

Plant Production of Veterinary Vaccines and Therapeutics

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Abstract Plant-derived biologicals for use in animal health are becoming an increasingly important target for research into alternative, improved methods for disease control. Although there are no commercial products on the market yet, the development and testing of oral, plant-based vaccines is now beyond the proof-of-principle stage. Vaccines, such as those developed for porcine transmissible gastroenteritis virus, have the potential to stimulate both mucosal and systemic, as well as, lactogenic immunity as has already been seen in target animal trials. Plants

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are a promising production system, but they must compete with existing vaccines and protein production platforms. In addition, regulatory hurdles will need to be overcome, and industry and public acceptance of the technology are important in establishing successful products.

Introduction

Veterinary pharmaceutical products generated US \$14.5 billion in worldwide sales in 2000, the leading biological products of which were vaccines against foot-and-mouth disease (Gay et al. 2003). For viral diseases, the most common antigen and route of vaccination are live attenuated or chemically inactivated whole viruses via parenteral injection. For diseases caused by bacteria, live attenuated or killed bacteria or outer membrane protein preparations elicit protective antibody responses when injected, given orally, or given intranasally (Bowersock and Martin 1999). Recent developments of subunit or peptide vaccines by incorporation of known protective antigenic proteins and/or serologically dominant epitopes into DNA plasmids or mammalian viral-based vectors for delivery have prompted investigation into the use of plant expression systems for production of candidate animal vaccines (Streatfield and Howard 2003a). In addition to the safety advantages through the reduced risk of contamination with animal or human pathogens, plant-based vaccines also offer the opportunity to deliver the vaccine cost-effectively and orally to large numbers of animals at the same time, thus saving time and the cost of immunizing animals individually by injection, as well as increasing client compliance, thereby contributing to overall herd health (Streatfield et al. 2001). There are new growth opportunities in veterinary applications of plant-based biologics, including vaccines and therapeutic products.

This chapter reviews the current status of the development of plant-derived biologics for administration to animals and for use as diagnostic tools. Development of stable, transgenic plants and plant viral-based vectors for transient expression of biopharmaceuticals for control of animal diseases has been carried out, with either full-length proteins or peptides, showing efficacy in animal trials.

Foot-and-Mouth Disease Virus

Foot-and-mouth disease virus (FMDV) is the causative agent of an economically important disease that affects meat-producing animals, including cattle, sheep, pigs, and other wild and domestic cloven-hoofed animals, and remains one of the most important pathogens of livestock (Mason et al. 2003). The disease, which is acute and highly contagious, is spread by contact with infected animals, and by movement of contaminated vehicles, humans, and nonsusceptible animals.

Current control measures include restrictions on the movement of animals and animal products, slaughter of affected animals, disinfection, and vaccination with chemically inactivated tissue culture-propagated FMDV (Mason et al. 2003); these vaccines require high-containment facilities for vaccine production.

FMDV is a member of the genus *Aphthovirus* of the family Picornaviridae. It is a nonenveloped, isometric virus that contains four capsid proteins (VP1–4; also known as 1D, 1B, 1C, and 1A, respectively) and a single molecule of polyadenylated RNA that is translated into a single polyprotein that undergoes proteolytic cleavage to mature, functional proteins (Levy et al. 1994). Studies have shown that VP1 carries epitopes responsible for induction of neutralizing antibodies. Immunization with VP1, synthetic peptides derived from VP1, or recombinant vaccinia virus expressing VP1, induces protection against challenge virus in natural and experimental hosts (Brown 1992; Berinstein et al. 2000, respectively).

Strategies that have been developed for the expression of FMDV antigens in plants include fusion of immunodominant epitopes of FMDV capsid protein VP1 with plant virus capsids. In addition, expression of the complete VP1 structural protein by stable incorporation in transgenic plants or by expression from a viral-based vector have been reported. These strategies have yielded promising results, which are summarized below.

The earliest attempt at producing a plant-derived vaccine to FMDV was by fusion of an immunodominant peptide epitope [amino acids (aa) 141–160 of the 211-aa protein] of VP1 from the O serotype into the β B– β C loop of cowpea mosaic virus (CPMV) S capsid protein (Usha et al. 1993), thereby incorporating the epitope into virus particles. Although CPMV containing the inserted epitope did not spread systemically in infected plants, the chimeric capsid protein isolated from plant protoplasts or inoculated leaves could be detected on Western blots using antiserum raised against the free homologous peptide. Since that time, optimization of the design of CPMV chimeric proteins has resulted in the expression of several FMDV epitopes (Porta et al. 2003), thereby demonstrating that the plant viral CP could be used as a presentation system for FMDV epitopes.

A tobacco mosaic virus (TMV)-based vector was also employed to express similar immunogenically dominant FMDV VP1 epitopes as a fusion with the TMV capsid protein (Wu et al. 2003). TMV recombinants, expressing either an 11- or 14-aa epitope of VP1, systemically infected tobacco plants, and the epitopes were stably expressed in the virus particles. The authors estimated that 0.3–0.4 g of the FMDV epitope was expressed per kilogram of infected leaf tissue. Guinea pigs either immunized parenterally or by oral administration of purified virus were protected at some level against an FMDV challenge given at 42 days after the first immunization dose. Although the oral presentation had some protective effects, it was less effective than parenteral administration.

TMV was also employed earlier to express the VP1 (serotype O1 Campos, O1C) complete structural protein from a separate mRNA utilizing the duplicate subgenomic promoter of the virus-based vector (Wigdorovitz et al. 1999b). *Nicotiana benthamiana* plants infected with the recombinant virus produced the protein in a

soluble form at 50–150 µg per gram of freshly harvested leaves, although there was some evidence of proteolytic degradation. Mice parenterally immunized with crude leaf extracts were protected from FMDV challenge.

