

Review

The coming of age of virus-like particle vaccines

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Abstract

Virus-like particles are supra-molecular assemblages, usually icosahedral or rod-like structures. They incorporate key immunologic features of viruses which include repetitive surfaces, particulate structures and induction of innate immunity through activation of pathogen-associated molecular-pattern recognition receptors. They carry no replicative genetic information and can be produced recombinantly in large scale. Virus-like particles thus represent a safe and effective vaccine platform for inducing potent B- and T-cell responses. In addition to being effective vaccines against the corresponding virus from which they are derived, virus-like particles can also be used to present foreign epitopes to the immune system. This can be achieved by genetic fusion or chemical conjugation. This technological innovation has greatly broadened the scope of their use, from immunizing against microbial pathogens to immunotherapy for chronic diseases. Towards this end, virus-like particles have been used to induce autoantibodies to disease-associated self-molecules involved in chronic diseases, such as hypertension and Alzheimer's disease. The recognition of the potent immunogenicity and commercial potential for virus-like particles has greatly accelerated research and development activities. During the last decade, two prophylactic virus-like particle vaccines have been registered for human use, while another 12 vaccines entered clinical development.

Keywords: Alzheimer's disease; bacteriophage Q β ; hypertension; immunotherapy; nicotine addiction.

Introduction

The merits of vaccination against infectious disease are well known and documented. Vaccines have been identified as a key contributor to the 30-year increase in average human-life span achieved during the 20th century (Cooper et al., 2002). The eradication of naturally occurring smallpox infection, near eradication of polio and greatly reduced incidences of diseases, such as measles, rubella, mumps, pertussis and diphtheria has contributed to the decrease in global mortality and morbidity from infectious diseases (Plotkin, 2005).

The greatest successes of vaccination have been established against pathogens that cause disease in a

relatively simple and direct manner. The most important characteristic of these pathogens is that neutralizing antibodies protect against infection or disease and that induction of such antibodies is relatively straightforward. Consequently, the vaccines employed so successfully against these pathogens have also been relatively simple (e.g., live attenuated, killed, detoxified) with a bias towards the induction of humoral immunity. In contrast, vaccination has been much less effective against organisms with less-tractable pathogen-host interactions, such as HIV, HCV, *Plasmodium* and *Mycobacterium tuberculosis*, which profit from undermining, evading or misdirecting the host's immune response. Simple antibody-mediated neutralization often is not effective.

Fundamental research in immunology, molecular-cellular biology and vaccinology has sought to elucidate the mechanisms of host-pathogen interactions with the aim of providing a basis for designing vaccines capable of evoking the types of immune responses required to prevent and control these more complex infectious diseases. Such knowledge is also essential for improving existing suboptimal vaccines and developing safe and effective vaccines to counter the threats of new and emerging pathogens. Moreover, these insights and advances may also be used to broaden the scope of traditional vaccines and develop immunotherapies for treatment of cancer, allergies and autoimmune diseases, such as multiple sclerosis or rheumatoid arthritis. It may even be possible to develop vaccines targeting new indications, such as Alzheimer's disease, hypertension or addiction to substances of abuse.

Vaccines targeting viral pathogens have traditionally been based around attenuated or inactivated viruses. Typically, these vaccines induce prompt and efficient immune responses in the host. Live vaccines have immunogenic properties that make them ideal immunogens; namely, they confer long-lived immunity, induce efficient B- and T-cell responses and usually require only a single immunization. While they are generally well tolerated in the overall population, there remains the real and present risk of transmission to non-target subjects and safety concerns for immune-compromised individuals (Goldman and Lambert, 2004). Recombinant subunit vaccines do not have these concerns, but their increased safety often comes at the cost of lower immunogenicity. Hence, to promote efficient induction of immune responses the addition of adjuvants may be required.

In recent years, mechanisms underlying the superb immunogenicity of viruses have been elucidated and four key viral parameters identified. These are 1) their highly repetitive and ordered structures, 2) their particulate nature, 3) their ability to induce innate immunity for appropriate conditioning of adaptive immune responses, and 4) their ability to replicate leading to distinct kinetics

and distribution of virus during infection. These features serve as a paradigm for rational vaccine design and many new-generation vaccines are attempting to harness these properties (Jennings and Bachmann, 2007). Such vaccines will be able to co-localize antigen and adjuvant to induce innate immune responses capable of directing the subsequent adaptive immune response of appropriate magnitude, quality and specificity.

Virus-like particles (VLPs) represent an advanced vaccine technology platform that is able to combine many of the elements above into one system making them extremely potent inducers of B- and T-cell responses. Consequently, during the last 20 years, VLP-based vaccines have been the subject of extensive preclinical and clinical research, the pace of which has dramatically accelerated in the last decade. As testimony to this, several highly efficacious VLP-based vaccines for prevention of infection with Hepatitis B or human papillomaviruses are today marketed for human use. Another 14 VLP-based vaccines aimed at inducing B- and/or T-cell responses against either microbial pathogens, disease related self-molecules or addictive agents have been tested in humans in clinical trials. This review will provide an outline of VLP technology, the underlying immunology and provide detailed examples of their clinical development.

The nature of virus-like particles

VLPs are supra-molecular structures that have the form of rods or icosahedrons with diameters in the range of 25–100 nm (Johnson and Chiu, 2000). They are composed of multiple copies of one or more recombinantly expressed viral structural proteins which spontaneously assemble into particles upon expression. In addition to being virus-like in structure, they are often antigenically indistinguishable from the virus from which they were derived. The genes encoding VLPs have been acquired from many different sources, including animal viruses, plant viruses, bacteriophages and yeast Ty retrotransposons. These genes can be recombinantly expressed in a variety of hosts, including, bacteria, yeast, plant, insect and mammalian cells. Due to their virus-like nature, VLPs are very immunogenic and their potential to be highly effective vaccines has long been recognized.

VLPs combine many of the advantages of whole virus vaccines and recombinant subunit vaccines into one system (Chackerian, 2007). Recombinantly expressed VLPs, while structurally similar to whole virus vaccines lack genetic information with replicative capacity. Hence, the safety issues of virus vaccines relating to reversion mutants and immunocompromised individuals are obviated. Their recombinant nature means they can usually be manufactured economically in large-scale following current good manufacturing practices. They display antigens in an ordered and repetitive way and thus induce rapid and fulminant humoral immune responses. Their particulate nature and dimensions means they are efficiently taken up by dendritic cells (DCs) and transported to lymph nodes for subsequent presentation and induc-

tion of optimal immune responses. The ability of VLP polypeptide subunits to spontaneously assemble allows them to be disassembled and reassembled *in vitro*, a process which permits stimulators of innate immunity, such as Toll-like receptor (TLR) ligands to be packaged within their interiors. Such VLPs are able to co-deliver both antigens and activators of innate immunity to DCs in a concentrated form and thus have the ability to induce efficient T-cell responses. Finally, they may be combined with depot forming adjuvants to better simulate the kinetics and distribution of natural viral infections.

