjuvants

Aluminum hydroxide has been used extensively as an adjuvant in veterinary and human medicine, predominantly because it is inexpensive and safe. Its activity is attributable to the physical characteristics of the formulation, including surface area, charge and morphology. Vaccines formulated with aluminum predominantly stimulate the Th-2-like immune response, inducing subclass immunoglobulin (Ig)G₁ and IgE antibodies; however, they only poorly induce cell-mediated responses (19). Aluminum's mechanism of action is not fully known, but it appears that it is not entirely dependent on antigen adsorption, as once thought (10). Vaccines formulated with aluminum cause inflammation at the injection site and in some instances have been thought to precipitate granuloma formation (10).

Mineral oil adjuvants

Vaccines formulated with either mineral-, plant- or animal-derived oils have proven to be very potent and as such have been used widely in veterinary vaccines. In general, mineral-based oils are more potent than plant- or animal-derived oils. However, regulatory authorities and livestock producers are apprehensive about the safety of products adjuvanted with mineral oil due to strong injection site reactions. Furthermore, mineral oils are not metabolised and are, therefore, present in tissues for a long time. Both alum- and oil-based adjuvants act as depot effectors either at the site of injection or in antigen presenting cells (APCs) (84).

Immunostimulatory complexes

The ISCOMs represent a significant step forward in the formulation of veterinary vaccines as they inherently

overcome many of the deficiencies that are characteristic of traditional alum- and mineral oil-based adjuvants. The ISCOMs are composed of hydrophobically associated cholesterol, phospholipids and quillaja saponins which form a small, stable, cage-like structure with a diameter of between 30-40 nm to which the antigen can be enclosed without altering its structure (18). As such, they are effective inducers of long-lasting cell-mediated and mucosal immunological responses, which are substantially greater than those elicited by traditional depot adjuvants.

Following parenteral immunisation, the T-cell response is first detected in the draining lymph node. At 50 days post vaccination, the majority of the antibody-producing cells are found in the bone marrow (74). It is extremely important to note that as a result of the dual processing of antigen in the endosomal vesicles and cytosol of the APCs, both cell-mediated and humoral immunological responses have been elicited by ISCOM-based vaccines (98). This explains why both T-helper and cytotoxic T-lymphocyte cells are simultaneously activated by ISCOMs (74). ISCOMs stimulate CD8+ and major histocompatibility complex (MHC) class I restricted T-cells and CD4+ helper T-cells.

For a long time it was suggested that only replicating agents could efficiently induce a mucosal immunological response due to their capacity to infect via the mucosal route. However, intranasal administration of influenza ISCOMs to mice not only demonstrated protection against challenge (59, 73), but more importantly induced a strong mucosal response and the induction of cytotoxic T lymphocytes (CTL). Oral administration of ISCOMs has resulted in the induction of a wide range of immune responses including serum antibodies, secretion of mucosal IgA, Th1, Th2 and CD4 T cell responses in addition to MHC class I restricted CTL activity. Furthermore, it has been shown that repeated low-dose oral administration of ISCOM-adjuvanted vaccines did not induce tolerance (78).

Either oral or intranasal administration of ISCOMs is capable of inducing a strong specific mucosal response in both local and remote mucosal surfaces, but amazingly within fifty days post-injection the majority of the antibody producing cells were found within the bone marrow (42), similar to the response seen via parenteral ISCOM injection. In contrast, there have been no reports of antibody production by bone marrow cells with alum- or oil-adjuvanted vaccines.

Several veterinary biologics formulated with ISCOMs, in addition to those discussed above, have demonstrated efficacy following challenge, including antigens against equine influenza virus (74), BHV-1 and *Pasteurella multocida* (72). An experimental ISCOM formulation has also been shown to elicit an immunological response in newborn mice and calves in the presence of passively acquired antibodies (74).