Alternative strategies for expression of FMDV antigens involved the development of transgenic plants where the foreign gene was stably integrated into the plant genome. The complete FMDV VP1 structural protein [serotype O1 Campos (O1C); ~23 kDa] was expressed in transgenic plants under control of the CaMV 35S promoter in *Arabidopsis* (Carrillo et al. 1998), alfalfa (Wigdorovitz et al. 1999a), and potato (Carrillo et al. 2001). Mice injected with leaf extracts from *Arabidopsis* plants expressing the VP1 protein were protected from FMDV challenge inoculation (Carillo et al. 1998). Although the levels of VP1 expression were low, mice either injected with leaf extracts of transgenic alfalfa or fed fresh transgenic leaf material were protected against FMDV challenge (Wigdorovitz et al. 1999a). In transgenic potato leaves, estimates of VP1 expression were between 0.005% and 0.01% of the total soluble protein (TSP). Mice immunized intraperitoneally with foliar extracts of transgenic potato plants displayed an immune response to the recombinant proteins as well as to purified FMDV particles (Carillo et al. 2001). When the immunized mice were experimentally challenged with infectious FMDV by intraperitoneal inoculation, 90% of the vaccinated mice were protected against infection as compared to the control group (Wigdorovitz et al. 2004).

The low expression levels of VP1 in transgenic plants found in earlier studies prompted Dus Santos et al. (2002) to investigate the possibility of increasing the vaccine epitope concentration by fusing the protective epitope (aa 135–160) of VP1 to the amino terminus of glucuronidase (gus A reporter gene). Under transcriptional control of the CaMV 35S promoter, protein levels of 0.5–1 mg per gram of TSP were realized (0.005%–0.01%) in alfalfa. These levels are similar to those found by Carillo et al. (2001) in transgenic potato. Crude extracts were used to immunize mice by intraperitoneal inoculation and there was complete protection against FMDV challenge.

In a report by Sun et al. (2003), accumulation of up to 3% TSP was obtained for a fusion of VP1 with the cholera toxin B subunit (CTB) and transformation of the green alga, *Chlamydomonas reinhardtii*, chloroplast genome. The fusion protein retained VP1 antigenicity, but no animal trials were conducted.

Finally, Dus Santos et al. (2005) reported the development of transgenic alfalfa plants expressing the complete FMDV structural polyprotein gene P1 and the 3C protease as experimental immunogens. A DNA fragment encoding the P1–3C protein was engineered under the control of the CaMV35S promoter. Foliar extracts of transgenic plant tissue elicited an anti-FMDV immune response in mice inoculated intraperitoneally. Vaccinated mice exhibiting an immune response and experimentally challenged with infectious FMDV were protected against the challenge infection.

The research described above demonstrates the feasibility of a plant virus-based vaccine against FMDV and indicates that its commercialization could be possible in the near future.

Transmissible Gastroenteritis Virus

Swine transmissible gastroenteritis virus (TGEV) is the causative agent of a highly contagious, severe, acute diarrhea of newborn piglets, resulting in high mortality rates of piglets under 2 weeks of age. Protective immunity against this disease must be developed in pregnant, TGEV-immune sows so that passive protection can be passed (mainly in the form of IgA antibodies capable of surviving the gastrointestinal tract) to piglets through colostrum and milk (Saif et al. 1994). Pigs that survive first infection are immune from subsequent infections. A commercially available modified live vaccine is used to control the disease.

TGEV, a member of the *Coronavirus* genus of the family *Coronaviridae*, is an enveloped, single-stranded RNA virus. It contains three structural proteins, M, N, and S. M is an integral membrane protein, N, a phosphoprotein that encapsulates the RNA, and S, the surface or spike glycoprotein (gS) (Laude et al. 1990). Neutralizing antibodies against the virus are directed mainly to the gS protein, and neutralizing epitopes have been mapped to the N-terminal domain of this protein. Four major antigenic sites have been mapped on the gS protein of which site A is immunodominant. The gS protein has been expressed in different mammalian expression vectors, including adenovirus, which promoted systemic and mucosal immunity and conferred protection to suckling piglets (Torres et al. 1996).

Transgenic plants have been developed to express the entire gS protein or portions thereof as candidate plant-based vaccines. Gomez et al. (1998) transformed *Arabidopsis* with cDNA constructs containing the N-terminal domain (aa 1–750) or the full-length gS protein under transcriptional control of the CaMV 35S promoter. Mice inoculated intramuscularly with leaf extracts containing 0.03%–0.06% TSP of gS from transgenic plants developed TGEV-specific antibodies which immunoprecipitated the virus protein and neutralized virus infectivity in vitro. Transgenic potato plants were also created that expressed the TGEV N-terminal domain of the glycoprotein S (N-gS), containing the major antigenic sites of the protein. Levels of expression, 0.02%–0.05% TSP of potato tubers, were similar to those achieved in *Arabidopsis*. Extracts of potato tubers expressing the protein were inoculated intraperitoneally to mice, and the vaccinated mice developed serum IgG specific for TGEV. When potato tubers expressing N-gS were fed directly to mice, they developed serum antibodies specific for gS protein, demonstrating oral immunogenicity of the plant-derived spike protein (Gomez et al. 2000). Similarly, antigens purified from transgenic tobacco plants expressing three different regions of the TGEV gS protein-induced TGEV-specific immune responses in pigs as determined by virus neutralization (Tuboly et al. 2000).

A corn-based production system has also been developed, primarily for the delivery of oral vaccines to TGEV (Lamphear et al. 2002, 2004; Streatfield et al. 2001, 2002). A synthetic, maize codon-optimized version of the TGEV gS protein was expressed in-frame with a maize codon-optimized version of the signal sequence for cell secretion from the barley α -amylase protein (Streatfield et al. 2001). Expression levels of up to 2% TSP were observed in transgenic corn seed

and the protein was shown to assemble into the active pentameric form in planta (Streatfield et al. 2002). When corn seed expressing the antigen was fed to piglets, partial protection against subsequent TGEV challenge was induced. Although TGEV-corn-fed piglets showed fewer overall symptoms compared to control unvaccinated piglets, they recovered more slowly from infection than piglets vaccinated with modified live virus.