Immunology of B-cell responses

VLPs are renowned for inducing rapid and strong antibody responses. This trait is attributed to their dense, highly repetitive, quasi-crystalline structures (Bachmann and Zinkernagel, 1997). The rationale behind this phenomenon is the following. Most viral genomes are small and encode relatively few gene products which limit the number of available structural proteins. Hence, viruses are constrained to use multiple copies of a few proteins to assemble their envelopes and cores. Consequently, the surface of a virus is composed of repeated, ordered subunits, a densely packed quasi-crystalline surface. This structure is distinct for viruses and certain microbes. Co-evolution of viruses and vertebrates has resulted in the adaptive immune system attaining the ability to rapidly detect, discriminate and respond to these repeated and ordered structures found on viral surfaces (Bachmann and Zinkernagel, 1996). At a molecular level, the repetitive epitopes on the viral surface cross-link specific immunoglobulins (Igs) that comprise the B-cell receptor (BCR) on the surface of B-lymphocytes. Cross-linking of the BCR and stabilizing BCR-signaling microdomains is a critical signal for B-cell activation and results in a prompt T-independent IgM response (Bachmann et al., 1995; Thyagarajan et al., 2003). Viral proteins expressed in an ordered and repetitive fashion are considerably more immunogenic than in soluble form and can even overcome B-cell tolerance (Bachmann et al., 1993; Schodel et al., 1993; Justewicz et al., 1995). The optimal spacing of antigens for B-cell activation has been determined using haptenated polymer molecules. It was found that a minimum of 20–25 epitopes spaced by 5–10 nm was sufficient for optimal B-cell activation (Dintzis et al., 1982). Immunogenic epitopes displayed on viruses (Figure 1) and VLPs have similar numbers and distance (Jegerlehner et al., 2002a).

Further enhancement of the immune response provided by repetitive surfaces is due to the fact that these structures are preferentially bound by components of the complement system and multivalent natural antibodies, resulting in enhanced phagocytosis (Fanger et al., 1996; Matsushita and Fujita, 2001). The proteinaceous nature of viruses and VLPs additionally induces T-cell-dependent IgG responses, germinal center formation, hypermutation and B-cell memory (Bachmann and Zinkernagel, 1997). The magnitude of the B-cell response is well illustrated by the observation that 3 weeks after vaccination with 50 µg of VLPs derived from the bacte-

riophage Q β , 50% of all isotype-switched B-cells are specific for the VLPs (Gatto et al., 2004). The power of VLP-based vaccination is further underscored by the fact that classical adjuvants, such as incomplete Freund's adjuvant (IFA), Alum or bacterial DNA rich in CpG dinucleotide motifs enhance antibody responses only marginally. Thus, VLPs seem to induce maximal B-cell responses even in the absence of adjuvants.

VLPs possess additional features which influence their ability to induce effective B-cell responses. One is their size; small particles with the dimensions of VLPs efficiently drain or diffuse to lymph nodes from the site of injection. Thus, VLPs can enter secondary lymphoid organs and interact directly with B-cells to trigger antibody responses. The fact that active transport and uptake is not required to present VLPs means conformation dependent epitopes can be presented and optimal B-cell responses induced. Another feature is that immune stimulating adjuvants, such as TLR ligands can be packaged within their lumens. TLR9 ligands have an adjuvant activity that directly affects B-cells. CpG oligodeoxynucleotides (CpG-ODNs) are known to stimulate antigen-specific B-cells due to synergy between TLR9 and the B-cell receptor, inhibit B-cell apoptosis and enhance class-switching (Krieg, 2006). VLPs packaged with CpG-ODNs have been shown to have a direct stimulatory influence on B-cells and influence isotype switching. Similar to CpG-ODNs, single-stranded RNA packaged within some VLPs can activate TLR7 present in B-cells and enhance B-cell responses and class switching when compared to VLPs devoid of TLR7 agonists (Jegerlehner et al., 2007).

In addition to VLPs, other antigen presentation platform technologies are being developed which include microparticles, virosomes and liposomes. VLPs are a

most effective means of presenting antigens to the immune system in an ordered and repetitive manner, and triggering antibody responses that are early and rapid, as well as lasting and sustained in nature. VLPs may be used in two different ways for vaccination: firstly, they can be used to immunize against the proteins comprising the VLP, or secondly, against any antigen linked to the VLP. The most straightforward application for VLPs is to use them for vaccination against the virus from which they have been derived. In some instances, the structural proteins capable of self-assembly into VLPs are derived from proteins with limited or no surface exposure, such as nucleoproteins. VLPs derived from internal viral proteins include gag proteins from retroviruses (HIV, RSV, etc.) and Hepatitis B core antigen. Antibodies induced by such VLPs are not able to recognize the native virus from which they were derived and hence are not able to interfere with the course of virus infection. However, these VLPs can be used as carriers of unrelated B- or T-cell epitopes which may themselves theoretically be able to induce anti-viral T-cell responses.

Of most interest for the development of prophylactic anti-viral vaccines are those VLPs composed of viral surface proteins, particularly when those proteins are recognized by virus neutralizing antibodies. Examples of VLPs that include such proteins are those derived from HBV (HBsAg), papillomavirus, Hepatitis C virus, Norwalk virus, HIV (gp 120), Rotavirus and Parvovirus. Many of these VLPs have been shown to induce neutralizing antibodies and to be protective in animal models (Grgacic and Anderson, 2006). VLP vaccines against infections from Blue Tongue virus, Rotavirus and Parvovirus are also being developed for veterinary applications and VLPs derived from papillomavirus and HBV are marketed for human use (see below).

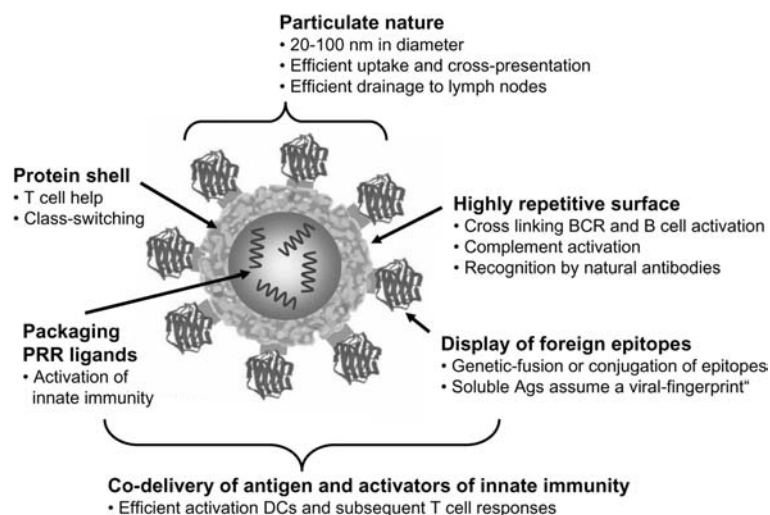


Figure 1 Immunogenic features of a VLP presenting foreign antigens.

VLPs can be viewed as a vaccine platform that combines key immunogenic properties of viruses into one system for efficient activation of T- and B-cell responses. Its highly ordered and repetitive surface leads to the direct activation of B-cells and of components of the complement cascade and binding of natural antibodies that can further enhance VLP uptake by APCs. Its proteinaceous composition provides T-cell help to activated B-cells and permits chemical conjugation or genetic fusion. Its particulate nature enhances uptake by DCs and antigen presentation on MHC class II and class I molecules. Its ability to self-assemble into nanostructures means that ligands for PRR can be incorporated within the lumen of VLPs, which leads to activation of innate immunity.