Liposomes

The liposome is another delivery system which has been widely employed for antigen delivery and presentation. Liposomes are microparticles that have been used in the pharmaceutical industry as drug delivery carriers for thirty years prior to being recognised as a potential antigen-delivery technology. Liposomes are bilayered vesicles composed of phospholipids and other sterols typically surrounding an aqueous centre where antigens or other products can be encapsulated. The liposome structure is highly versatile and over the past forty years many types have been developed, such as large unilameral vesicles which range in size from 100-500 μm , small unilameral vesicles which range in size from 25-100 nm, and biphasic liposomes which have been found to be effective in delivering therapeutic agents to dermal and mucosal surfaces (28). Liposomes can be modified for targeted delivery by incorporating specific antibodies into the surface membrane, or altered to encapsulate hydrophilic or corpuscular structures such as bacteria, viruses or parasites. The average survival time of the intact liposome structure has been significantly extended with the inclusion of polyethylene glycol, allowing for prolonged release *in vivo* (1).

Multilameral vesicles and biphasic delivery systems have been used successfully in veterinary vaccinology as carriers for the BHV-1 antigens. In 1997, Baca-Estrada *et al.* (9) reported an increase in antigen specific IgG_{2a} antibodies and interferon gamma (INF- γ) secreting cells when gD and interleukin (IL)-12 antigens were co-encapsulated, and an increase in antigen specific IL-4 secreting cells when gD and IL-4 antigens were co-encapsulated. While a substantial amount of bacteria, virus and parasitic antigens have been evaluated, to date no liposomal-based commercial veterinary vaccines have been licensed (10). It is thought that the major obstacle for liposomal-based antigen delivery is the fact that organic solvents are used in their preparation. This complicates the vaccine registration process and negatively affects protein stability, while alternative encapsulation processes (which result in increased protein stability) are currently cost-prohibitive.

Microparticles

Microparticles are another promising technology which employs aliphatic polyester microspheres, where copolymers of polylactide and polyglycol esters are used to synthesise the small biodegradable spheres which act as depots for vaccine delivery (24). The major advantage that polymer microspheres possess over other depot-effecting adjuvants is that they are extremely safe and have been approved by the Food and Drug Administration in the United States of America for use in human medicine as suitable sutures and for use as a biodegradable drug delivery system (55, 91). It

is important to emphasise that microparticle formulations can be lyophilised and that the rates of copolymer hydrolysis are very well characterised, which in turn allows for the manufacture of microparticles with sustained antigen release over prolonged periods of time (82).

Parenteral administration of microparticles elicits long-lasting immunity, especially if they incorporate prolonged release characteristics. The rate of release can be modulated by the mixture of polymers and their relative molecular weights, which will hydrolyse over varying periods of time. Furthermore the formulation of different sized particles (1 μm to 200 μm) may also contribute to long-lasting immunological responses due to the fact that large particles must be broken down into smaller particles before being available for macrophage uptake. In this manner a single-injection vaccine could be developed by integrating various particle sizes, thereby prolonging antigen presentation and greatly benefiting livestock producers. An excellent example of this concept would be in the employment of polymer microparticles in an immunocastration product, where the duration of immunity is the key factor to efficacy. However, there is evidence to suggest that certain polymers create an acidic micro-environment when degraded *in vivo*, potentially altering protective epitopes and possibly inactivating the vaccine (95).

Unfortunately, microsphere technology is currently cost-prohibitive for use as a veterinary adjuvant and there are still problems with antigen stability during manufacture and long-term storage. No polymer microparticle-based veterinary vaccines have been licensed to date.

Stimulation of mucosal immunity

Vaccines have traditionally been administered to cattle in the rump via intramuscular injection. However, this part of the carcass produces the highest cuts of meat, so the lesions caused by vaccination and broken needles mean that this route of administration is no longer attractive. The Canadian Beef Survey, an annual evaluation of packing industry losses, has determined losses related to this intramuscular injection site to be Can\$8 per carcass side due to the loss of these choice cuts of meat (111). As a result of these studies, both producers and processors are promoting subcutaneous injections in order to reduce these losses and increase carcass value.

However, both intramuscular and subcutaneous injection routes share one significant disadvantage in the induction of significant systemic responses, while inducing poor mucosal immunity. Mucosal immunity and the production of local IgA antibodies are central to preventing pathogen

penetration of mucosal surfaces, the major route of infection for numerous diseases. Administration of vaccine to mucosal surfaces such as the nose, eye, lung and gastrointestinal tract is an effective way of inducing mucosal immunity. It is important to emphasise that all mucosal sites are interconnected by a common mucosal immune system (CMIS) and that the administration of protective antigens at one primary site will stimulate antigen-specific lymphocytes which migrate and provide immunity through the body, regardless of the site of induction (10, 60, 67).