Commercial processing methods were tested in order to examine the distribution of antigen in the major seed compartments—germ, grits, and bran (Lamphear et al. 2002). TGEV-S antigen was enriched in the germ fraction. Storage of the germ meal at 4°C or whole seeds at higher temperatures for up to 1 year had negligible effects on antigen levels. These results showed that the antigen could survive standard grain processing. Furthermore, the antigen could be enriched in a particular fraction (up to 500 µg/g of germ), thereby reducing the volume necessary per dose of vaccine and allowing it to be easily incorporated into animal feed.

Subsequent studies of prime/boost vaccination and efficacy of the corn-derived oral vaccine showed that rapid induction of neutralizing antibodies occurred in piglets, which previously did not contain detectable neutralizing antibodies in the serum. This suggests that there was a memory response to previous oral ingestion of the antigen (Lamphear et al. 2002). The swine feeding studies were extended to evaluate the ability of the corn-based oral vaccine to boost immune responses in young sows previously sensitized with the commercially available modified live viral vaccines (Lamphear et al. 2004). It was found that the oral immunization could boost neutralizing antibody levels in the young sows in the serum, colostrum, and milk (lactogenic immunity), with the potential to confer protection to suckling piglets. In light of the results obtained by Walmsley et al. (2003) showing the passive immunization of mice pups through oral immunization of the mother, commercial application of a plant-based oral vaccine as a boost to large herds of swine shows great potential.

Canine Parvovirus and Mink Enteritis Virus

Canine parvovirus (CPV) and mink enteritis virus (MEV) are members of the *Parvovirus* genus of the family Parvoviridae, which are nonenveloped, single-stranded DNA viruses (Levy et al. 1994). The virus possesses three structural proteins, VP1, VP2 (the major structural protein of CPV), and VP3. VP1 and VP2 are splicing products from the same gene. Only VP2 is required for viral particle formation. CPV, MEV, and feline panleukopenia virus (FPLV) are host-specific variants of CPV (sharing 98% amino acid sequence homology) that can infect dogs, mink, cats, or raccoons. These viruses are of great economic importance and clinical manifestations of the disease include diarrhea, severe inflammation of the intestine, and anorexia. In cats, FPLV induces a severe febrile disease. The disease can be controlled by vaccination with chemically inactivated or live, attenuated virus.

It is known that protective antibodies produced during CPV infection are specific for the structural proteins. Extensive studies of surface epitopes of VP2 and synthetic peptide vaccines against CPV based on the the surface epitopes have been described. Among these, the amino terminus of VP2 contains peptides (linear peptide epitopes) that have been shown to induce neutralizing activities against CPV in mice, rabbits, and mink (Langeveld et al. 1994, 1995; Casal et al. 1995).

The development of antigen presentation systems in plants for creation of CPV and related candidate vaccines has been primarily based on the fusion of the parvovirus neutralizing epitopes, shown previously to provide full protection against challenge infection, with plant virus capsid proteins. Dalsgaard et al. (1997) first reported the fusion of peptide 3L17 (aa 3–21 of VP2; DGAVQPDGGQPAVRNER) to the capsid protein of CPMV. The chimeric virus particles were produced by infection of black-eyed bean and 50–60 mg virus could be purified from 50 g of leaf material. Subcutaneous injection of mink with purified virus particles conferred protection against clinical disease and abolished shedding of virus after challenge with virulent MEV (Dalsgaard et al. 1997). This was the first demonstration that an experimental vaccine produced in plants was able to confer protection against infectious disease challenge in an animal. The reduction or abolishment of virus shedding is an important step toward containing the virus, as it is often difficult to isolate infected animals to prevent spread to other animals in a hospital, home, pet store, or dog parks.

In a separate study, Fernández-Fernández et al. (1998), fused a peptide corresponding to aa 2–21 (2L21) of VP2 to the amino terminus of the plum pox potyvirus capsid protein. Antigenicity of the chimeric coat protein virus was demonstrated by immunization of mice and rabbits, and although the antibodies showed neutralizing activity, the antibody titers were very low.

An alternative expression strategy was reported by Gil et al. (2001), who described the high-yield production of a CPV peptide vaccine in transgenic plants. A 21-mer linear antigenic peptide (2L21) was expressed as an amino terminal fusion with the GUS protein and under transcriptional control of the CaMV 35S promoter. Expression levels of the recombinant protein in *Arabidopsis thaliana* were up to 3% TSP, a production yield comparable to that obtained with the same epitope expressed by chimeric viruses. The immunogenicity of the plant-derived peptide was demonstrated in mice immunized either intraperitoneally or orally with transgenic plant extracts.

Finally, in follow-up studies using chimeric plant virus particles as immunogens for CPV, Langeveld et al. (2001) and Nicholas et al. (2002, 2003), report improved safety/delivery and immunization protocols, respectively. CPMV particles expressing the 3L17 linear epitope of VP2 (Dalsgaard et al. 1997) were inactivated with UV light to remove the possibility of replication of the purified virus in a plant host after manufacture of the vaccine (Langeveld et al. 2001). Parenteral vaccination with the inactivated virus was able to protect dogs from a lethal challenge with CPV. The dogs displayed no signs of clinical disease, did not shed CPV particles, and had high titers of peptide-specific neutralizing antibodies. Using the same experimental immunogen, combinations of systemic and mucosal routes for priming

and boosting immunizations were tested for their influence on the immune response in mice (Nicholas et al. 2003). Serum antibody responses were greatest in animals receiving subcutaneous prime and boosting; the response was least in mucosally vaccinated animals. The route of administration did not alter antibody ratios; intranasal administration following subcutaneous priming was effective in inducing mucosal IgA responses. These studies have implications for the development of effective immunization strategies using chimeric virus particles for protection against mucosally acquired viral infections.

Rabies Virus

Rabies virus, a member of the genus *Lyssavirus* of the family Rhabdoviridae, causes an acute and deadly viral infection of the central nervous system. It remains a significant threat to human and animal health. Although rabies in humans is rare in the United States, as many as 18,000 Americans get rabies shots each year because they have been in contact with animals that may be rabid. In 1998, according to the United States Centers for Disease Control and Prevention (CDC), only one person died of rabies in this country. In other parts of the world, however, many people die of rabies each year. The World Health Organization (WHO) estimates that around the world more than 40,000 people die every year from rabies. WHO also estimates that 10 million people worldwide are treated after being exposed to animals that may have rabies (<http://www.niaid.nih.gov/factsheets/rabies.htm>). The virus, which is in the saliva of infected animals, is usually transmitted by bites from infected animals. All warm-blooded animals can get rabies, and some may serve as natural reservoirs of the virus. Therefore, control of the infection in wild animals will reduce the risk of infection in humans and domesticated animals.

