Display of Peptides on the Surface of Tobacco Mosaic Virus Particles

M.L. Smith, W.P. Fitzmaurice, T.H. Turpen, and K.E. Palmer

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Abstract In this review, we focus on the potential that tobacco mosaic virus (TMV) has as a carrier for immunogenic epitopes, and the factors that must be considered in order to bring products based on this platform to the market. Large Scale Biology Corporation developed facile and scaleable methods for manufacture of candidate peptide display vaccines based on TMV. We describe how rational design of peptide vaccines can improve the manufacturability of particular TMV products. We also discuss downstream processing and purification of the vaccine products, with particular attention to the metrics that a product must attain in order to meet criteria for regulatory approval as injectable biologics.

Keywords tobacco mosaic virus, vaccine, plant, virus-like particle, papillomavirus

M.L. Smith
Genentech, Inc. 1000 New Horizons Way Vacaville, CA 95688, USA

W.P. Fitzmaurice
Novici Biotech., Vacaville, CA 95688, USA

T.H. Turpen
Technology Innovation Group, Inc., 7006 Firewheel Hollow, Austin, TX 78750, USA

K.E. Palmer(✉)
Owensboro Cancer Research Program, University of Louisville, 1020 Breckenridge Street, Owensboro, KY 42303, USA
e-mail: kenneth.palmer@louisville.edu

A.V. Karasev (ed.) *Plant-produced Microbial Vaccines*.
Current Topics in Microbiology and Immunology 332

Introduction

It is now well established that immunogenic peptides are most efficiently presented to the immune system in a highly ordered, repetitive, quasicrystalline array such as by a virus-like particle (VLP). By their structure, some VLPs are thought to be capable of stimulating proliferation of dendritic cells and other antigen-presenting cells. Thus, antigen-specific B and T cell responses are markedly enhanced when epitopes are coupled to VLPs. The regular array of epitopes on the surface of chimeric VLPs is thought to allow efficient crosslinking of antigen-specific immunoglobulins on B cells, leading to B cell proliferation and production of antibodies. Exposure of the immune system to a repetitive array of self-peptides is an effective mechanism of breaking immunological tolerance and producing auto-reactive therapeutic antibodies. Therefore, there is currently significant interest in VLP-epitope display systems for induction of antibodies for disease therapy and prophylaxis, as well as for induction of peptide-specific T cell responses for immunotherapy of cancer and chronic disease. There are well-established methodologies for recombinant production of VLP-epitope display systems that use self-assembling capsid proteins of several different viruses, most notably papillomaviruses, hepatitis B core and surface antigens, and various bacteriophages, including the leviviruses MS2 and Qβ. Many of these are easily produced in various eukaryotic expression systems, and some may also be manufactured in bacteria. The scientific literature is replete with examples of plant-produced recombinant virus particle and VLP epitope display-based vaccines that show utility, mainly as prophylactic vaccines against infectious diseases.

Research on the use of recombinant virus-like particles as epitope carriers started in the mid-1980s, where in addition to tobacco mosaic virus (TMV) (Haynes et al. 1986), hepatitis B surface and core antigen particles, yeast Ty Gag particles and poliovirus virions were shown to be effective carriers that significantly enhanced the immunogenicity of linked epitopes (Valenzuela et al. 1985; Delpeyroux et al. 1986; Adams et al. 1987; Clarke et al. 1987; Burke et al. 1988; Delpeyroux et al. 1988; Martin et al. 1988, 2003; Clarke et al. 1990). However, VLP epitope display technology has only recently moved toward practical development of vaccines for use in human and veterinary medicine. It is interesting to note that two promising malaria vaccines based on epitope display on the surface of VLPs are being tested in humans. The product that is most advanced in clinical evaluation is a promising malaria vaccine based on display of a large part of the malaria parasite *Plasmodium falciparum* circumsporozoite protein, in chimeric recombinant HBsAg particles. This vaccine, RTS,S, which is being developed by GlaxoSmithKline Biologicals, shows promising efficacy in human vaccinee volunteers challenged with *P. falciparum*, and a phase III efficacy trial in children in Mozambique is planned for the near future (Moorthy et al. 2004). The Malaria Vaccine Initiative, in collaboration with biotechnology company Apovia Inc., is conducting a phase I trial with recombinant HBcAg particles displaying a B cell epitope from the circumsporozoite protein fused to the major immunogenic domain of the core protein, and a universal T-helper epitope at the carboxy-terminus (Birkett et al. 2002; Moorthy et al. 2004). These are good examples of how

VLP-epitope display products are starting to show their promise for addressing infectious diseases. Thus far, only one plant VLP-based candidate vaccine has been tested in humans: a candidate rabies virus vaccine (Yusibov et al. 2002), and as far as we are aware there are no plant-produced VLP products that are nearing commercialization. The focus of this review is on some of the practical issues that must be considered in order to bring plant-produced virus particle and VLP-based epitope display systems into commercial use. In light of the fact that our primary interest is in TMV-based VLP and virion peptide display, we will concentrate our discussions on the TMV platform technology, and review some of the scientific, manufacturing, and regulatory issues that must be addressed in order to commercialize this technology.

Overview of Technologies for Display of Peptides on the Surface of Tobacco Mosaic Virus

TMV has been studied as a model antigen for over 50 years (reviewed in Van Regenmortel 1999). The viral particles are excellent immunogens, and much of the foundation of modern immunology was laid by TMV serological research, for example, neutralization of virus infectivity by antibodies was demonstrated in the TMV system 15 years before similar results were obtained in animal viral systems. Years before the mechanism of presentation of antigen to cells of the adaptive immune system by professional antigen-presenting cells (APCs) was understood, Loor (1967) demonstrated in rabbits that 14C-labeled TMV virions were rapidly and effectively transported from the site of injection to proximal lymph nodes and then to the spleen. With remarkable foresight, both Loor (1967) and Marbrook and Matthews (1966) showed that the particulate nature of viruses is essential for their immunogenicity because disassembled plant virus coat protein induced lower titer antibodies and poorer immune memory in animals immunized with equivalent doses of intact and disassembled viruses. In the ^{14}C-labeling experiments referred to above, Loor also showed that the particulate nature of viruses is important for uptake by and activation of APCs since far lower amounts of labeled coat protein were transported by APCs to lymphoid organs in animals immunized with disassembled virus in comparison with intact virus particles (Loor 1967).