In veterinary vaccinology, the delivery of live attenuated organisms to mucosal surfaces has proven to be very effective, as demonstrated by the intranasal presentation of BHV-1 and parainfluenza-3 followed by successful challenge within 60 h to 72 h (104). Additionally, the intranasal delivery of *Bordetella bronchiseptica* to dogs induced protection to kennel cough (49), and the aerosol administration of *P. multocida* vaccine to chickens proved to be both practical and effective in preventing disease (38).

Since prolonged antigen presentation appears essential in eliciting effective mucosal immunity, the employment of ISCOMs, liposomes, and microparticle adjuvants, live vector vaccines, or the feeding of plant-based oral vaccines hold significant promise in the continued development of effective mucosal vaccines.

Deoxyribonucleic acid vaccines

The molecular breakthroughs in cell transformation and gene therapy induction has created the new field of DNA vaccinology and its enormous potential to provide safe, inexpensive and effective DNA-based vaccines. The basic concept of DNA vaccines is to deliver plasmid DNA encoding for protective proteins into the cells of the host animal where they can direct transcription and translation, effectively transforming the vaccinee into a mammalian bioreactor for the production of its own vaccine. The fact that the protein is produced within the mammalian host means the vaccine should be correctly modified post-translationally and as such possess the authentic conformational structure and thereby a functional epitope.

Owners of livestock and companion animals vaccinating with inactivated or killed vaccines have justified concerns regarding the severity of injection site reactions. However, these concerns may become worries of the past since DNA vaccines are not formulated with chemical adjuvants, the leading contributor to injection site reactions. Since DNA vaccines consist solely of nucleic acids, they lack the ability to replicate, infect or induce disease. As such, they are considered extremely safe, yet they not only have the ability to provide protection from pathogens which pose environmental or human health risks, but they also have the

potential to provide protection from organisms which are either difficult to cultivate or have not even been isolated.

Despite the fact that the antigen is produced by the host's cellular machinery, the immune system recognises the protein as being foreign and mounts both a cellular and humoral immune response very similar to one induced by live vaccines or in animals recovered from natural infection (112). Although CpG motifs commonly present in DNA vaccines induce a wide range of cytokines, it seems that Th1-like or Th2-like responses are not equal, with Th1 induction exceeding Th2-like responses. The type of immunological response following vaccination depends not only on the gene being introduced but also on the site of administration and the mechanism of delivery. Delivery of DNA vaccines via intramuscular injection tends to promote an immune response that is characterised by the production of INF- γ and IgG_{2a} isotype immunoglobulins of the Th1 phenotype (92). In contrast, delivery via gene gun to the skin tends to promote a Th2 lymphocyte response predominantly characterised by IL-4 and IgG1. In order to balance the immune response, genes encoding various cytokines and co-stimulatory molecules can be included or co-administered with the gene of interest (56). A large number of studies have been performed to date in order to evaluate the efficacy of co-administered plasmids encoding biological adjuvants, including INF- γ (41, 114), IL-2 (81) and IL-12 (31, 36).

Delivery of deoxyribonucleic acid vaccines

The effectiveness of DNA vaccines is largely dependant on effective uptake of the DNA by the cell and more importantly by the nucleus. Numerous approaches used to enhance plasmid uptake, include chemical transformation, electroporation and gene gun delivery.

Poly(lactic-co-glycolide) (PLG) microparticles used in delivery of protein-based vaccines hold great promise for delivery of DNA vaccines either by systemic or oral delivery. This hypothesis was confirmed by the successful induction of an immunological response when PLG encapsulated DNA was taken up by gastrointestinal M cells (47). While the magnitude of this response was lower than expected – primarily believed to be as a result of DNA damage due to the traditional encapsulation process – subsequent construction of cationic PLG microparticles with surface adherent DNA resulted in a 1,000 fold increase in response as compared to free DNA (83). The preliminary studies in mice, guinea pigs and rhesus monkeys have demonstrated enhanced cellular and humoral enhanced duration of immunity following vaccination with PLG-DNA particles and suggest that large-animal immunisation may be possible.