The virion of rabies virus consists of a lipid-rich envelope that covers a helical ribonucleocapsid core consisting of a negative-sense RNA genome (Levy et al. 1994). The genome encodes five proteins, one of which is a glycoprotein (G) that forms approximately 400 spikes that are tightly arranged on the surface of the particle. Fusion of the rabies virus particle to the host cell membrane initiates the infection process, which may involve interaction of the G proteins with specific cell surface receptors. The G protein possesses hemagglutinin activity and is the target of neutralizing antibodies. Oral immunization with bait containing a vaccinia virus-rabies glycoprotein recombinant has been shown to protect raccoons and foxes against the disease (Rupprecht et al. 1986; Brochier et al. 1990, respectively).

Efforts have been made to develop a cheap, safe, oral plant-based vaccine to control rabies in animals and humans. McGarvey et al. (1995) engineered tomato plants to express the viral G protein. The protein, which was expressed in leaf and fruit tissues, was immunoreactive with anti-G antibodies. Yusibov et al. (1997) expressed a B cell epitope from rabies glycoprotein (G) G5-24 and a T cell epitope from rabies nucleoprotein (N) 31D as chimeric protein fusions with the N terminus of the coat protein of the plant virus Alfalfa mosaic virus (AMV). This allowed the recombinant epitopes to be displayed on the surface of spherical AMV particles.

The chimeric virus proteins were translated from subgenomic RNAs expressed from a TMV-based plant virus expression vector. AMV virus-like particles (VLPs) were purified from infected plant tissue and used to immunize mice intraperitoneally. High titers of rabies-specific antibodies were detected in mice immunized with the purified particles and sera from the immunized mice neutralized the CVS-11 strain of rabies virus *in vitro*.

In a follow-up study, Modelska et al. (1998) reported on the development of local and systemic immune responses in mice, immunized either intraperitoneally or orally by feeding with virus-infected spinach leaves containing the engineered virus displaying the rabies antigen. Forty percent of the intraperitoneally immunized mice were protected against challenge with a lethal dose of rabies virus; orally immunized mice (feeding with raw, virus-infected spinach leaves or by gastric intubation) showed stimulation of both IgG and IgA production and weakened signs of the disease.

Yusibov et al. (2002) reported that recombinant virus or VLPs expressing the G and N protein epitopes, produced using two additional expression strategies, protected mice from challenge infection with rabies when they were immunized parenterally. In addition, virus-infected, unprocessed, raw spinach leaves were orally administered to human volunteers who had either been preimmunized with a conventional rabies vaccine or had not been immunized. Those who had been previously vaccinated showed a response against the peptide antigen after ingesting the spinach leaves. Five of nine individuals who had not been previously immunized demonstrated significant antibody responses to the antigen, and, when given a dose of conventional vaccine, three of the individuals showed detectable levels of rabies virus-neutralizing antibodies.

These results demonstrate that recombinant, subunit-based, plant-manufactured rabies vaccines can be delivered by injection or orally and show promise for veterinary applications. For example, raccoon rabies is common in parts of the Eastern United States. Raccoon rabies spreads rapidly and infects large numbers of raccoons. The disease often spreads to other wildlife and pets, making human exposure a real concern. To address this problem, an oral vaccination program using recombinant vaccinia virus containing the rabies antigen is incorporated into bait. A plant-derived vaccine could easily be incorporated into a bait. Management of rabies in other animals, including dogs, cats, ferrets, and livestock, involves parenteral injection of inactivated virus, necessitating the production of large quantities of rabies virus in cell culture. A plant-based subunit vaccine would eliminate the risk of exposure to vaccine producers. It would also eliminate the chance of infection in the vaccinated animal if the inactivated virus vaccine retains some viability.

Rabbit Hemorrhagic Fever Virus

Rabbit hemorrhagic disease is a rapidly spreading, lethal infection of adult animals in the wild rabbit population and affected farms. Infected rabbits usually die within 48–72 h of necrotizing hepatitis. Current vaccines are based on formalin-inactivated liver homogenates of infected animals (Peeters et al. 1992).

The causal agent of the disease, rabbit hemorrhagic disease virus (RHDV), is a member of the family *Caliciviridae*, that also includes feline calicivirus, Norwalk virus, and swine vesicular exanthema virus. It is nonenveloped virus with a single-stranded RNA genome that contains one long open reading frame encoding structural and nonstructural proteins (Parra and Prieto 1990). The major RHDV structural component of the capsid is a 60-kDa protein known as VP60.

Active immunization with purified viral VP60 or the VP60 fusion protein (oral or parenteral vaccination) expressed in a baculovirus system has been shown to induce protection of rabbits against a lethal challenge with RHDV (Parra and Prieto 1990; Plana-Durán et al. 1996, respectively).

Plant-based production of VP60 was first achieved by expression using a plum pox potyvirus (PPV)-based vector (Fernández-Fernández et al. 2001). The foreign sequence was cloned between the N1b replicase and the coat protein cistrons of PPV, where posttranslational proteolytic cleavage releases the VP60 polypeptide during virus infection in the plant. Immunization of rabbits subcutaneously with leaf extracts (with an oily adjuvant) of *Nicotiana clevelandii* plants infected with plant virus chimera induced an effective immune response that protected animals against a lethal, intranasal challenge with RHDV.

The VP60 protein has also been produced in transgenic potato plants under control of the CaMV 35S promoter or a modified 35S promoter that included two copies of a strong transcriptional enhancer. Both promoters produced detectable levels of recombinant VP60, with higher levels being produced with the modified promoter (Castañón et al. 1999). Rabbits immunized parenterally with leaf extracts from plants carrying the modified promoter showed high anti-VP60 antibody titers and were fully protected against the hemorrhagic disease (Castañón et al. 2002).