Immunology of T-cell responses

Due to the general difficulties of inducing and maintaining effective T-cell responses, the aim of current T-cell vaccine strategies is to produce the strongest possible T-cell responses. VLPs represent a promising technology for achieving this goal, because they possess numerous characteristics thought to enhance the induction of potent T-cell responses. DCs circulating in the periphery actively take up antigen and transfer it to lymph nodes for subsequent presentation to T-cells. The processes of uptake into the DC, trafficking of the DC to the lymph node and triggering of DC maturation are key. Presentation of antigen by DC expressing co-stimulatory molecules and cytokines is necessary to generate strong and effective T-cell responses with lasting memory.

The particulate nature and dimensions of VLPs means they are efficiently taken up by antigen presenting cells and, in particular, DCs (Figure 1). Uptake of antigens by DCs depends upon a number of antigen associated properties, such as size, shape, surface charge, hydrophobicity/hydrophilicity and receptor interactions (Ahsan et al., 2002). The size of an antigen is a key factor for efficient uptake by DCs. Molecular aggregates, such as VLPs, have extended surfaces exhibiting charge, hydrophobic or receptor interacting properties, and thus have superior ability to interact with cellular surfaces than soluble proteins. Antigen-presenting cells (APCs) of the immune system are able to efficiently take up antigens with pathogen-like dimensions (10 nm to 3 µm). VLPs have dimensions that fall within this range which accounts for their efficient uptake by endocytosis/phagocytosis. Indeed, it has been demonstrated that DCs optimally take up antigens with diameters of approximately 40 nm (Fifis et al., 2004).

Antigens taken up by DCs are processed by proteases in endosomal compartments to peptides which can be presented on major histocompatibility complex (MHC) class II molecules. DCs in addition also have the property to cross-present endocytosed antigens. This means that exogenous antigens are shuttled into the MHC class I processing pathway and are presented on MHC class I molecules (Figure 1). Cross-presentation of antigens derived from particulate structures, such as VLPs, occurs much more efficiently than cross-presentation of soluble antigens and is comparable in its magnitude to presentation on MHC class II molecules (Kovacsics-Bankowski et al., 1993; Harding and Song, 1994; Storni and Bachmann, 2004).

VLPs are only processed by professional APCs and not by other cell types, such as fibroblasts or T-cells (Ruedl et al., 2002). Because antigens presented by non-professional APCs can turn T-cell responses off rather than on, this property probably facilitates induction and maintenance of effector T-cell responses.

As with most recombinant antigens, VLPs typically induce poor T-cell responses in the absence of additional stimuli, despite the fact they efficiently deliver and present VLP-derived peptides on MHC class I molecules. The reason for this is that they do not deliver additional stimuli to the innate immune system and in particular to APCs. In the absence of such stimuli, cytotoxic T lymphocyte

(CTL) responses remain inefficient and abortive. Live viruses replicating inside cells also facilitate loading of MHC class I molecules, but in contrast to recombinant antigens, they stimulate the innate immune system by various means. These include induction of cell lysis and production of single- and double-stranded or uncapped 5'-triphosphate RNA which trigger TLRs, retinoic acid-inducible gene I-like helicases and PKR (IFN-inducible dsRNA-dependent protein kinase), resulting in production of type 1 interferons and proinflammatory cytokines (Takeuchi and Akira, 2007). If DCs and macrophages are stimulated while simultaneously immunizing with VLPs, then very strong CTL responses may be induced (Storni et al., 2002). In essence, co-administration of VLPs with stimuli of innate immunity system closely mimics the course of events during a viral infection.

Several types of VLPs have been reported to directly induce phenotypic and functional maturation and activation of DCs. Human papillomavirus (HPV) has been reported to directly activate DCs leading to the expression of co-stimulatory molecules cytokines necessary for activation of CTLs (Lenz et al., 2001). However, this is not the case for most VLPs, and typically, the addition of stimulators of innate immunity are required. We have developed and tested VLPs that co-deliver TLR ligands and T-cell epitopes to DCs and established proof-of-concept in animal models. We have shown Q β -VLPs devoid of any TLR ligands essentially failed to induce cytotoxic T-cells (Storni et al., 2004). Q β VLPs recombinantly expressed in *Escherichia coli* spontaneously package host cell RNA during their assembly process and thus represent a ready-made delivery system for this normally unstable TLR ligand. Co-delivery of ssRNA and T-cell epitopes by such VLPs activates TLR7 present in DCs and enhances T-cell-dependent responses when compared to VLPs devoid of TLR agonists, but the responses are still fairly weak (Schwarz et al., 2005). Numerous ligands for Toll-like receptors (including those for TLR2, -3, -4, -5, -7 and -9) have been tested. DNA containing CpG motifs, a ligand for toll-like receptor 9, were by far the most potent adjuvants (Schwarz et al., 2003). Robust T-cell responses have been obtained by co-administration of TLR9 ligand (CpG-ODN) packaged within VLPs (see above) linked with the p33 epitope from lymphocytic choriomeningitis virus (LCMV). T-cell responses induced by this vaccine have been shown to protect from infection with the virus and eradicate established fibrosarcomas (Storni et al., 2004). The power of VLPs to induce T-cell responses is further underscored by the fact that VLPs not only prime T-cell responses well but that they are also able to boost pre-existing responses. Importantly, while most prime-boost strategies require a change in carrier between the priming and the boosting, VLPs could induce and maintain T-cell responses if given repeatedly. Thus, if VLPs are used for induction of T-cell responses, it is not necessary to change carrier to boost or maintain T-cell responses, but one in the same VLP can be used for both (Schwarz et al., 2005).

In addition to improved immune responses, packaging activators of innate immunity within VLPs offers important technical advantages. The often labile TLR ligands, such as CpG-ODNs and ssRNA, are resistant to degra-

dation when packaged within VLPs. Non-specific binding of these polyions to serum proteins is also reduced, which drastically improves their pharmacokinetic profile compared to when they are administered alone. Furthermore, packaging and consequent targeted delivery to the requisite APCs limits systemic distribution and reduces side effects, such as splenomegaly (Storni et al., 2004).

Several VLP-based vaccines which incorporate T-cell epitopes have been tested in the clinic for their ability to induce protective/therapeutic T-cell responses. The vaccines CVLP (chimeric virus-like particles; Medigene AG, Martinsried, Germany), Malarivax (Apovia, San Diego, USA) and p24-VLP (British Biotech plc, Oxford, UK) targeted the pathogens HPV, *Plasmodium falciparum* and HIV, respectively. In all instances, only relatively weak T-cell responses against the respective T-cell epitopes were generated and not surprisingly clinically relevant primary end-points were not met (Lindenburg et al., 2002; Walther et al., 2005; Kaufmann et al., 2007). The negative outcomes of these trials may have been influenced by the fact that new-generation activators of innate immunity, such as CpG-ODNs, were not utilized and perhaps sub-optimal dosing regimens were employed.

With these shortcomings in mind, we have initiated 3 three-phase IIa studies with MelQbG10, a therapeutic vaccine for the treatment of malignant melanoma. MelQbG10 consists of a modified fragment of the Melan-A/MART-1 protein coupled to VLPs derived from the bacteriophage Q β . Packaged within the VLP are immunostimulatory CpG-ODNs. The studies include patients with malignant melanoma at different disease stages (stages II–IV) and are designed to explore safety, tolerability and immunogenicity of the vaccine. In addition, T-cell functionality will be evaluated and disease progression will be monitored. All three studies are designed as single-centre, open-labeled clinical trials and will compare different routes of administration and dose regimens.