The electroporation of cells is an effective method of DNA transformation and is a standard molecular tool.

Through the use of a non-invasive surface electrode and an invasive needle electrode, a DNA vaccine encoding for BHV-1 gD has been successfully used in pigs. Not only did electroporation result in effective protein expression, but a significant and proportional increase in both IgG1 and IgG2 was observed, demonstrating both cell-mediated and humoral immunity (4).

In molecular biology, the most efficient transformation method is that of the gene gun, whereby gold particles are coated with DNA and explosively propelled through the skin and directly into the target cell cytoplasm and nucleus. Delivery of DNA plasmid vaccines via a gene gun is much more efficient at achieving transformation and induction than an equivalent amount of plasmid injected with a needle (88). In fact, gene gun administration can achieve an equivalent immunological response with 100-fold less DNA than that required via needle delivery (8). The efficient transformation of Langerhans cells and dendritic cells with gene gun technology has been demonstrated in mice and cattle using green fluorescent protein as the gene product (58), which is significant as these cells rapidly migrate from the mucosa to the lymph nodes (25, 105, 107).

One of the major problems of current vaccines is their inability to elicit active immunity in neonates possessing passively acquired maternal antibodies. However, DNA vaccines have been shown to effectively circumvent maternal antibody interference (37, 71, 113). Furthermore, since the mucosal immunological system functions as a common inter-related system, the delivery of a DNA vaccine at one mucosal site should induce at least partial immunity at other sites and may in fact serve to prime the immune system prior to vaccinating with a traditionally delivered, killed or subunit vaccine. This concept was confirmed by Loehr *et al.* (58) who demonstrated that immunisation with as little as 6 µg of plasmid DNA delivered with a gene gun to the vagina of cattle was capable of priming a mucosal immunological response in the respiratory tract. This work lays the ground for an entirely new approach to vaccination.

A large number of experimental DNA-based veterinary vaccines have been evaluated in a number of species with varying degrees of immunological response and efficacy against challenge (3, 8, 23). Despite the promising results, some questions have been raised regarding the safety of DNA vaccination. Possible integration of plasmid DNA into the host genome and potential risk of malignancy and integration of foreign genes into germ cells could potentially lead to vertical transmission. Additionally, the potential exists for the development of antibodies against the DNA vaccine itself, with the putative consequence of an autoimmune disease developing. There is, however, no evidence to date to suggest that an animal can produce antibodies to DNA-based vaccines, even when milligram quantities are successively injected (112). Despite the lack of scientific evidence to substantiate plausible risk from

DNA vaccination, it would be unwise to rule them out entirely and it is reasonable to expect regulatory authorities to impose strict measures in order to avoid these situations.

Live vaccines

The classification of live vaccines can be applied to the following technologies:

- a) conventional attenuated live vaccines
- b) live gene-deletion attenuation vaccines
- c) recombinant non-pathogenic live vectored vaccines.

Live attenuated vaccines

Attenuated live vaccines delivered to mucosal surfaces are able to induce effective long-lasting cell-mediated and humoral immunological responses (39, 61). The advantages of live attenuated vaccines are their low reactivity, their induction of a systemic response with subsequent protection against disease, their induction of a mucosal response with subsequent protection against infection, and their low manufacturing costs due to minimal downstream processing combined with the lack of adjuvants in the formulation. However, the overall safety profile of conventional attenuated live vaccines is far from that of an ideal vaccine (6).

Conventional live bacterial and viral vaccines are produced by selecting attenuated mutants which have the capacity to induce infection but have reduced, or completely lack, ability to induce disease. These mutations are usually created with chemicals, heat or spontaneous mutagenesis. It is important to realise that mutational attenuation is an uncontrollable process, and the induced mutations are rarely fully characterised at the genomic level. As such, it is very difficult to control the degree of attenuation: if only a single point mutation occurs, there is a high probability that the mutation may revert in the field. Several vaccines have demonstrated reversion of attenuation, including an experimental porcine reproductive and respiratory syndrome virus vaccine (75, 106), and an oral polio vaccine for children (68). Reversion of attenuation is the greatest risk attributed to attenuated live vaccines: not only does it cause disease in vaccinated animals, but the animal will shed the organism into the environment, thereby serving as a continuous source of infection. However, even with these risks, there are many examples of very effective and safe, live attenuated vaccines currently in use in veterinary medicine.