Martín-Alonso et al. (2003) reported the development of transgenic potatoes producing up to 3.5 µg VP60/ mg of TSP in the tuber, levels of expression significantly higher than that of the TGEV glycoprotein S(N-gS) in transgenic potato tubers found by Gomez et al. (2000), but similar to levels reported by Mason et al. (1996) for transgenic potato tubers expressing Norwalk virus capsid protein. Oral immunization with potato tuber extracts (four doses of reconstituted lyophilized extracts in water; extracts containing either 100 or 500 µg of VP60) was performed using a syringe. Only two animals who received the 500-µg dose were seropositive for anti-VP60 antibodies after the third dose. Those receiving the 100-µg dose produced no detectable immune response, even though the equivalent amount of antigen produced an immune response when presented intramuscularly (Castañón et al. 1999, 2002). Rabbits were challenged with RHDV to evaluate the protective efficacy of the vaccination regime; only the rabbit having the highest anti-VP60 antibody titer survived, therefore achieving only a low level of protection. Several other animals that received the high (500 µg) dose of antigen did not survive the virus challenge; however, they survived longer than those not receiving the vaccine.

Additional Diagnostic Reagents and Vaccines for Viral Diseases

Group A Rotavirus

Group A rotavirus is one of the most important causes of severe viral diarrhea in humans and animals. It is a member of the *Rotavirus* genus of the family Reoviridae and is a multicomponent, double-stranded RNA virus that has wheel-like capsids in which spikes radiate from the inner capsid to the smooth viral outer capsid (Levy et al. 1994). The virions are not enveloped and three proteins make up the outer capsid, and four to six proteins, the core. VP6, the major capsid protein of the virus, is located on the inner capsid, and contains the common antigens of each rotavirus serogroup. VP6 has also been shown to be a protective antigen in a mouse infection model (Choi et al. 1999).

It has previously been shown that VP6 could be expressed from a Potato virus X-based vector in *N. benthamiana* plants (O'Brien et al. 2000). Matsumura et al. (2002) reported the first production of an immunogenic VP6 protein of bovine group A rotavirus in transgenic potato plants as a candidate diagnostic reagent for disease detection. The maximum level of antigen production was 0.1% TSP in leaf tissue. When potato tuber extracts containing recombinant VP6 were injected intraperitoneally into mice, anti-VP6 antibodies were detected in mouse serum. Sera were able to detect the purified GAR 22R strain in ELISA and in Western blots. In addition to its potential use as a diagnostic reagent to detect rotavirus serogroups, the VP6 antigen could also be developed into a subunit vaccine.

Wigdorovitz et al. (2004) reported on the development of an edible peptide vaccine for bovine rotavirus, but in this case an immunodominant peptide derived from the VP4 protein of bovine rotavirus (BRV) was expressed in transgenic alfalfa plants as a translational fusion with β -glucuronidase, which acts as a carrier protein. VP4 is an outer capsid protein forming spikes that emerge from the virion surface layer and is implicated in absorption of the virus to epithelial cells. Between 0.4 and 0.9 mg of fusion protein per gram of TSP of leaf extracts was produced. Mice were vaccinated intraperitoneally with crude leaf extracts or orally with freshly harvested transgenic leaves. Both sets of mice developed an immune response to BRV. Pups born to dams of both sets of mice showed a significant degree of protection when challenged with BRV.

Bovine Herpesvirus Type 1

Bovine herpesvirus type 1 (BHV-1) is the causative agent of a group of respiratory and reproductive disorders in cattle, commonly referred to as infectious bovine rhinotracheitis (Kahrs 1977). It affects adult and young animals and is common worldwide. The herpes virion consists of a dense core that is covered by an icosahedral

capsid. The structure is also covered by a lipid bilayer envelope with short glycoprotein spikes on the surface (Levy et al. 1994). The genome is a double-stranded DNA that is linear but is in a circular form in the capsid. Several proteins are present on the surface of the virus particle, of which half are glycoproteins. Current vaccines for BHV-1 are formulated from inactivated or modified live virus and have the disadvantages of being poorly immunogenic or producing clinical disease if poorly inactivated, respectively. Alternative vaccination strategies using viral components, including glycoprotein D (gD) to induce protective immune responses, have been explored (van Drunen Littel-van den Hurk et al. 1993).

In a report by Pérez-Filgueira et al. (2003), the TMV viral-based expression system was used to produce a truncated, cytoplasmic subunit form of BHV-1 gD protein in plants. The amount of recombinant protein was estimated at 15–20 µg per gram of fresh leaf tissue. Crude extracts of *N. benthamiana* leaves inoculated with the recombinant virus were used to parenterally vaccinate mice (0.2 g per dose containing 2 µg of antigen) and cattle (5 g per dose). Both humoral and cellular-specific responses recognizing the gD antigen were induced, and the candidate vaccine was able to induce protection in the natural bovine host to challenge, intranasal viral infection with the BHV-1 LA strain. Protection in cattle was manifested by reduced amounts of excreted virus in nasal fluids, as compared to BHV-1 vaccinated cows, and later and milder clinical symptoms of the disease in vaccinated animals.

Rinderpest Virus

Rinderpest is an acute highly contagious, often fatal, disease of cattle and other domestic and wild ruminants. The disease affects the gastrointestinal and respiratory systems. It is caused by rinderpest virus (RPV), a member of the genus *Morbillivirus* of the family Paramyxoviridae. A highly effective live, attenuated virus vaccine is available and the disease has been successfully eradicated from most parts of the world (Plowright 1962). However, a few foci of the disease still exist in parts of Africa, the Middle East, and South Asia. The disease remains a threat to livestock in developing countries. The difficulty in maintaining a cold chain for the vaccine results in failure of vaccination in the hot regions where rinderpest is endemic. Attempts at making thermostable whole virus vaccines have been made using animal virus vectors (Romero et al. 1994).