The first demonstration that VLPs derived from TMV coat protein could display antigenic epitopes was reported by Haynes et al. (1986), who displayed a poliovirus epitope on the surface of VLPs assembled from TMV coat protein expressed in *Escherichia coli*. With the advent of the first infectious clones of TMV (Dawson et al. 1986; Meshi et al. 1986), it became possible to manipulate the genome in vitro and thus to construct recombinant TMV vectors and fuse epitopes to the surface of the coat protein. These technologies were the foundation of a biotechnology company, now called Large Scale Biology Corporation (LSBC), dedicated to production of vaccines and therapeutics for human and animal health through exploitation of recombinant TMV (reviewed by Turpen 1999). Since 1987, we have developed and successfully implemented technologies and infrastructure for rapid, cost-effective

expression and extraction of recombinant proteins from plants, including the world's only operating, commercial-scale biomanufacturing facility for recombinant protein extraction from plant tissues. Commercial-scale biomanufacturing allows translation of the body of data providing proof of the concept that TMV can function as an effective carrier for antigenic peptides into vaccine products for human and veterinary applications (Pogue et al. 2002).

The tobamovirus virion is a rigid rod of about 18 nm in diameter and 300 nm in length. The structures of the virion and coat protein have been determined by x-ray diffraction (Watson 1954; Namba and Stubbs 1986). The virion contains approximately 2,130 coat protein subunits, each approximately 17.5 kDa, arranged in a right-handed helix with 16.3 subunits per turn. Each subunit of the coat protein tolerates insertion of epitopes at one of three solvent-exposed positions: the N-terminus, at or near the C-terminus and in a surface-exposed loop corresponding to amino acids 59–65. The density with which epitopes may be displayed on rod-shaped viruses such as TMV is unmatched by any competing VLP system. For example, it is possible to display greater than 2,100 copies of an epitope on the surface of TMV, compared to 180 on a T=3 particle such as HBcAg VLP or 420 on a T=7 particle such as papillomavirus VLP. Table 1 summarizes the most significant reports of display of immunogenic peptides on the surface of TMV.

Table 1 Vaccine epitopes produced on the surface of TMV

Vaccine model	Summary of results	Reference
Malaria B cell epitope	Successful display of epitope from malaria on the surface of TMV. High yield in field production	Turpen et al. 1995
Mouse zona pellucida ZP3 epitope, a model contraceptive vaccine	Vaccination was able to break B cell tolerance and induce autoreactive antibodies that recognized zona pellucida located in mouse ovaries. This study is the first and only published account of the use of a plant virus particle to break immune tolerance	Fitchen et al. 1995
Murine hepatitis coronavirus neutralizing epitope	Five of six vaccinated mice were protected from challenge with murine hepatitis virus	Koo et al. 1999
Feline panleukopenia parvovirus epitope	Vaccinated cats were partially protected against challenge with feline panleukopenia parvovirus	Pogue et al. 2004
Pseudomonas aeruginosa OMPF peptide	Vaccinated mice had reduced lesion number and disease severity when challenged with *Pseudomonas*	Staczek et al. 2000
Foot and mouth disease virus neutralizing epitope	Guinea pigs and swine protected against challenge with FMDV	Wu et al. 2003
Rabbit papillomavirus L2 Imer epitopes	Full protection against homologous virus challenge; some cross-protective immunity between papillomavirus types	Palmer et al. 2006
Mouse tumor T Cell epitopes	Induced T cell responses that protected mice against terms challenge	McCormick et al. 2006

Overall, these data show that TMV-based peptide display vaccines can be applied for prevention of infectious disease. Of all of these publications, the results presented by Fitchen et al. (1995) are perhaps the most important, since they show that TMV particles displaying a repetitive array of self-peptides can break B cell tolerance and allow production of autoreactive antibodies. In the following sections, we propose some solutions to the manufacturing and regulatory issues associated with bringing TMV, and TMV-based VLP carriers into commercial production.

Manufacture and Purification of Tobacco Mosaic Virus Particle-Based Vaccines

To date no vaccine based on a chimeric plant virus has been delivered by parenteral injection to human subjects. However, LSBC has developed a patient-specific vaccine for the treatment of non-Hodgkin's lymphoma (NHL) (McCormick et al. 2003). The idiotype regions of the tumor-specific IgG were successfully expressed as single chain Fv proteins in tobacco plants using the TMV-based GENEWARE expression platform. These vaccines were tested in a phase I clinical trial with 16 patients, and an excellent safety profile was demonstrated, together with encouraging immune response profiles. This study was the first to test a plant-derived biologic delivered parenterally (McCormick et al. 2008). The quality control (QC) and quality assurance (QA) framework implemented for this phase I trial will facilitate the transition of plant virus vaccines from the laboratory to evaluation in humans.

The patient-specific nature of the NHL vaccine lent itself to a manufacturing process on the milligram scale, with purification from growth room-cultivated plants. In contrast, for vaccines targeting human and animal pathogens, a yield of 1–10 kg of final product per manufacturing run can be anticipated. In a recent review (Pogue et al. 2002), production at this scale was discussed, in addition to the regulatory issues surrounding the use recombinant TMV vectors in field-based production. Herein we will focus on the processing and purification considerations and challenges that must be addressed, to permit TMV fusions to be produced and formulated as human and veterinary vaccines. We will use data from a number of LSBC investigational vaccine programs to illustrate key points.