Gene-deleted attenuated vaccines

Recent advances in genetic engineering techniques enable us not only to identify the genes associated with the virulence of

a pathogenic organism, but also to delete or inactivate these genes, thereby increasing the safety profile of a vectored vaccine. The introduction of two independent mutations which are not chromosomally linked, either as functional mutations or gene deletions, will dramatically reduce the risk of pathogenic reversion.

In 1985, Kit *et al.* (50) demonstrated that the herpesvirus genes for the structural gC and nucleic acid metabolism thymidine kinase (TK) could be deleted without significantly affecting virus ability to replicate *in vitro*. Subsequently, it has been demonstrated (48) that the TK, gC and gE genes of the BHV-1 virus could be deleted without significantly altering its immunological protective characteristics. The first commercial live gene-deletion attenuated vaccine was a gE⁻ pseudorabies vaccine that is currently used for eradication programmes in Europe and United States of America (51). More recently a gE⁻gG⁻TK⁻ live attenuated vaccine pseudorabies vaccine has been developed (117). It seems highly likely that these types of vaccines will become more prevalent in the future as people accept that they are safer than conventional attenuated vaccines and that the degree of attenuation can be managed more effectively. Some regulatory bodies are expressing concern that these vaccines may recombine with field strains, but exactly the same can occur with conventionally attenuated vaccines. Indeed, conventionally attenuated vaccines can be even worse, since we do not even know which genes are altered in the conventionally attenuated vaccines.

Recombinant live vectored vaccines

The delivery of heterologous antigens via a recombinant live vector offers significant advantages as a vaccination strategy in that the recombinant organism would only induce a mild infection and subsequently induce immune responses to the gene from the pathogenic organism (100). Regardless of the vector organism, expression of the protective antigen by the recombinant vector is key to effective presentation of the antigen to the cells and the induction of the mucosal immune system (29). Currently, it is possible to use viruses, bacteria and even parasites as live vectors.

Gram-negative bacteria can elicit strong and long-lasting immune responses to foreign antigens by establishing limited infections which resemble mild forms of the natural infection (57), and the LPS rich cell walls of Gram-negative bacteria act as natural adjuvants. Surface expression on bacteria of a vaccine antigen was first described by Charbit *et al.* (15) with the report of expression of the C3 epitope of poliovirus, and subsequently with the fimbriae presentation of a foot and mouth viral protein by van Die *et al.* (110). The most ubiquitous recombinant Gram-negative bacteria is *E. coli* which, although it has been used extensively as a recombinant live vector, does not induce a significant

undesired immune response to itself due to the fact that it is a natural inhabitant of the colo-rectal tract. *Salmonella typhimurium* is another Gram-negative bacterium which has been extensively used in the development of experimental recombinant live vectored oral vaccines against bacterial, viral and parasitic diseases (16, 30, 35). *Salmonella* is closely related to *E. coli*, and is an intracellular pathogen which colonises the intestinal tract and proliferates in the gut-associated lymphoid tissue and in advanced enteric infections will systemically spread to the liver and spleen. As an intracellular enteric pathogen *S. typhimurium* elicits a potent cellular response to the delivered antigen, and has induced mucosal immunity in mice immunised with recombinant *S. typhimurium* expressing bacterial and viral antigens (57). Despite the extensive research efforts in the last two decades, *Salmonella* sp. have not met vaccinologists' expectations as a recombinant live-vectored delivery system, predominantly due to virulence reversions and the induction of disease in immunocompromised individuals.