The lipid envelope of RPV contains two glycoproteins, F (fusion, 65 kDa) and H (hemagglutinin, 80 kDa), which form spike-like structures on the surface of the particle. Virus infection is initiated by the action of these two proteins: H mediates the attachment of the virus to the host cell membrane and F mediates virus penetration into the host cell and virus-induced cell fusion and hemolysis. These proteins, which are known to be highly immunogenic, are important targets for the host cell response and confer protective immunity. Efforts have been made to develop subunit vaccines of recombinant vaccinia virus or capripox expressing the H and F

proteins, and long-term immunity with these vaccines has been demonstrated (Yamanouchi et al. 1993; Romero et al. 1994, respectively). Although these vaccines are effective, their use may become prohibitively expensive due to the high cost of the cell culture used to produce the recombinant animal virus vaccines.

Khandelwal and colleagues (Khandelwal et al. 2003a, b, 2004; Satyavathi et al. 2003) have reported attempts to produce a less expensive, yet effective, vaccine by expression of the H protein in transgenic tobacco, peanut, and pigeon pea and oral delivery of the candidate vaccine in a mouse model and in cattle. The H protein expressed in tobacco was immunogenic and elicited a specific humoral response in an experimental mouse model (Khandelwal et al. 2003a).

Pigeon pea, also known as regram, is used as a food and fodder crop, with the foliage being used for animal feed after the seeds have been harvested for human consumption. Satyavathi et al. (2003) transformed pigeon pea with the H protein under transcriptional control of the CaMV 35S promoter. Levels of H protein in transgenic leaves reached 0.49% TSP. In peanuts transformed with the same construct, the expression level of H protein was in the range of 0.2%–1.3% TSP in leaf extracts (Khandelwal et al. 2003b). The peanut-derived H protein was immunologically active when delivered orally or parenterally in the absence of adjuvant in an experimental mouse model system (Khandelwal et al. 2004). When cattle were fed with transgenic peanut leaves at weekly intervals for 3 weeks with 5–7.5 g of leaf tissue, H-specific antibody was detected in serum of immunized cattle, and the serum neutralized RPV virus infectivity in vitro (Khandelwal et al. 2003b).

Classical Swine Fever Virus

Classical swine fever virus (CSFV), also known as hog cholera virus, is related to bovine viral diarrhoea virus and belongs to the genus *Pestivirus* of the family Flaviviridae (Meyers and Thiel 1996). CSFV is a highly contagious viral disease of swine that occurs in an acute, subacute, chronic, or persistent form. In the acute form, the disease is characterized by high fever, severe depression, multiple superficial and internal hemorrhages, and high morbidity and mortality (Kleiboeker 2002). In the chronic form, the signs of depression, anorexia, and fever are less severe than in the acute form, and recovery is occasionally seen in mature animals. Transplacental infection with viral strains of low virulence often results in persistently infected piglets, which constitute a major cause of virus dissemination to noninfected farms.

CSFV is an enveloped RNA virus with a viral nucleocapsid containing a single molecule of genomic, positive-sense RNA complexed with a single polypeptide. The viral capsid is surrounded by a lipid bilayer that contains two proteins, one of which is the 51- to 60-kDa E2 protein, which determines the serological specificity of the virus. Legocki et al. (2005) sequenced the E2 gene from two strains of CSFV and constructed transgenic lettuce and alfalfa plants in which the gene was under the control of the CaMV35S promoter. One gram of lyophilized lettuce contained

10 µg of E2 antigen; when coupled to ubiquitin, the yield increased to 160 µg of antigen per gram of dry tissue. Preliminary testing of antigenicity by oral administration to mice and rats revealed an increase in IgG and IgA antibodies against the antigen after the second immunization.

Hantavirus

Hantaviruses (HV) are an emerging threat to animal and human health as new outbreaks of hantavirus infections are occurring with more frequency (Elliott et al. 1994). Hantaviruses cause the clinical syndromes in humans known as hemorrhagic fever with renal syndrome, and hantavirus pulmonary syndrome with high fatality worldwide. The individual hantavirus genotypes are carried by specific rodent hosts that do not exhibit clinical signs of infection. The major mode of transmission is by aerosolized excreta of HV-infected rodents. Anyone who comes into contact with the virus is susceptible to the disease, and those especially at risk for infection include infants, the immunocompromised, farmers, veterinarians, rodent breeders, and zoo/wildlife/primate and other animal healthcare workers. There is no commercially available hantavirus vaccine; however, inactivated virus vaccines are currently being developed and tested (Cho et al. 2002; Hjelle 2002). Although cats and dogs are not known to be hosts for hantavirus, they may bring infected rodents into contact with humans and, therefore, development of oral, plant-based vaccine as bait in rodents would seem an effective strategy to control the disease in both rodents and humans.

Hantaviruses are negative-sense, single-stranded RNA, enveloped viruses that belong to the genus *Hantavirus* of the family Bunyaviridae. The three-segmented genome encodes RNA-dependent RNA polymerase on the L-RNA segment, two surface glycoproteins G1 and G2 on the M-RNA segment, and the viral nucleocapsid protein (N) on the S-RNA segment. Major antigenic domains are located on the N protein (Gött et al. 1997). The N protein and the G1 and G2 glycoproteins are promising candidates for development of subunit vaccines using plant-based expression strategies. Kehm et al. (2001) recently described the development of transgenic tobacco and potato plants expressing the Puumala virus N protein. The recombinant protein was expressed at 1 ng/4 µg of dried leaf and root tissues of transgenic potato. Rabbits immunized intraperitoneally with leaf extracts from tobacco and potato plants produced anti-N protein serum. Oral immunization of mice is under investigation. It has not yet been reported if the recombinant proteins are able to elicit effective protective immune responses in test animals.

Infectious Bronchitis Virus

Infectious bronchitis virus (IBV) is the pathogen causing chicken infectious bronchitis (IB), an acute, highly contagious respiratory disease of young chicks. The disease is controlled by serotype-specific vaccines. Outbreaks of IB occur due to

the lack of cross-protection of the vaccines between serologically distinct viruses. Zhou et al. (2004) developed transgenic potato lines expressing the full-length spike protein of IBV. IBV, like TGEV above, is a member of the family *Coronaviridae*, order Nidovirales, and its genome encodes three major structural genes, one of which is the spike (S) protein. The entire 120-kDa (S) protein was expressed in potato tubers, and the plant-derived S protein retained reactivity to IBV antisera on Western blots. Following oral or intramuscular inoculations of chicks with potato tuber extracts containing the S protein (2.5 µg/g tuber tissue), detectable levels of serum neutralizing antibodies were seen. In addition, the chicks were protected against challenge with virulent IBV.