We carry out the majority of the developmental work associated with a new coat protein fusion with *Nicotiana benthamiana* as the host plant for the recombinant virus, since *N. benthamiana* is very susceptible to TMV infection and is easily cultivated under growth-room conditions. At scale and under field conditions, *N. benthamiana* is a suboptimal host as its growing season is limited to the cooler months because elevated summer temperatures adversely affect biomass yield (Fig. 1).

Some cultivated varieties of tobacco are highly susceptible to wild-type TMV, but the yield of recombinant virion can be reduced significantly when foreign epitopes are displayed on the coat protein surface. The requirement for host susceptibility coupled with acceptable biomass yield under field conditions prompted LSBC to embark on a breeding program to develop improved plant hosts (Fitzmaurice 2002).

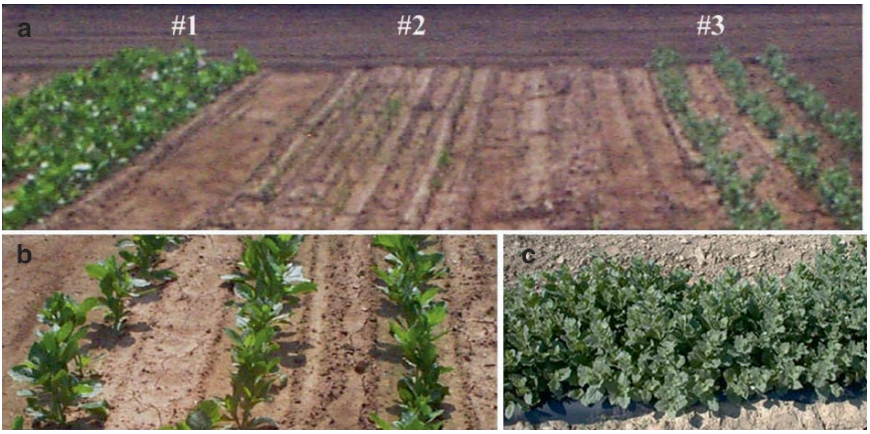

Fig. 1 **a–c** Plant host growth characteristics under field conditions. **a** Comparison of biomass yield for an improved host, *N. excelsiana* (*#1*) and two different accessions of *N. benthamiana* (*#2*, plants mostly dead and *no. 3*, grown under field conditions in Kentucky, mid-summer). **b** Close-up of *N. excelsiana* (*#1*), 13 days after transplanting, cultivated during the spring. **c** Close-up of *N. benthamiana* (*#3*), 28 days after transplanting, cultivated during the spring. Plants shown in **b** and **c** were cultivated in Kentucky

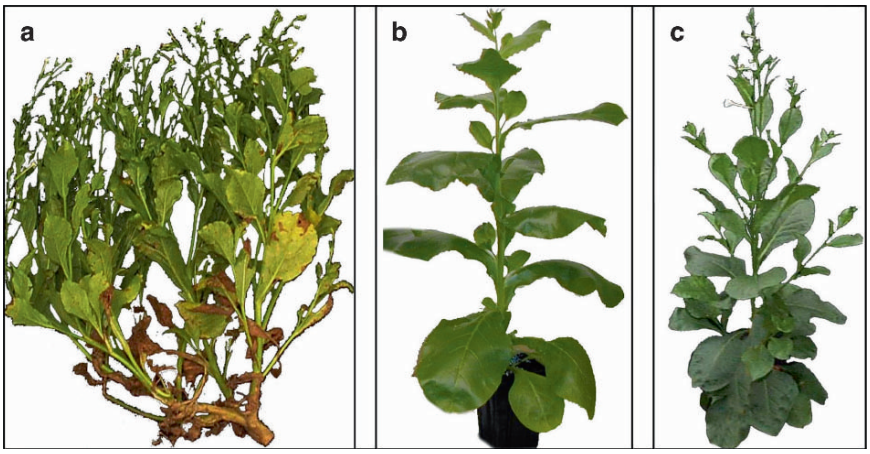

Fig. 2 **a–c** Effect of cultivation conditions on *N. excelsiana* plant morphology. **a** Field-grown plants, harvested 52 days after transplanting. **b** Growth room cultivated plant, 48 days after sowing. **c** Greenhouse grown plant, 43 days after sowing

The improved performance of the alternative plant hosts upon field cultivation is clear. Figure 2 illustrates the superior growth characteristics of a novel LSBC-proprietary host (*N. excelsiana*), an interspecific hybrid between *N. excelsior* and *N. benthamiana*, in the field, greenhouse and under growth-room conditions, at a typical age of harvest.

Since growing conditions clearly alter the host plant morphology, the effect of the growth environment on host susceptibility to TMV, as well as product yield and

quality, must be addressed. In particular, the host response to the pathogen will differ with the growth conditions. One outcome of this may be altered accumulation and stability of epitope fusion virions due to the complement of proteases upregulated within the host upon infection. For example, screening for fusion accumulation in different hosts, cultivated under growth-room conditions, illustrated that epitope stability was a function of the particular host/epitope combination (Fig. 3a). Field growth conditions may also impact this relationship. Promising vaccine candidates whose production requirements dictate production in the field should, therefore, be evaluated under actual growth conditions early in the product development cycle. The availability of a number of virus-susceptible field-adapted cultivars for screening is clearly desirable, should the initially selected host prove suboptimal. LSBC has a portfolio of production host candidates that may be screened for optimal performance with particular coat protein fusion vectors (Fitzmaurice 2002). By being cognizant of the biological parameters that affect the relationship between a recom-