Many Gram-positive bacteria including *Staphylococcus aureus* and bacillus Calmette-Guérin have been investigated for their potential to act as recombinant live vectors in the delivery of heterologous antigens, with *Listeria monocytogenes* and *Mycobacterium tuberculosis* showing the most promise. Both, *M. tuberculosis* and *L. monocytogenes* are intracellular pathogens which elicit a mucosal immunological response similar to that of *Salmonella* sp. (27, 101), with *Listeria* stimulating antigen-specific CD8⁺ and CD4⁺ T cells, and *Mycobacterium* stimulating Th1 helper response, a strong IFN- γ production, and cytotoxic CD8⁺ lymphocytes (22). However, recent development emphasis has been shifting towards non-pathogenic food grade or commensal bacteria as they are immunologically quiescent and non-infective, thus eliminating clinical and virulence issues.

The first recombinant live viral vector evaluated was the vaccinia virus (62, 86) and it has since been used to express viral, bacterial and parasitic antigens, which have been reported to elicit protective immunity in animal disease models providing both cellular and humoral response (44, 63, 76). Vaccinia virus has demonstrated efficacy as a recombinant live viral vector in experimental trials when virus expressing rabies gG as a surface antigen was orally delivered to foxes and other wild animals (12, 13, 87); vaccinia virus expressing pseudorabies gD as a surface antigen delivered intramuscularly induced an effective immunological response very similar to a conventional killed vaccine (66). Currently, a number of pox-based vectored vaccines are being marketed. To further improve the safety of these vaccines, avian poxviruses are being used to deliver antigens to mammals. Since avian poxviruses cannot replicate in mammals, this provides a further level of safety. Bovine adenovirus expressing the protective gD of BHV virus was evaluated as a recombinant live vector in laboratory animals and cattle (69, 121) and demonstrated effective

mucosal and systemic immunity when delivered intranasally (7). Viruses with large genomes, such as vaccinia and adenovirus, are better candidates for recombinant viral vectors than smaller genome viruses due to the fact that they can accommodate substantially larger inserts of foreign DNA while retaining their infectivity. These viruses present a cost-effective vaccination strategy due to the fact that their genomes have been sequenced and that commercial expression vectors are available, combined with the fact that they can be cultured to very high titres resulting in reduced production costs.

Despite very promising results with live vectored vaccines there are also some concerns. The major concern is that live vector vaccines will not induce adequate immunological responses in animals that have pre-existing antibodies against the vector. However, it has been demonstrated that these vector vaccine can elicit immunological response in seropositive animals (7) if given mucosally, most likely due to the fact that mucosal immunity levels are not as long-lived as serum antibody level. Similarly, the use of avian poxviruses to immunise mammals should dramatically remove this concern since mammals would not normally have antibodies to avian disease. This does not remove the concern that repeated immunisation with the same vector will reduce the efficacy of subsequent immunisation to different antigens. Thus, it may require the use of various types of vectors and vaccines over the life of the animal to maximise protection. This will require a very good method of vaccination record keeping and adherence to vaccination recommendations by both the veterinarian and the client.

Marker vaccines and disease eradication

In order to evaluate the effectiveness of a disease eradication programme, a detection method is required in order to differentiate vaccinated (immune) animals from naturally infected (carrier) animals (116). This is achieved by developing vaccines that lack one or more antigens, thereby causing the antibody response produced by vaccinated animals to be serologically distinct from that induced by a wild-type infection. In essence, this could be considered as a vaccine fingerprint.

The key to negative markers is that an antigenic protein which is present in the wild-type pathogen fails to elicit an immune response following vaccination. All vaccines can serve as positive marker vaccines by adding a foreign immunogenic antigen. However, in disease eradication programmes the identification of vaccinates (positive marker animals) is less important than the identification of disease carriers (negative marker animals), which forms the basis of the concept for differentiating infected from vaccinated (DIVA) individuals (115). The use of a negative marker, or

DIVA vaccines, is an important tool in assessing the effectiveness of vaccination programmes, without the time-consuming and near impossible task of individually evaluating infectivity, transmissibility and susceptibility within a vaccination programme.