VLPs as carriers of B-cell epitopes

The ability of VLPs to serve as carriers of B-cell epitopes derived from either the parental virus or foreign sources has further enhanced and broadened their potential as prophylactic and therapeutic vaccines. The poor immune response of many soluble antigens can be overcome by rendering them highly repetitive. This can be achieved by incorporating them onto or into the surface of VLPs. In this way, the underlying immunogenic ‘viral fingerprint’ of the VLP is imparted to the attached epitope making it as potent an immunogen as the VLP itself. Target epitopes may either be genetically fused into subunit proteins of the VLP to form chimeras or attached to the surface of the VLP by covalent or non-covalent means (see below). A diverse range of molecules have been incorporated into VLPs, including proteins, polypeptides, capsular polysaccharides and small organic molecules (haptens). Not only can VLPs act as carriers of immunologic epitopes derived from microbial pathogens but they have also been successfully used to present self-antigens to the immune system and overcome B-cell unresponsiveness.

For the most part, the remainder of this review will focus on B-cell vaccines.

Genetically fused epitopes

VLP chimeras have been extensively explored as vaccine candidates since the mid-1980s (Ulrich et al., 1998; Pumpens and Grens, 2001). The development of recombinant DNA engineering techniques combined with structural knowledge from X-ray crystallography and electron cryo-microscopy and immunology has permitted the design of hundreds of chimeric VLPs. VLPs derived from both double- and single-stranded DNA and RNA viruses encompassing 14 different families of virus have been successfully used for the production of chimeras (for a detailed description, see references directly above). Chimeras are produced by cloning the DNA encoding either linear or conformational epitopes into the genes encoding the self-assembly competent polypeptides of VLPs. Upon assembly of the hybrid subunit proteins into supra-molecular structures, the introduced epitopes are presented in a repetitive and ordered fashion, at relatively high density (1 copy per VLP subunit) and ideally with optimal accessibility and conformation.

The Hepatitis B core antigen (HBcAg) is the most extensively investigated chimeric carrier of B-cell epitopes to date (Pumpens and Grens, 2001). HBcAg VLPs can be expressed with high yields in numerous eukaryotic, prokaryotic and plant expression systems and are relatively easily purified. There are several sites within the HBcAg subunit that permit the insertion of foreign epitope sequences which include the N- and C-termini and the tip of the spike of the HBcAg particle, the major immunodominant region (MIR). The MIR is the most exposed region of the assembled VLP and also the most favored location for introduction of insertions. However, this site is also the most difficult in which to introduce foreign sequences and have the native structure maintained. Nevertheless, some relatively large epitopes have been successfully inserted into the MIR of HBcAg, including 120 amino acids of the immunoprotective region of the Hantavirus nucleocapsid (Koletzki et al., 2000) and 238 amino acids of green fluorescent protein (GFP) (Kratz et al., 1999), but these tend to be exceptions rather than the rule.

Mosaic VLPs have been developed to aid in the assembly of complex VLP chimeras and have been produced with HBcAg and Q β VLPs. Mosaic VLPs are typically formed by co-expression of a wild-type VLP subunit along with a chimeric VLP subunit. The resultant VLPs contain copies of both wild-type and chimeric subunits which are incorporated with differing proportions. The use of mosaic VLPs has permitted the formation of HBcAg VLPs containing fragments of up to 213 amino acids from the Hantavirus nucleoprotein and 163 amino acids from the Hepatitis B virus (HBV) presS domain (Kazaks et al., 2002, 2004). Alternatively, co-expression of VLP subunit proteins derived from different virus strains may be used to assemble mosaic VLP vaccines that generate antibodies that protect against multiple pathogen serotypes. Toward this end, a diploid

yeast strain that co-expresses the L1 and L2 capsid proteins of both HPV-6 and HPV-16 has been reported to produce VLPs comprising all 4 four co-assembled subunit proteins (Buonamassa et al., 2002).

Numerous chimeric VLP vaccines have been tested in preclinical research. The genetically fused B- and T-cell epitopes used in these vaccines have been derived from viral, bacterial eukaryotic parasites and disease related self-molecules. The application for these vaccines encompasses human to veterinary use and prophylactic to therapeutic treatment. Four chimeric vaccines entered clinical trials and include an anti-influenza A M2-HBcAg VLP vaccine (described in detail below) and the anti-HIV p24-Ty VLP. The remaining two clinically tested chimeras are anti-malaria vaccines. Glaxo Smith Kline is developing RTS,S; a peptide comprising amino acids 207–295 of the circumsporozoite (CS) protein fused to the amino terminus of HBsAg. The physical nature of this RTS,S is similar to the Hepatitis B vaccine and is a complex lipoprotein structures (Gordon et al., 1995). Apovira developed Malarivax ICC-1132, which is composed of B- and T-cell CS epitopes fused with HBcAg surface protein (Nardin et al., 2004).

Coupled epitopes

From the perspective of commercial vaccine development, the use of chimeric VLPs has limited general applicability for the following reasons: self-assembly of chimeras is highly unpredictable, non-peptide antigens cannot be incorporated, the length of the fused antigen is typically restricted, folding of the introduced antigen in association with the VLP subunit proteins is problematic,

which means conformational epitopes may not be preserved, it is nearly impossible for the incorporated antigen to adopt quaternary structure and yields during expression and purification are often reduced. In contrast, modular approaches provide a more robust and flexible means for displaying antigens on the surface of VLPs. The basis of this approach involves separate synthesis of the native VLP and target antigen. The two components are subsequently assembled *in vitro* using either covalent or non-covalent binding to link the antigen to the surface of the preassembled VLP. An added advantage of this approach is that the size and structure of the recombinant target antigen is not limited by the folding constraints of the VLP monomer and by particle assembly. Independent synthesis of the recombinant antigen markedly improves the chances of combining a full-length, correctly folded target protein with the VLP and thus maximizes vaccine quality. We have demonstrated the utility of the modular approach by successfully producing more than 150 polypeptide-VLP conjugate vaccines using a single VLP carrier, all of which induced high antibody titers against the respective antigens (see below and unpublished data). A total of 25 of these conjugates were either biologically active domains or full-length proteins with sizes ranging from 6.6 to 63 kDa. Many of these protein antigens were expressed and coupled in their native multimeric forms. Furthermore, we have also used covalent coupling to attach non-peptide antigens, such as capsular polysaccharides and small organic molecules.