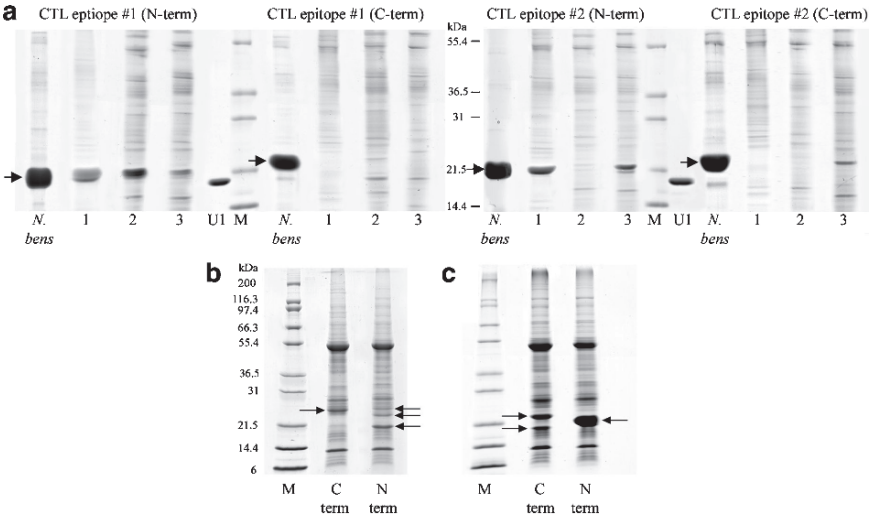

Fig. 3 a–c TMV coat protein fusion stability and expression as a function of the plant host employed and fusion location. **a** Screening of partially purified TMV peptide fusions from *N. benthamiana (N. bens)* and a series of field-adapted cultivars (*1, 2* and *3*). Plant host was screened under growth room conditions. Two 9 amino acid HLA-A2*01-restricted cytotoxic T lymphocyte (*CTL*) epitopes (CTL epitopes *#1* and *#2*) were tested as fusions to either the N-terminus (*N-term*) or located at the C-terminus of coat protein, internal to the last four residues (*C-term*). The coat protein fusion for each is identified by an *arrow*. *M* Mark 12 molecular-weight ladder, *U1* wild-type U1 TMV control. **b** Example of peptide fusion which showed stability when located at the C-terminus of coat protein, internal to the last four residues, but was subject to proteolytic cleavage in vivo at the N-terminus. **c** Example of *i*n vivo peptide fusion cleavage when located at the C-terminus of coat protein, internal to the last four residues, while peptide stability was obtained as an N-terminal fusion. For **b** and **c** the identity of both the full-length and the truncation products (identified by *arrows*) was confirmed by MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) analysis

binant virus and the host, we have been able to manipulate the system to ensure that any given product can be manufactured in an optimal fashion.

The stability of the peptide displayed on TMV is also dependent on the location of the fusion peptide in the coat protein. Published reports have focused predominantly on peptide display at the C-terminus and within the surface loop, with certain studies favoring peptide insertion preceding the four C-terminal residues, presumably to prevent proteolytic cleavage (Fitchen et al. 1995; Sugiyama et al. 1995; Turpen et al. 1995; Koo et al. 1999; Staczek et al. 2000; Wu et al. 2003). During preliminary screening for expression, we recommend that fusion to the N-terminus of TMV U1 also be considered. Although cases have been noted where the internal C-terminus location prevented in vivo proteolytic degradation when compared to N-terminal display, we have also observed the reverse (Fig. 3b, c).

Yet a further consideration is the influence of the peptide fusion on the virus–host interactions. For TMV, epitopes with a high isoelectric point and positive charge were found to result in a necrotic response by the host (Bendahmane et al. 1999). A high isoelectric point was also shown to be deleterious for the systemic infection of cowpea mosaic virus fusions (CPMV), with epitope length being another factor (Porta et al. 2003). Both studies noted that the addition of acidic residues counterbalanced the undesirable properties associated with the epitope fusions. The beneficial effect of compensatory acidic residues has also been noted for TMV-displayed epitopes in our laboratory (Fig. 4). The addition of compensatory residues can, therefore, be viewed as a strategy to rescue the expression of certain coat protein

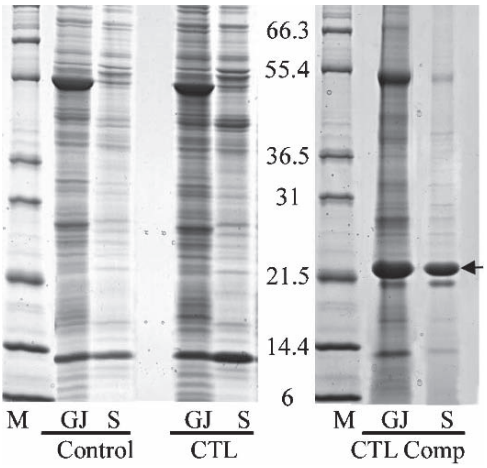

Fig. 4 Recovery of TMV coat protein fusion expression by the addition of compensatory acidic acid residues. A mouse CTL epitope was expressed as an N-terminal fusion to the U1 coat protein. The epitope contained one basic residue and no acidic residues. *M* Mark 12 (Invitrogen) molecular-weight marker, *GJ* starting green juice (crude extract), *S* Supernatant following green juice processing, *Control* Uninfected *N. benthamiana* control, *CTL* original CTL epitope coat protein fusion, *CTL Comp* CTL epitope coat protein fusion with compensatory residues. *Arrow* indicates coat protein fusion, the identity of which was confirmed by MALDI-TOF analysis. For the GJ and S CTL Comp, samples were diluted 5× prior to loading. For S Control and S CTL, samples were loaded at 1×

fusions. However, examples exist for the successful expression of positively charged peptides on the surface of TMV (Wu et al. 2003), suggesting that the peptide–host relationship is more nuanced than initially proposed (Bendahmane et al. 1999).