Vaccines and Therapeutics Against Bacterial and Parasitic Diseases

Swine Edema

Swine edema, also called gut edema, can cause morbidity and mortality in piglets. It is caused by certain serotypes of enterotoxigenic *Escherichia coli* (0138, 0139, 0141) that are able to produce a powerful, Shiga-like toxin. The toxin damages the walls of small blood vessels, including those of the brain, and causes fluid, or edema, to accumulate in tissues of the stomach and the large bowel. Damage to blood vessels in the brain results in characteristic nervous signs. Flexible filaments, known as F18 fimbriae, are expressed by various strains of *E. coli* causing disease in humans and animals, and have been reported to be an important virulence factor related to edema (Rippinger et al. 1995). The backbone of fimbriae consists of about 1,000 copies of a polypeptide that are arranged in an open helix. Colonization of pigs with the bacterial strains results in high levels of antifimbriae antibodies, especially IgA. As a result, there have been attempts made to vaccinate with the fimbrial proteins.

Rossi et al. (2003) reported the isolation of the F18 fimbriae gene from genomic DNA of *E. coli* isolated from pigs that died of edema. The gene was cloned into plant transformation vectors, and transgenic tobacco seeds expressing up to 0.1% TSP of F18 adhesive fimbriae were produced. No animal tests were conducted in this study. The tobacco seeds may offer a source of oral vaccine to protect against the disease.

Porcine Taenia Solium

Porcine *Taenia solium*, the pork tapeworm, is a long, flat, ribbon-like parasite that averages 6–10 feet long but can reach 30 feet in length. It causes a major parasitic disease known as taeniasis/cysticercosis that affects humans and pigs and is preva-

lent in poor sanitary conditions and rustic rearing of pigs. Cysticerci (one stage of the life cycle) may localize in the central nervous system of humans, causing neurocysticercosis, a major health problem in developing countries. Pigs play the role of obligatory intermediate host in the life cycle of the parasite; therefore, control of the disease in pigs by vaccination may reduce human infection. A number of vaccines have been investigated, including the use of native or recombinant antigens, or peptides, derived from different stages of the tapeworm life cycle (Plancarte et al. 1999). Three well-defined peptides of 18, 12, and 8 amino acids have been developed into a synthetic vaccine, SPvac, that is very effective under field conditions (Huerta et al. 2001), but is costly to produce. In a search for less costly alternatives, Sciutto et al. (2002) engineered the peptides to be expressed in recombinant filamentous phage (M13) and in transgenic carrot and papaya plants. Candidate antigens were identified from cDNA expression libraries of a related parasite, *T. crassiceps*, by screening with sera from *T. solium*-cysticerci-infected pigs. Three peptides were identified from three of the expression products as being antigenic, and were shown to give high levels of protection in a mouse model. These protective peptides are distributed among the different life stages of the *T. solium* parasite. No data are yet available on expression levels in plants or effectiveness of the plant-based vaccine.

Bovine Pneumonic Pasteurellosis

Bovine pneumonic pasteurellosis (PP), also known as shipping fever or bovine respiratory disease complex, is a major cause of sickness in transported cattle (Yates 1982). The bacterium, *Mannheimia (Pasteurella) haemolytica* serotype A1 is the principal microorganism responsible for the disease. It inhabits the upper respiratory tract of healthy, unstressed calves. The disease occurs due to a combination of the presence of the bacteria, respiratory disease viruses, and stress. Traditional vaccination may involve subcutaneous injection of live virus, tissue culture-derived killed virus, orally administered bacterial culture supernatants, outer membrane protein preparations, and subunit vaccines (e.g., Sreevatsan et al. 1996). These vaccines provide various degrees of protection; however, they all involve herding and restraint of animals.

A noninvasive alternative vaccine candidate for PP has been developed by Lee et al. (2001) and is based on the expression of one of the major virulence factors of *M. haemolytica* A1. The leukotoxin (Lkt) is a protein that is secreted by the bacterium and acts as a pore-forming cytolysin that inserts into the plasma membrane of host cells, leading to cell lysis. Resulting tissue damage leads to pneumonia and death of animals. A recombinant derivative of Lkt, Lkt50, lacking the putative hydrophobic transmembrane domains, was engineered as an N-terminal fusion with modified green fluorescent protein (mGFP) and containing an N-terminal signal sequence and C-terminal endoplasmic reticulum retention signal. This chimera was used to generate transgenic white clover (*Trifolium repens* L.) expressing the chimeric

protein from the CaMV 35S promoter at levels of 1% Lkt50-GFP (equivalent to 18 $\mu\text{g/g}$ fresh weight tissue). Lkt-GFP-enriched fractions prepared from leaf extracts were able to induce an immune response to authentic Lkt in rabbits when delivered by intramuscular injection. The resulting antibodies were able to neutralize Lkt. According to the report, experiments are in progress to assess the immunogenicity of the candidate vaccine in cattle and to test the efficacy of feeding the transgenic clover to cattle in stimulating a mucosal immune response to Lkt.

Fasciola Hepatica

The liver fluke, *Fasciola hepatica*, is a common parasite of sheep and cattle, and also infects humans worldwide. Fascioliasis in sheep, cattle, and goats results in animals which show low productivity and higher mortality, causing severe economic losses. Among the proteins released by flukes are potent proteases required for parasite metabolism. Previous reports suggested that cysteine proteases could be used as protective antigens when injected intramuscularly into rats (Wedrychowicz et al. 2003). Legocki et al. (2005) engineered a DNA fragment encoding the catalytic domain of the cysteine protease of *F. hepatica* into plant transformation vectors and used to transform lettuce. When fused to a ubiquitin protein sequence, antigen expression was 100 μg of catalytic domain per gram of dried tissue. Both IgG and IgA antibodies were present in serum of mice that had been fed the transgenic plant material.