A further advantage of the coupling approach is that the location for coupling the antigen to the VLP can be optimized to achieve the best exposure of the conjugated antigen. For example, when the extra cellular M2 domain

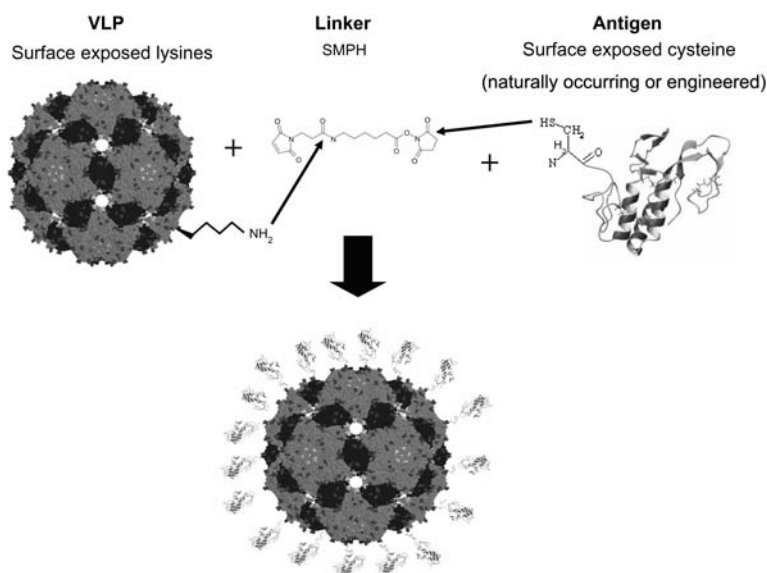


Figure 2 Covalently linking a polypeptide to a VLP by chemical coupling.

The VLP carrier (in this instance a VLP derived from the RNA phage, Q β) is first reacted with a hetero-bifunctional cross-linking reagent, such as succinimidyl-6 [(β -maleimidopropionamido)hexanoate]. Surface-exposed amines (from the amino-terminus of VLP subunit proteins and ϵ -amines of lysine residues) on the VLPs react with the amine reactive NHS-ester of the cross-linker (see arrow). Next, the derivatized VLP is reacted with a peptide antigen (in this example phospholipase A2 from bee venom – not to scale) engineered to contain an exposed cysteine residue. This reaction is performed under conditions permitting conjugation of the maleimide group of the cross-linker (see arrow) to the sulfhydryl group of the antigen. In this example, directional coupling is achieved by introducing an amino-terminal cysteine group.

of influenza A virus was coupled to the surface of the Hepatitis B core antigen, higher antibody titers were reached than when the same domain was genetically fused to the N-terminus of the VLP (Jegerlehner et al., 2002b).

The most adaptable and flexible way of displaying antigens on the surface of VLPs is via covalent linkage. This can be achieved by the use of chemical cross-linkers, and numerous conjugation strategies exist to achieve this end. One way in which this can be achieved is to use hetero-bifunctional conjugation reagents that contain two distinct reactive groups which couple to different and distinct functional targets; one on the VLP (typically amines or sulfhydryl residues) and the other on the antigen (Lechner et al., 2002). By way of example (see Figure 2), the ϵ -amines of exposed lysine residues of VLPs derived from the bacteriophage Q β are first reacted with an amine-reactive N-hydroxysuccinimide (NHS) ester of the cross-linker succinimidyl-6-[(β -maleimidopropionamido)hexanoate], whilst preserving the activity of its sulfhydryl-reactive maleimide group. Following the first reaction, the derivatized VLP is reacted with the target antigen under conditions permitting conjugation of the maleimide group of the cross-linker to a sulfhydryl group of the antigen. To ensure that the antigen is coupled to the VLP in a directed and oriented fashion, peptide antigens may be engineered to contain either an amino- or carboxy-terminal amino acid linker sequence containing a free cysteine group. Similarly, VLPs from any origin may be mutated to incorporate suitable reactive residue for coupling. Examples of this include engineering surface-exposed cysteine residues into bacteriophage MS2 or cowpea mosaic virus (Wang et al., 2002; Peabody, 2003). We have used a chemical conjugation strategy for both laboratory-scale production of preclinical research material and for large-scale GMP manufacture processes of vaccines for clinical trials, which is demonstrative of its convenience, utility and adaptability.

There are several examples of non-covalent attachment of proteins to the surface of VLPs. A method developed by Schiller and co-workers takes advantage of the strong interaction between streptavidin and biotin (Chackerian et al., 2001). Recombinantly produced streptavidin-tumor necrosis factor α (TNF α) peptide fusions were successfully bound to biotinylated HPV VLPs and used to generate neutralizing anti-TNF α antibodies. Experimental VLP based vector delivery systems have also utilized non-covalent interactions to attach targeting molecules. Tumor specific antibodies have been coupled to polyoma VLPs chimeras containing the antibody binding domain of Protein-A fused into the HI loop (Gleiter and Lilie, 2001). While not developed for vaccines per se, this design has the potential to be used for presenting B-cell epitopes. It should be noted that in both these examples the target antigen must be expressed as a fusion protein which potentially limits the overall utility.

Targeting self-molecules using VLPs

A novel application for VLP vaccines utilizes the principle of ordered and repetitive antigen presentation to overcome B-cell tolerance and induce auto-antibodies to

self-molecules that mediate disease. The use of VLPs to present self-antigens to the immune system offers great promise for treatment of chronic diseases, such as cancer, allergy, arthritis and central nervous system and cardiovascular disorders. Many of the molecules targeted by the numerous monoclonal antibodies currently in development may also be used in conjunction with VLPs for therapeutic vaccination. Potential advantages of such an active vaccination strategy over passive administration of antibodies include lower costs, avoidance of allotypic responses, less frequent administration, and better suitability for chronic treatment. Important safety issues, such as the difficulty in readily reversing the antibody response and duration of neutralizing antibody titers must be carefully assessed.

The feasibility of using VLPs conjugated with self-molecules for active vaccination has been demonstrated on numerous occasions. High titers of IgG auto-antibodies can be induced against self-antigens attached in a highly ordered manner to the surface of viruses or VLPs (Fehr et al., 1997; Chackerian et al., 1999). Furthermore, the auto-antibodies have been shown to be either blocking or neutralizing and have therapeutic effect. Chimeric VLPs comprising the first external loop of the murine CCR5 chemokine receptor cloned into the immunodominant neutralizing epitope of bovine papillomavirus L1 coat protein were constructed and immunized in mice. CCR5 is the co-receptor for macrophage tropic HIV strains. CCR5-specific auto-antibodies capable of blocking human RANTES binding and recognizing CCR5 on the cell surface were generated (Chackerian et al., 1999).

We have used covalent conjugation to produce numerous experimental immunotherapeutic VLP vaccines for the treatment of chronic diseases. Efficacy has been demonstrated in numerous preclinical models, including rheumatoid arthritis, osteoporosis, experimental autoimmune encephalitis, systemic lupus erythematosus, myocarditis, atherosclerosis, hypertension, Alzheimer's disease and obesity (Spohn et al., 2005, 2007, 2008; Röhn et al., 2006; Sonderegger et al., 2006; Staufienbiel et al., 2006; Ambuhl et al., 2007; unpublished data).

These promising preclinical data have served to validate the concept that vaccination with self-antigens, rendered highly ordered within the context of VLPs, can overcome tolerance and neutralize the activity of target molecules in disease settings. An important consequence of this has been the initiation of clinical trials for VLP-based therapeutic vaccines targeting self-molecules, such as β -amyloid, angiotensin II (Ang II), TNF α and ghrelin.

Clinical experience with VLP vaccines

VLP vaccines are in various stages of development spanning preclinical evaluation to market. Vaccines in clinical development include those of the type where the VLP itself represents the target epitope and those where the VLP is used to present a non-VLP derived epitope to the immune system. The majority of VLP-based vaccines tested preclinically have been designed to produce antimicrobial responses, and while many have been tested, relatively few have entered clinical testing. Of those that

have, several (typically aimed at inducing therapeutic T-cell responses), such as Malarivax, have ceased development due to insufficient immune responses.