Multiple procedures have been developed for the purification of viruses from infected plant extracts on a laboratory scale (Corbett 1961; Dunn and Hitchborn 1965; Timian and Savage 1966; Gooding and Hebert 1967). Several of these employ an organic extraction or high-speed centrifugation step, which are undesirable when processing is done on a production scale (2,000–4,000 kg infected tissue per day). Large Scale Biology Corporation has developed a method compatible with the processing of large masses of plant material (Garger et al. 2000, 2001). The process was validated through the purification of a TMV coat protein fusion displaying a malarial epitope (Turpen et al. 1995; Pogue et al. 2002). This procedure is illustrated schematically in Fig. 5a. Briefly, the infected plant tissue was harvested and homogenized in the presence of 0.5 l water/kg of biomass. Removal of the plant fiber yielded a crude extract, or "green juice" that was adjusted to pH 5 and heated to 45–50°C. After incubation for approximately 10 min, the juice was cooled to below 10°C and a low-speed centrifugation step employed to separate the coagulated Fraction I proteins from the virus-containing supernatant. Ultrafiltration was employed to concentrate the supernatant 40- to 50-fold, prior to virus precipitation by the addition of polyethylene glycol (*M*r 8,000) and sodium chloride, each at 4% w/v. The precipitated virus was recovered as a paste by centrifugation, with the soluble host proteins remaining in the discarded supernatant. Operating at scale, this process yielded 0.6–1 kg of purified TMV fusion product per acre of infected plant material. The final purity of this TMV malarial epitope fusion was greater than 95% (Pogue et al. 2002). To evaluate the generalizability of this process, we have used a scaled-down version of the procedure, which faithfully mirrors the principal parameters for the production process, e.g., centrifugation conditions and holding times at the different temperatures. The process (outlined in Fig. 5a) has yielded good recoveries, with final purities comparable to the malarial epitope coat protein fusion, for 30%–40% of the peptide fusions evaluated. The remaining epitope fusions partitioned with the Fraction I proteins following centrifugation of the pH-adjusted and heat-treated green juice (Fig. 5b). These fusions can be recovered by resuspension of the Fraction I pellet under slightly alkaline conditions, followed by an additional centrifugation step. These process modifications, which are readily scaleable (Garger et al. 2000, 2001), effectively release the TMV fusion into the supernatant, with good recoveries, while the Fraction I proteins remain in an aggregated form. The processing of this supernatant can then proceed as indicated in Fig. 5A and yields a final product that meets purity requirements.

The displayed peptide may alter the purification characteristics of the recombinant virus at other stages of the process as well. For example, we have noted cases where the peptide prevents complete precipitation of the virus following the addition of NaCl and polyethylene glycol (Fig. 5c), a condition that can be remedied by the alteration of the ionic strength and polyethylene glycol concentration employed. The resistance of each fused peptide to proteases is an additional characteristic that must be evaluated. In vivo proteolysis has been alluded to above (Fig. 3); however, this is

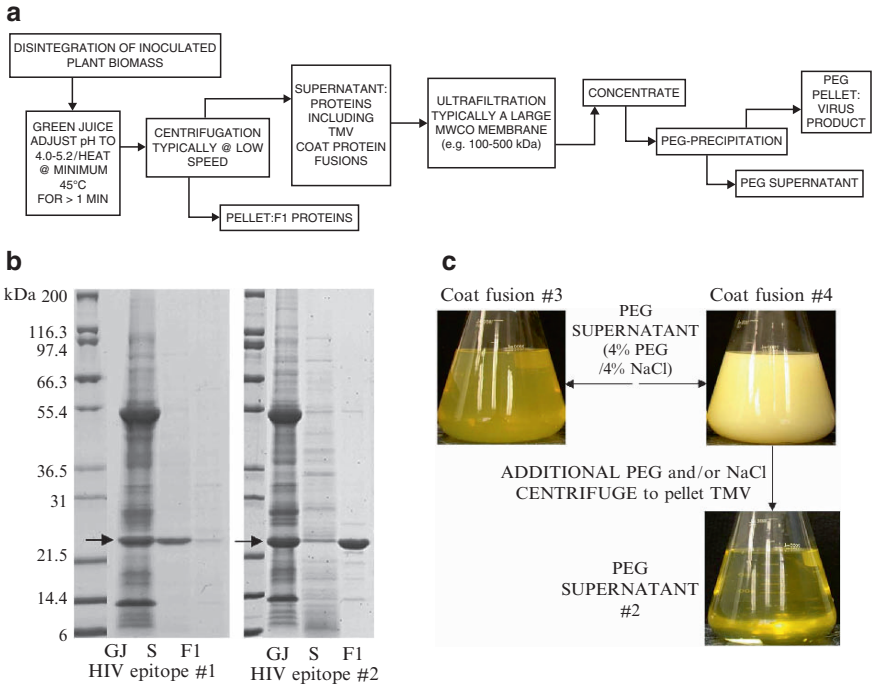

Fig. 5 **a** Simplified process flow diagram for the extraction and purification of TMV coat protein fusions on a multimetric ton per day scale (adapted from Garger et al. 2000). **b** Illustration of the differential partitioning observed between the pH adjusted and heat-treated supernatant and the coagulated Fraction I proteins for two TMV coat protein fusions, displaying peptides derived from HIV envelope proteins. *GJ* initial green juice (crude extract), *S* supernatant following green juice processing, *F1* processed F1 fraction. *Arrow* indicates TMV coat protein fusion. **c** Incomplete precipitation of TMV fusions under standard NaCl/polyethylene glycol (4% w/v each) conditions. Image shows the PEG supernatants (see **a**) following the centrifugation step to precipitate the virus fusion. Coat fusion *#3* precipitated as expected, leaving a clear supernatant, while incomplete precipitation was observed with coat fusion *#4*. For coat fusion *#4*, higher polyethylene glycol concentrations were required to obtain full precipitation of the virus, leaving a clear supernatant (PEG SUPERNATANT *#2*)

not necessarily predictive of stability during processing, as the loss of cellular compartmentalization with homogenization will expose the TMV fusion to additional proteolytic activities. One approach consists of incubating in-process samples, e.g., the initial supernatant, at 20–22°C (room temperature) for 2 h and evaluating stability by gel analysis, upon completion of the purification. For the peptide fusion shown in Fig. 6, excellent stability was obtained at bench-scale under standard processing conditions, where temperatures were maintained below 10°C (with the exception of the heat-treatment step). In contrast, with the room temperature incubation, substantial truncation was observed. By defining the process parameters critical to the stability of a particular fusion, the appropriate precautions can be implemented during scaled-up production, where the processing and holding times encountered