Coliform Mastitis

Coliform mastitis is one of the most common forms of environmental mastitis in dairy cows, accounting for 40%–50% of all clinical cases of mastitis (Hogan and Larry Smith 2003). Significant losses also occur in goats, sheep, and pigs. Important Gram-negative organisms in mastitis include *E. coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, and *Pseudomonas aeruginosa*. *E. coli* is shed from the intestinal tract. All of the Gram-negative organisms can also be found in the environment such as in soil, water, and bedding, and are common in dairy operations. If left unchecked, coliform mastitis can cause severe economic losses from a number of factors: reduced milk production and quality, increased labor and treatment costs, and increased culling rate and death losses. In the absence of effective vaccines, current control strategies rely heavily upon antibiotics and topical germicidal chemicals.

The bovine CD14 antigen is a high-affinity receptor for the complex of lipopolysaccharide (LPS, endotoxin) and LPS-binding protein. The secreted form of CD14 (sCD14) binds and neutralizes LPS from *E. coli* and other coliform bacteria and prevents development of acute endotoxin shock in cows as well as intramammary infection by coliform organisms (Lee et al. 2003). A CD14 recombinant gene was

incorporated into a plant virus vector for transient expression in *N. benthamiana* (Nemchinov et al. 2006). Western blots probed with CD14-specific antibodies demonstrated that crude plant extracts as well as affinity-purified samples contained immunoreactive recombinant protein of predicted molecular mass. Biological activity of the plant-derived sCD14 was demonstrated in vitro by induction of apoptosis and interleukin-8 production in bovine endothelial cells and in vivo in bovine udders as shown by an increased leukocyte response in the presence of LPS.

Immunocontraceptive Vaccines and Follicle Stimulating Hormone

Fertility control by vaccination is a management practice that has been used on captive wildlife species, such as feral, or wild, horses (Kirkpatrick et al. 1992). The product in this case was injected whole porcine zona pellucida (ZP), a thick extracellular matrix surrounding the mammalian ovum (egg) which binds sperm, harvested from pigs at slaughter (Barber and Fayrer-Hosken 2000). Antibodies are induced against reproductive self-antigens present on the ZP, resulting in a reduction in fertility.

Smith et al. (1997) proposed the use of oral, plant-derived immunocontraceptive vaccines for management of free-ranging wildlife species. In New Zealand, the introduced brushtail possum (*Trichosurus vulpecula*) causes major economic and environmental damage. In a recent report by Polkinghorne et al. (2005), the feasibility of using a plant-based immunocontraceptive composed of recombinant antigens derived from the ZP for control of possums was investigated. Females injected with porcine ZP showed reduced fertility and production of anti-ZP antibodies. Recombinant antigens (glycoproteins ZP2 and ZP3) from the ZP were cloned and expressed in bacteria; females injected with the purified proteins showed 75%–80% reduction in fertility. Although the recombinant ZP antigens have not yet been produced in a plant, female possums fed with transgenic potato tubers expressing the model antigen LT-B (heat-labile *E. coli* enterotoxin) (Mason et al. 1998) expressed systemic antibodies and antibody-secreting cells against LT-B. Future research includes the expression of the ZP antigens in transgenic carrot root and delivery of the vaccines in an oral bait.

At the other end of the spectrum, superovulation and embryo transfer is widely used to improve success of reproduction in economically important animals, including cattle. Superovulation is usually induced using pregnant mare serum gonadotropin or pituitary-derived follicle stimulating hormone (FSH), both of which have the disadvantage of potentially infectious agents in the purified preparations. Dirnberger et al. (2001) used TMV transient expression in *N. benthamiana* to produce the single-chain version of bovine FSH, a protein that requires extensive N-glycosylation for proper folding, activity, and stability. The protein was secreted to the extracellular compartment and up to 3% of TSP was produced. Although the recombinant protein contained plant glycans, it retained significant bioactivity in mice, though much lower than that of pregnant mare serum gonadotropin.

Regulatory Issues and Future Prospects

The regulatory considerations for products made in bioengineered plants, using either engineered viruses or transgenic plants, are, for the most part, the same as those for other therapeutics or vaccines, except for issues that may be unique for production of the products in plants (Stein and Webber 2001; Peterson and Arntzen 2004). Compliance of plant-based vaccines to Good Manufacturing Practices (GMP), and control of toxicity, dose, lot-to-lot consistency, possible allergic responses and immune tolerance are all to be considered for testing and commercialization of plant-based biologics. Draft guidance for drugs, biologics, and medical devices derived from engineered plants has been issued by the United States Food and Drug Administration Center for Veterinary Medicine and is currently in the comment phase (http://www.fda.gov/cvm/biotechnology/bio_feeds.html). Other issues that may need to be addressed relate to the use of bioengineered feed in animals where meat or milk is destined to be used as human food.

Future directions for veterinary vaccines include the development of oral, multivalent vaccines to replace injectable vaccine mixes. As an example, multivalent vaccines for poultry viruses, expanded vaccine targets to reduce human exposure to the pathogens that may be present in meat products (e.g., *E. coli* 0157:H7 and *Salmonella*) or for diseases that are currently uncontrollable by vaccination. Oral neutralizing antibodies, similar to those produced as a prophylaxis against rabies in humans (Ko et al. 2003), are also potential therapeutic reagents for animal disease control. Among other potential vaccines that are currently being investigated, but not yet reported in the literature, are oral vaccines for farmed fish, and vaccines for equine herpesvirus.

The potential for the use of plant-derived biologics in animal health is high. However, in spite of the potential of plants as bioreactors, one would also need to show that the plant-derived product is clearly advantageous either for efficacy, ease of delivery and/or cost. Several of the reports reviewed in this chapter provide the proof of principle that the products are efficacious. However, cost is clearly a factor when replacements are considered for traditional veterinary vaccines, some of which are currently available for pennies per dose. With that said, the most likely near-term possibilities for commercialization of plant-derived vaccines and therapeutics will likely be veterinary products.

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