Fortunately, the failures have been balanced by some astounding positive clinical results. These successes have served to demonstrate the great potential of VLP vaccines for inducing effective B-cell response and will be detailed below. Two VLP-based antiviral vaccines have reached the market and a number of clinical development programs using VLPs to target non-pathogen antigens are currently underway. Clinical trials with VLP-based vaccines have shown they are well tolerated and can be administered by a number of routes, including intramuscular, subcutaneous, oral or intranasal. VLP vaccines have also been demonstrated to be highly immunogenic and capable of stimulating protective immunity in a number of instances when administered with or without adjuvants.

Hepatitis B virus VLP vaccine

Hepatitis, caused by the HBV represents one of the world's most common and serious infectious diseases. Currently, approximately 350 million people are persistent carriers of the HBV and each year nearly 1 million people die from the chronic sequelae of infection, such as liver cirrhosis and hepatocarcinoma. The most widely used preventative vaccines against HBV are VLP-based vaccines. The vaccines are actually 22 nm complex irregular lipoprotein structures rather than the 'classic' highly ordered protein complexes typically thought of as VLPs. HBV vaccines are produced for use in developed countries principally by GlaxoSmithKline (GSK) and Merck and Co. and are marketed under the names of Engerix and Recombivax, respectively. Both vaccines are safe and highly efficacious and are one of the highest revenue generating vaccines in the world today. The discovery of the vaccine was prompted by observations that non-infectious particles consisting of membrane phospholipids and the major 24 kDa surface antigen HBsAg were found in the serum of infected individuals. These so-called 'Dane particles' were highly immunogenic and elicited antibodies capable of neutralizing the authentic virus. The currently marketed form of the vaccine comprises the HBsAg recombinantly expressed in yeast from which immunogenic particles composed of HBsAg and yeast phospholipids are harvested by gradient centrifugation and subsequently properly disulfide linked *in vitro* (Wampler et al., 1985). Responder rates to both vaccines exceed 90% with seroprotection being achieved for between 88% and 96% of vaccinees (Coates et al., 2001). Moreover, the protective response to these vaccines is long-lasting, with effective titers being measured more than 10 years after vaccination.

Research and development of HBV-VLP vaccines has focused on improving responder rates in immunocompromised individuals and increasing protective titers and reducing the number of required doses. This has partly been achieved by combining the vaccines with adjuvants, such as CpG-ODNs, MF59 or a combination of 3-O-desacyl-4'-monophosphoryl lipid A (MPL) and Alum.

This latter combination has the trade name Fendrix and has received marketing authorization for use in patients suffering from renal insufficiency. Expression of HBV-VLPs in transgenic plants with the aim of producing edible vaccines is also being pursued.

Human papilloma VLP vaccines

HPV is estimated to infect 24–40 million men and women in the United States. Symptomatic infection by the virus is associated with a variety of clinical disorders ranging from relatively benign wart-like lesions of the anogenital urinary tract to lethal tumors. Almost invariably, cervical squamous cell carcinomas harbor DNA from high risk HPVs. Clinical and molecular epidemiologic studies have shown that sexually transmitted HPV infections only rarely lead to malignancy, but HPV infection is the central cause in the development of nearly 100% of all cervical carcinomas; 50% are caused by a single serotype, HPV16. Worldwide, this corresponds to nearly half a million new cases of cervical cancer caused by HPV every year. Vaccination strategies targeting HPV have the potential to be a highly effective means of reducing the incidence of HPV infection and associated neoplasias and carcinomas.

The discovery that the L1 major capsid protein of papillomaviruses could self-assemble into VLPs that are structurally and antigenically similar to the original virion represented a watershed in HPV vaccine development (Kirnbauer et al., 1992). Thus, the potential to generate neutralizing antibodies against conformational epitopes of the L1 protein with the potential to prevent anogenital infection was realized. Early assessment of L1 VLP vaccines in rabbits, dogs and cows demonstrated the feasibility of a prophylactic vaccination approach when greater than 90% protection against high-dose challenge with species-specific mammalian papillomaviruses was observed (Schiller and Lowy, 2001). The exact role of various immune mechanisms in the protective efficacy of the HPV L1 VLP vaccines remains to be determined. However, it is believed that the vaccine provides protection by inducing type-specific antibodies that interfere with HPV transmission by binding to and neutralizing contaminating virus prior to entry into basal cells.

Several factors render the design of prophylactic VLP-based HPV vaccines difficult. There is no systemic infection with the virus which is manifest as lesions and warts and it evades immune surveillance by shedding to external surfaces. The etiology of HPV infection is complex with more than 75 different types of HPV of which 12–15 types have an association with cervical cancers. Within HPV types, there is considerable variation of L1 epitopes and vaccines designed for one region of the world may not be as well suited as for other locations. Variations of epitopes within HPV types also exist. Nevertheless, only few HPV types are implicated in the majority of cases of genital warts or cancer: type 16 and 18 cause approximately 70% of cervical cancer. Indeed, clinical trials with multivalent vaccines have demonstrated that protection against multiple disease-causing HPV serotypes could be achieved (Garland et al., 2007).

Both Merck and Co. and GSK have developed prophylactic HPV vaccines. Merck's vaccine, Gardasil, contains VLPs of the recombinant major capsid (L1) protein of HPV types 6, 11, 16 and 18 adjuvanted with aluminum hydroxyphosphate sulfate. The recombinant proteins forming the VLPs are produced by separate fermentation in recombinant *Saccharomyces cerevisiae*. Four major studies involving more than 20,000 women have been conducted. Garland and colleagues (2007) recently reported the results from one of these studies, a randomized, placebo-controlled, double-blind phase III study involving 5455 women between the ages of 16 and 24 years. One-half of the women received Gardasil at day 1, month 2 and month 6, while the other half received placebo. They were followed for an average of 3 years after administration of the first dose. Vaccine efficacy was 100% for the co-primary composite end points which included the incidence of genital warts, vulvar or vaginal intraepithelial neoplasia, or cancer and the incidence of cervical intraepithelial neoplasia, adenocarcinoma *in situ*, or cancer associated with HPV type 6, 11, 16 or 18. An analysis of intention to treat showed vaccination reduced the rate of vulvar or vaginal perianal lesions regardless of the causal HPV type by 34%. From all four major clinical studies, it was concluded that Gardasil is effective against cervical dysplasia and external genital lesions related to HPV types 6, 11, 16 and 18. For women not previously infected with HPV types 6, 11, 16 and 18, and who received the full course of vaccination, none vaccinated with Gardasil (8487) developed high-grade dysplastic cervical lesions due to HPV16 or -18, whereas 53 of the 8460 women on placebo had lesions. The results of three large studies reported 1 woman in the Gardasil group (7897) compared to 91 women in the placebo group (7899) had genital warts attributable to HPV type 6, 11, 16 or 18. These results contributed to Gardasil receiving market authorization for Europe, United States and numerous other countries.