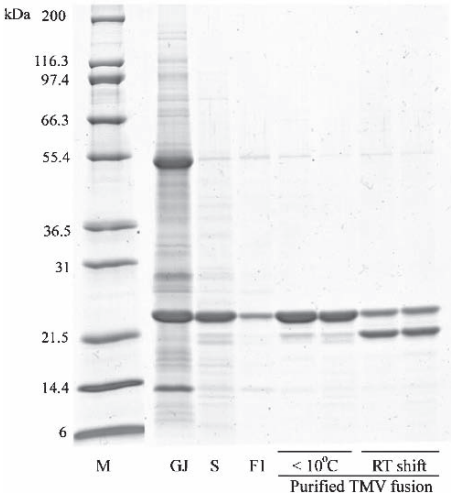

Fig. 6 Evaluation of TMV peptide fusion stability during processing. *M* Mark 12 (Invitrogen) molecular weight marker, *GJ* starting green juice, *S* supernatant following green juice processing, *F1* processed F1 fraction; *<10°C* purified TMV fusion (displaying a peptide from the L2 protein of human papillomavirus) obtained from S, with processing steps performed under chilled conditions, *RT shift* purified TMV fusion obtained from the supernatant S, which was incubated for 2 h at room temperature prior to processing under standard conditions

are typically of a longer duration. In summary, while each TMV peptide fusion modifies the particle surface properties and consequently its processing and purification characteristics, our experience has been that with the appropriate modifications, the majority of fusions evaluated can be made to conform to a general and scaleable production process, yielding a final product of acceptable purity.

For those cases where limited solubility persists despite processing modifications, alterations to the peptide itself may prove beneficial. Koo et al. (1999) noted that TMV displaying a ten amino acid peptide from the S2 glycoprotein of murine hepatitis virus was relatively insoluble, even though the recombinant coat protein expressed effectively and formed virus particles. When this peptide was flanked by additional S2 glycoprotein sequence, the TMV fusion was soluble and readily purified. This suggests that tiling around problematic peptides of interest may yield sequences with improved solubility characteristics when displayed on the surface of TMV. An alternative approach would be the introduction of compensatory sequences, in a manner similar to that described earlier in the context of improving virus–host compatibility, see for example Smith et al. (2006). For both approaches, immunogenicity testing in the appropriate model systems would be required to ensure the altered TMV fusions retain their desired properties.

Final Product Release and Stability

Once the bulk purified vaccine has been obtained, it must be formulated with excipients and/or adjuvants, sterilized and filled (Patro et al. 2002). The final form of the vaccine must also be considered. Is it to be distributed as a liquid or as a lyophilized powder, to be reconstituted with diluent prior to use? The filled product must also be submitted to extensive stability testing under the intended storage conditions to ensure that the product retains both integrity and immunogenicity.

There are a great number of potential excipients compatible with the formulation of parenteral drugs (Powell et al. 1998). For TMV fusions, which can be resuspended following purification in the required formulation buffer, e.g., phosphate buffered saline or Hank's buffered saline solution, formulation normally involves only a protein concentration adjustment and may include the incorporation of an adjuvant. However, issues relating to sterilization and TMV inactivation, discussed below, will impact the formulation steps, in particular relating to final product pH, ionic strength, and possibly the timing of adjuvant incorporation. An area where additional excipient testing may be of interest relates to the solubility of the TMV fusion. Wild-type TMV is inherently soluble and can be stored at concentrations above 20 mg/ml for several years at 4°C, with no visible signs of aggregation, degradation, or precipitation. However, the solubility characteristics of the virus can be changed dramatically by the addition of peptide fusions, and we have noted several examples where visible precipitation occurs with storage at 4°C, even at relatively low concentration (1–2 mg/ml). Addition of the appropriate excipient may prevent this from occurring, and we have found that aggregation state can be modulated and controlled through addition of excipients. Protein–protein aggregation is typically viewed as a negative for protein biologics since it may adversely affect activity and half-life (Patro et al. 2002). The impact, if any, of aggregation on a vaccine product needs to be considered. Given that aggregates can enhance the immune response, the outcome may ultimately be a beneficial one if potency is controlled.

Following the formulation of protein biologics, sterile filtration through a 0.2 μm filter is typically performed, to ensure the product is free from bioburden. However, the physical dimensions of TMV (18×300 nm) results in the rapid fouling of 0.2-μm filters. Although the methods that we have developed for purification of TMV generally yield product with very low endotoxin and bioburden loads, the implementation of a method or combination of methods to permit TMV fusion vaccine sterilization, to eliminate residual bioburden, is a priority. One possibility is serial 0.45-μm sterile filtration, since 0.2-μm filtration is typically employed with tissue culture-derived biologics to ensure the absence of mycoplasma, which is not a concern for plant-derived products. TMV passes readily through 0.45-μm membranes. However, the introduction of 0.2-μm filters was prompted by the identification of diminutive bacteria, that consistently penetrated 0.45-μm filters (Bowman et al. 1967). These pathogens are widely distributed in nature and have been isolated from water (Howard and Duberstein 1980; Sundaram et al. 1999). Exclusion of these pathogens from the buffers used during extraction, purification, and formula-

tion can be achieved, but the field or greenhouse-cultivated infected plant material remains a potential source.

One approach for the sterilization of TMV fusion vaccines is to employ UV irradiation. UV treatment was applied to a canine parvovirus (CPV) vaccine, based on CPMV, and the vaccine preparation remained effective, protecting dogs from lethal challenge with CPV (Langeveld et al. 2001). When tested with a TMV fusion displaying a peptide from the VP2 capsid protein of CPV, the conditions required to inactivate bioburden resulted in cleavage of the coat protein, producing an ill-defined product that was difficult to qualify. As a result, we have not pursued this technique further. Other sources of ionizing radiation, e.g., gamma radiation, when combined with excipients to prevent protein aggregation (Assemand et al. 2003) and fragmentation (Moon and Bin Song 2001) may provide an alternative to UV treatment. Gamma irradiation is also an attractive route to sterilization, as it can be performed on the final vialed product.