The first gene-deleted live attenuated pseudorabies vaccine was a naturally occurring mutant, lacking glycoprotein E (gE⁻), known as the Bartha vaccine and has been used for decades in controlling pseudorabies virus in pigs. The Bartha vaccine was subsequently recognised for its ability to differentiate vaccinated animals from naturally infected animals, effectively making it the world's first marker vaccine (116). With the availability of recombinant technologies and sequenced pathogenic genomes the identification of potential negative markers is more feasible than ever before. It seems logical that vaccines developed in the future will embrace the DIVA principle in order to provide a much needed tool for agricultural disease eradication programmes.

Currently, agricultural disease surveillance and eradication programmes are largely based on the serological confirmation of infection and the destruction of herds to limit the spread of disease. Due to this reliance on serological confirmation, a complementary vaccination programme is actually incompatible with surveillance. Thus vaccination programmes are often banned along with animals originating from countries vaccinating for these diseases. The application of the DIVA negative marker technology creates compatibility between surveillance and vaccination programmes, allowing vaccination to play a large and overwhelmingly significant role in the eradication of these diseases. Several marker vaccines are already commercially available, and their role and contribution in disease eradication appears promising. However, for them to be embraced by the industry there needs to be support by governments and the livestock industry so as to ensure that the biopharmaceutical industry develops these vaccines. The move to eradicate diseases is not only driven by direct economics to the producer, but is often politically motivated: once a country is declared disease-free, the country can use this disease-free status as an effective trade barrier, making the vaccines even more attractive.

Conclusions and future prospects

Despite the enormous advances in vaccinology during the last three decades, the perfect vaccine has yet to be developed. This vaccine would be multivalent and induce specific immunity against the protective antigens, would protect against both disease and possibly infection, would protect newborn animals regardless of passively acquired antibodies, would have a long duration of immunity, would not require booster doses, would be free

of adverse reactions, and would be administered easily without needles.

Now that the perfect vaccine has been defined, the real question is: can scientists deliver such a vaccine? Based on

the progress of the last three decades we should positively predict that the foundation has been laid to build such a vaccine. However, the question remains: will this vaccine be economical to produce for use in veterinary medicine? ■

Nouveaux vaccins issus de la biotechnologie

D. Rogan & L.A. Babiuk

Résumé

La vaccination reste la principale méthode permettant de protéger les animaux contre les maladies infectieuses. Jusqu'à une date récente, tous les vaccins ayant obtenu une autorisation étaient développés en utilisant des technologies classiques. Cela étant, l'introduction des outils modernes de la biologie moléculaire et de la génomique, conjuguée à une meilleure connaissance non seulement des antigènes qui sont essentiels à l'induction d'une immunité protectrice, mais aussi des défenses de l'hôte qui doivent être stimulées, a ouvert de nouvelles possibilités de développement de vaccins plus sûrs et plus efficaces. Les auteurs décrivent les tendances actuelles et futures en matière de développement de vaccins et soulignent qu'en plus de l'identification et la production des antigènes protecteurs, la formulation et l'administration appropriées de ces vaccins sont cruciales pour tirer le meilleur parti des progrès récents accomplis en pathogénèse et en vaccinologie.

Mots-clés

Administration vaccinale – Immunité – Marqueur vaccinal – Vaccin sous-unité – Vaccin vivant – Vaccination à acide désoxyribonucléique.



Nuevas vacunas producidas por métodos biotecnológicos

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Resumen

La vacunación sigue siendo el método más utilizado para proteger a los animales contra las enfermedades infecciosas. Hasta hace poco tiempo, todas las vacunas autorizadas se producían con tecnologías convencionales. Sin embargo, la aparición de nuevos medios biológicos moleculares y de la genómica, a los que se sumó un conocimiento más profundo de los antígenos que inducen la protección y de las defensas que es preciso estimular en el huésped, abrió una nueva vía para la elaboración de vacunas más seguras y eficaces. En este artículo se describen las perspectivas, presentes y futuras, de la producción de vacunas y se hace hincapié en que, para sacar todo el

provecho posible de los avances contemporáneos en materia de patogenia y vacunas, no es suficiente identificar y producir antígenos protectores; su formulación y administración adecuadas también revisten una fundamental importancia.

Palabras clave

Administración de vacunas – Inmunidad – Inmunización con ácido desoxirribonucleico – Vacuna de subunidades – Vacuna de virus vivos – Vacuna marcadora.



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