Glaxo Smith Kline has developed a bivalent HPV L1 VLP vaccine Cervarix that is produced in insect cells and encompasses strains HPV16 and HPV18. The vaccine is formulated with MPL and Alum. The results of a large international phase III clinical trial (18 644 subjects) to assess the efficacy Cervarix against infection with human HPV types 16 and 18 in young women have recently been reported (Paavonen et al., 2007). Vaccine efficacy against cervical intraepithelial neoplasia grade 2+ containing HPV16/18 DNA was 90.4%. This demonstration of prophylactic efficacy prompted regulatory filing for market authorization for cervical cancer prevention. To date, licensure has only been granted in the European Union; however, the US Food and Drug Administration (FDA) is also expected to grant marketing authorization.

VLP vaccines for influenza A

Influenza is one of the main viral diseases afflicting humans. Typically 10–20% of the world population is infected during seasonal epidemics resulting in three to five million cases of severe illness and 250 000–500 000 lethal cases per year (WHO, 2003). In the United States,

influenza kills an average of 20 000–40 000 people per year, while an average of over 100 000 influenza-related hospitalizations results in an economical cost of \$12 billion per year (Poland et al., 2001).

Development of protective vaccines against influenza virus is problematic due to the high mutation rate of the viral surface proteins. Moreover, occasionally novel influenza strains containing new hemagglutinin (HA) and/or neuraminidase genes acquired from animal influenza viruses appear in the human population causing pandemics ('genetic shift'). In the last century three major pandemics occurred, the worst of which (1918–1920) caused 50–100 million deaths worldwide. The last pandemic occurred in 1968. The threat of a new influenza pandemic has dramatically increased in the last years with the currently circulating, highly pathogenic avian flu strain H5N1.

Conventional vaccination strategies have proven efficacious in preventing epidemic infections. Antigenically matched vaccines derived from the circulating strains have protected healthy adults by up to 90% from influenza illness. Conventional flu vaccines are composed of an inactivated virus, administered as whole virions, split vaccines or purified viral subunit proteins. These vaccines are targeted to primarily induce neutralizing antibodies directed against the major viral envelope protein HA, as well as the neuraminidase. Due to antigenic drift, vaccine formulations need to be evaluated on a yearly basis to match the circulating strains, and accordingly people have to be immunized annually. Additional complications of these vaccines are that they require long production times and they are produced in embryonated chicken eggs.

An alternative target protein for vaccination that is not subject to antigenic shift and drift is the highly conserved M2 protein (Kaiser, 2006). M2 is a tetrameric transmembrane protein that functions as an ion channel needed for viral uncoating in endosomes and as such is essential for productive infection. M2 is present in low copy numbers in mature virions and is readily detectable on influenza-infected cells. The 23 amino acid extracellular domain of M2 has shown remarkable conservation and has remained essentially unchanged since the first influenza strains were isolated (1918), despite numerous epidemics and two major pandemics since then. Evidence that antibodies targeting M2 protected against influenza infection was established more than a decade ago (Slepushkin et al., 1995; Treanor et al., 1990). Hence, influenza vaccines targeting M2 have the potential to circumvent the problems associated with the need to annually produce strain-matched vaccines. Additionally, M2-based vaccines may be recombinantly produced using microbial fermentation systems which address the difficulties of vaccine supply.

Recombinantly produced VLPs are an excellent antigen delivery platform for vaccines engineered to target M2. Neirynck and coworkers (1999) showed that M2-HBcAg VLPs, based on the genetic fusion of the extracellular part of M2 to the N-terminus of HBcAg protein, induced protection against lethal homologous and heterologous influenza A challenge. Using a chemical coupling strategy, we produced a more immunogenic

M2-HbcAg VLP vaccine by chemically conjugating M2 the major immunodominant region. Furthermore, we demonstrated that protection from live virus challenge is mediated exclusively by antibodies, but the anti-M2 antibodies neither bound efficiently to the free virus nor neutralized virus infection (Jegerlehner et al., 2004). Instead, antibodies bind to M2 protein expressed on the surface of virus-infected cells. The presence of natural killer (NK) cells is important for protection, whereas complement is not. This established that protection is mediated via antibody-dependent, cell-mediated cytotoxicity. An M2-HPV VLP conjugate vaccine has also been assessed. The vaccine comprises the M2 peptide covalently conjugated to the HPV 16 L1 VLPs using a hetero-bifunctional chemical cross-linker (Ionescu et al., 2006). The vaccine adjuvanted with an aluminum adjuvant demonstrated immunogenicity and dose-dependent protection from lethal influenza virus challenge.

A phase I clinical trial has been initiated with a VLP-based M2 vaccine. The vaccine, being developed by Acambis, comprises three tandem copies of the M2 peptide genetically fused to the NH₂-terminus of the HBcAg protein. The use of the M2 sequence in triplicate attempts to address the lower immunogenicity attained when the antigen is linked to the less exposed amino-terminus relative to the major immunodominant spike (discussed previously). The clinical trial is a randomized, double-blind, placebo-controlled multicenter trial involving 80 healthy subjects. The study will compare two different formulations involving different adjuvants.

Norwalk virus VLP vaccines

It is estimated that Norwalk virus (NV) accounts for more than 90% of outbreaks of non-bacterial acute gastroenteritis throughout the world with approximately 23 million cases occurring per year in the United States alone. Outbreaks are usually epidemic in nature and associated with crowding and poor sanitation. The potential for prophylactic vaccines based on NV-VLPs was realized when the 58 kDa NV capsid protein was shown to spontaneously assemble when expressed in insect cells infected with baculovirus (Jiang et al., 1992). NV-VLPs are very stable and have also been expressed in plants, such as potatoes and tomatoes. Preclinical studies demonstrated the VLPs are morphologically and antigenically similar to the native virus and induce systemic and mucosal immune responses in mice.

Several phase I clinical trials have been performed in which NV-VLPs were either orally administered to volunteers in water without adjuvant or via ingestion of transgenic potatoes. In all studies, the vaccines were well tolerated. Vaccine delivered in water induced serum IgG responses in a dose-dependent manner and 15 of the 18 subjects responded with serum IgG titers at least 4-fold higher than pre-vaccination levels. Titers could not be boosted with a second dose of vaccine (Ball et al., 1999). A dose-ranging trial of NV-VLPs administered orally demonstrated 90% of subjects had increases in serum anti-VLP IgG, but neither the rates of seroconversion nor

geometric mean titers increased with 500 nor 2000 µg doses. Only 30–40% of volunteers developed mucosal anti-VLP IgA responses. Studies to increase immunogenicity using mucosal adjuvants are planned (Tacket et al., 2003). NV-VLP vaccine administered on 2–3 occasions by ingestion of 150 g of transgenic potatoes was estimated to deliver 215–751 µg of recombinant vaccine per dose. The immune response was, however, weak with less than half of the volunteers developing specific IgG or IgA titers (Tacket et al., 2000).

VLP vaccine for nicotine dependence

It is estimated that 5 million deaths occur annually due to tobacco use which makes smoking the leading cause of preventable-death worldwide. The principal addictive component of tobacco is nicotine. Its addictive effect is caused by its action in the brain. Nicotine binds to the acetylcholine receptors of neurons in the ventral tegmental area, which induces dopamine release in the nucleus accumbens. This is the key immediate reward signal of the central nervous system (CNS). Continued stimulation of the mesolimbic reward system quickly leads to establishment of repetitive drug-seeking behavior. Successful quit rates without medicinal aid are typically as low as 10%. The reason most individuals fail in their attempts to quit smoking is that they experience withdrawal symptoms and a strong craving for nicotine. Consequently, the abstinent smoker seeks nicotine and relapse occurs. Lapse results in uptake of nicotine, reinstatement of the CNS reward signal and the positive reinforcement of the addiction and return to regular smoking.