Another sterilization methodology that may be applicable is the use of inactivating agents, such as formaldehyde, β-propiolactone and the aziridines. These agents are typically employed to inactivate viruses and bacterial toxins employed as vaccines, but they should prove equally effective at eliminating residual bioburden. Formaldehyde, which acts as a microbicide due to its peptide cross-linking activity, is employed in the manufacture of at least eight vaccines licensed for use in the United States (Offit and Jew 2003). Following treatment, the remaining free formaldehyde may be neutralized by the addition of sodium bisulfite (Martin et al. 2003). In spite of the widespread use of formaldehyde, it is not considered the ideal inactivant, as the inactivation reaction is neither linear nor first order (Wesslen et al. 1957) and extended inactivation periods are required (up to 60 days). Furthermore, since the nucleic acid remains functional, incomplete inactivation is possible, as has occurred with a foot-and mouth disease (FMD) vaccine and an early inactivated polio vaccine (Bahnemann 1990; Brown 2001). Consequently, inactivants that target nucleic acids may be more appropriate. One option is β-propiolactone (BPL), which functions by alkylating nucleic acids, thereby abolishing replication. It is currently employed in the inactivation of the cell culture-derived rabies virus vaccine (Perez and Paolazzi 1997) that is currently licensed in more than 20 countries and gained FDA approval in the United States in 1997 (Dreesen 1997). BPL is considered a possible carcinogen in humans, but it undergoes rapid hydrolysis in aqueous solution, and the breakdown products are nontoxic (Perrin and Morgeaux 1995). Of greater concern is the fact that BPL reacts with several amino acids, which could impair the immunogenicity of the coat protein-displayed epitopes, a point that has also been raised with regard to formaldehyde inactivation (Brown 2001).

A promising alternative is the use of aziridine compounds, such as binary ethylenimine (BEI), which also function through the alkylation of nucleic acids. Following virus inactivation, the residual BEI is hydrolyzed by the addition of sodium thiosulfate, which is itself innocuous. Although ethylenimines have been shown to react with proteins (Kasermann et al. 2001), their impact on epitope conformation and accessibility was substantially less than either BPL or formaldehyde

treatment (Blackburn and Besselaar 1991). LSBC has evaluated BEI as a means of eliminating bioburden from investigational vaccine preparations. To date, approximately ten different TMV epitope fusions have been tested with the BEI procedure. We have determined conditions that effectively inactivate bioburden and in all cases the displayed epitopes retained antigenicity (Palmer et al., 2006). This procedure can be readily incorporated into veterinary vaccine production, since BEI inactivation is already used in the preparation of the current FMD vaccine, which is distributed globally and is the highest volume viral vaccine manufactured (Bahnemann 1990). BEI inactivation has not yet been approved for use with human biologics; however, no toxicity or tumorigenicity has been observed in the billions of livestock vaccinated with aziridine-inactivated products (Brown et al. 1998). In addition, a chemically related compound, PEN110, is currently in phase III clinical trials as a method of pathogen eradication in red blood cells, for patients requiring acute and chronic transfusions (Wu and Snyder 2003). For these reasons, the aziridines have been proposed as a universal method for pathogen inactivation in blood products, as well as in biopharmaceutical manufacture (Brown et al. 1998), and appear, from our experience, to be readily applicable to the preparation of TMV peptide vaccines. BEI treatment also eliminates TMV infectivity (LSBC, unpublished data). Inactivation of TMV may be important in developing these products for use in veterinary medicine, since state Departments of Agriculture regulate movement of infectious plant pathogens. We, and others, have shown that TMV is incapable of replication in mammalian cells, so elimination of infectivity is not a human or animal safety issue per se, but rather a plant health concern.

Once formulated, sterilized and vialed, the TMV fusion enters a stability study to ensure that protein integrity is maintained and to evaluate shelf life. For example, one ongoing stability study at LSBC is for a TMV fusion displaying a 13 amino acid peptide from the VP2 protein of canine parvovirus. This fusion constitutes a potential veterinary vaccine against canine parvovirus, feline panleukopenia virus and mink enteritis virus, as the neutralizing epitope is conserved across all three viruses (Dalsgaard et al. 1997). The vialed vaccine was stored as a 2-mg/ml solution at 4°C. Data for the 14-month time point is shown in Fig. 7a, b. Excellent long-term stability for this fusion was observed as evidenced by SDS-PAGE analysis and from MALDI-TOF, which confirmed the presence of the full epitope.

In another stability study for a series of investigational papillomavirus vaccines, consisting of epitopes from the L2 surface protein fused to TMV, the vialed products were stored at –20°C. Coat protein fusion purity, defined as the percentage of full-length product, was determined by SDS-PAGE, and MALDI-TOF analysis was performed to identify all species. The initial and 6-month data for four of the TMV fusions is summarized in Fig. 7c. Excellent stability was observed for L2 peptides #1 and #3, whereas cleavage occurred with the remaining two TMV fusions over the course of the study. This example highlights the need to completely characterize each coat protein fusion. Stability of the frozen products is also important since, during vaccine production, the purified biologic is often frozen prior to formulation to permit inventory build-up and for storage prior to formulation in a campaign mode (Patro et al. 2002). The cause for the observed

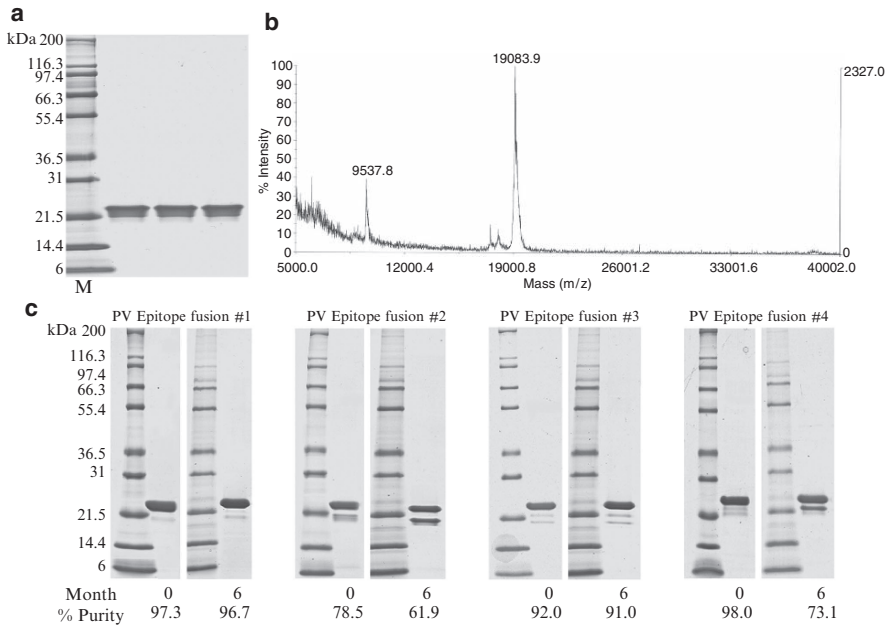

Fig. 7 **a–c** Stability of TMV fusion vaccines with storage. **a** Stability study for TMV fusion displaying a 13 amino acid peptide from the VP2 protein of canine parvovirus. Vialed vaccine, at 2 mg/ml, was stored for 14 months at 4°C. SDS-PAGE analysis of vaccine, analyzed in triplicate: purity estimated at more than 98%. **b** MALDI-TOF mass spectrometry spectrum for TMV fusion vaccine shown in **a**. The expected MW was 19,081 Da and the observed MW was 19,084 Da. **c** Stability study for four papillomavirus (*PV*) L2 peptide TMV fusions. SDS-PAGE analysis for the initial vialed vaccine and the vaccines after storage for 6 months at −20°C. Percent purity (full-length species) is indicated. For analysis each sample was run in triplicate. The identity of the coat protein fusions and any observed cleavage product was determined by mass spectrometry

reduction in full-length coat protein fusion for certain fusions is currently under investigation. One possibility is that the hydrolysis of the protein backbone is catalyzed by a host-derived protease, present in the purified vaccine preparation at below Coomassie-stainable levels (<0.5%). If this is the case, a polishing chromatography step or steps could potentially be employed to separate the proteolytic activity from the TMV fusion.

Summary

Many groups have developed vaccines based on peptide display on the surface of plant viruses that show exciting potential for use as prophylactics and therapeutics. However, the challenges associated with bringing new biological products into practical use in human and animal healthcare are underappreciated. Since plant virus peptide display vaccines have not yet been administered to humans, except in crude

edible vaccine form, many of the manufacturing, quality control, and quality assurance methodologies necessary to bring the technology into more general use have not been described.

From a commercial perspective, development of vaccines that can prevent infectious diseases poses challenging business questions. Infectious disease poses the greatest risk to human health in poorer areas of the world, resulting in the requirement for low cost-of-goods, and lower profit margins than are traditionally attractive to larger companies. For a plant-based system such as the TMV peptide display platform, the exceptionally low cost-of-goods and outstanding environmental stability of TMV vaccines may make this a viable system for delivery of vaccines in resource-poor conditions. Data on safety and immunogenicity of these vaccines in humans are urgently required to move the product concept forward.

For companies developing new vaccine technologies, such as VLP epitope display platforms, it is also attractive to consider immunotherapeutic products that have lower development costs than prophylactic vaccines against infectious diseases, which require very large clinical trials over long periods of time. The concept of using vaccination to break immune tolerance to self-antigens and induce antibodies that may act therapeutically is a new and exciting one (Bachmann and Dyer 2004). It is well known that the best way to achieve this is through linkage of critical B cell epitopes to virus-like particles (Fehr et al. 1998; Chackerian et al. 2002). The report by Fitchen et al. (1995) provides data showing that TMV particles may be useful for the display of self-peptides in the development of therapeutic vaccines for induction of autoantibodies for the treatment of chronic diseases, such as Alzheimer's disease, rheumatoid arthritis, Crohn's disease, psoriasis, allergy, obesity, and drug addiction, and various infectious diseases, among others. These are drug targets of many vaccine and pharmaceutical companies. Therapeutic vaccines against chronic diseases also frequently require functional T cell immunity, directed against chronically infected or cancerous tissues, TMV displayed peptides do indeed promote functional T cell responses (McCormick et al. 2006). A fundamental requirement for therapeutic vaccines to be effective is that they activate professional APCs that can prime T cells reactive against target cells and tissues, and induce a strong Th1-biased immune response. The data published by Loor (1967) suggest that TMV particles are engulfed by APCs and rapidly transported to lymphoid organs for presentation to T cells. However, recent data suggest that different VLPs have very distinct properties when their abilities to activate the innate immune system are compared, and that the use of adjuvants, as well as elegant molecular engineering strategies can be applied to modulate the activity of epitope-display vaccines (Chackerian et al. 2002; Storni et al. 2004). Investigation of the interaction of plant virus particles with the innate immune system is an area of research that must be addressed in order to make plant virus-based epitope carriers competitive with other systems. When these data are coupled with industrial-scale methods for manufacturing, plant virus capsid-based epitope display systems could provide valuable contributions to human and veterinary health care in the near future.

Acknowledgements The authors thank Gregory Pogue, Stephen Garger, and Kathleen Hanley for insightful discussions and suggestions in the preparation of this manuscript. We also acknowledge the scientific staff at LSBC, who provided data for this review. In particular, we thank Tiffany Bliss, Amanda Lasnik, Alison McCormick, Michael McCulloch, Michael Mullican, Long Nguyen, and Sarah Venneman.

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