Current therapies for treating nicotine addiction targeting craving and withdrawal include nicotine replacement therapies (e.g., nicotine patches, gum and inhalers), Bupropion (an antidepressant inhibitor of norepinephrine and dopamine reuptake and nicotinic receptor antagonist) and Varenicline (a partial agonist of the nicotinic α4β2 AChR subtype). These therapies help smokers quit, but cessation rates do not exceed 23% for any of the above treatments.

Active vaccination with the goal of inducing anti-nicotine antibodies is an alternative therapy, one which acts by preventing reinstatement of the positive reinforcement resulting from relapse. The mechanism for this is relatively simple: anti-nicotine antibodies sequester nicotine in the blood, the nicotine-antibody complex is too large to cross the blood-brain barrier, and consequently the flux of nicotine to the brain is altered. By altering the pharmacokinetics, the reward obtained from drug intake is decreased, the pleasure from reinitiating smoking is removed and the positive reinforcement cycle disrupted.

Nicotine is a small organic molecule, a typical hapten and as such is not immunogenic: induction of antibodies necessitates that nicotine be linked to a larger protein carrier molecule. This conjugation promotes clustering of the B-cell receptor and can provide the cognate T-cell help required for class switching from IgM to IgG. The feasibility of using conjugate vaccines against substances of abuse was first established by Bonese and

colleagues (1974), who showed that vaccination of monkeys with a morphine-bovine serum albumin (BSA) conjugate reduced heroin self-administration. Numerous nicotine-conjugate vaccines have been developed in the last decade and preclinical proof of concept established in a variety of animal models. Rodents immunized against nicotine and subsequently administered an intravenous nicotine bolus exhibited strongly reduced nicotine levels in the brain compared with control animals (Hieda et al., 1997; Maurer et al., 2005). Immunization of rats has also suppressed nicotine-induced dopamine release in the brain, prevented reinstatement of nicotine-seeking behavior and attenuated the peripheral effects of nicotine on locomotor activity and blood pressure (Pentel et al., 2000; Malin et al., 2001; de Villiers et al., 2002; Lindblom et al., 2002). However, many of the above studies were performed with complete Freund's adjuvant (CFA); an adjuvant not permitted for human use due to its unacceptable tolerability and side effects. Hence, the challenge for developing anti-nicotine conjugate vaccines for human use has been to induce high anti-nicotine antibody responses using acceptable formulations. A difficult task since vaccine efficacy seems to correlate with the antibody response.

The excellent B-cell responses generated against antigens conjugated to VLPs has been utilized to overcome this difficulty. A nicotine-VLP vaccine has been produced by covalently coupling nicotine to the surface of recombinantly produced VLPs derived from the bacteriophage Q β via a succinate linker. The resultant vaccine, containing approximately 580 nicotine molecules per VLP particle, was used to establish safety and immunogenicity in a phase I clinical trial in non-smokers (Maurer et al., 2005). Subsequently, a multicenter, double-blind, placebo-controlled phase II smoking cessation study was conducted with moderate to heavy smokers. Subjects were immunized five times with 100 μ g NicQ β formulated with the human compatible adjuvant Alhydrogel or placebo at monthly intervals. The vaccine was safe and generally well tolerated. As with the phase I study, a 100% responder rate was achieved. The target quit date was 1 month after the first immunization. Abstinence from smoking was assessed by self-reported smoking status and by measuring CO in exhaled air. In the intention-to-treat analysis, which comprised 340 subjects, continuous abstinence between month 2 and month 6 was higher in smokers, which were randomized to the active arm, than for placebo-treated subjects (28% versus 21%), but the difference did not reach significance. However, when smokers in the per protocol population were stratified according to their anti-nicotine antibody titers (equally divided into three groups according to their titers), a robust significant effect was observed for the highest tertile. In this group, 57% were continuously abstinent compared to 31% of subjects who received placebo, this effect was highly significant ($p=0.004$). The increased abstinence persisted for at least 12 months by which time 42% of the subjects with high antibody titers had not relapsed compared to 21% in the placebo group (Maurer and Bachmann, 2006). Novartis Pharmaceuticals has recently acquired a manufacturing and commercial-

ization license for this compound and is continuing its development.

VLP vaccine for hypertension

Hypertension is the most important risk factor for coronary heart disease and stroke, the first and third leading cause of death in North America. It is also a principle risk factor for congestive heart failure, end-stage renal disease and peripheral vascular disease. Approximately one billion adults worldwide suffer from elevated blood pressure. The highest prevalence is in the United States and Eastern Europe where approximately 30% of adults are hypertensive (Kearney et al., 2004). While primary arterial hypertension is a multifactorial disease (Crook, 2002), the renin-angiotensin-aldosterone system (RAAS) is probably the single most important regulator of systemic blood pressure. Binding of the 8 amino acid peptide, Ang II, to its receptor, the angiotensin receptor subtype 1 (AT1R), leads to vasoconstriction and aldosterone secretion, which contribute to its pressor effect. Ang II is generated in two steps, first through cleavage of the precursor protein angiotensinogen (Ang-N) by the enzyme renin, yielding angiotensin I (Ang I), which is in turn cleaved to Ang II by angiotensin converting enzyme (ACE). Pharmacological treatment of hypertension with small molecule inhibitors of ACE and AT1R blockers (ARBs) has proven very successful. Nevertheless, only approximately one-third of hypertensive patients in the United States are presumed to have adequate control of blood pressure, i.e., blood pressure maintained below 140/90 mm Hg (Elliott, 2003). In addition to inadequate treatment, low compliance to daily application of medication and adherence to prescribed treatment accounts for a high percentage of patients with arterial hypertension (Burnier, 2000). Hence, improving patient compliance by appropriate therapy is a primary goal of newly developed antihypertensives.

Immunotherapy represents an alternative approach to small-molecule pharmaceuticals with the likelihood of vastly improved patient compliance profiles. Active immunization, targeting the peptides derived from Ang-N, has been identified as an alternative means of down-regulating blood pressure via the RAAS. The binding of these peptides by specific antibodies would serve ultimately to prevent the binding of Ang II to its receptor thereby reducing blood pressure. In addition to improved compliance, other potential advantages, such as lower side effect profiles, slow and progressive onset of action, improved diurnal control and low probability of undesirable interactions with other drugs and lower costs could be expected.

Initial vaccination strategies targeting components of the RAAS proved either unsafe or unsuccessful (Michel, 2004). By way of example, an Ang I-keyhole limpet hemocyanin-conjugate vaccine formulated in Alhydrogel reduced the pressor effects of exogenous Ang I in rats, but failed in clinical trials to reduce blood pressure in patients with essential hypertension (Brown et al., 2004).

It was concluded that higher antibody titers were required.

This limitation has been overcome by utilizing the high immunogenicity provided by repetitive VLP-conjugate vaccines. A VLP-based anti-Ang II vaccine known as AngQb has been shown to reduce blood pressure in pre-clinical models of disease and in humans in a phase IIa clinical trial (see below). AngQb is composed of a modified Ang II peptide covalently conjugated at high density (2–3 copies per subunit) via its N-terminus, to recombinant VLPs derived from the coat protein of the bacteriophage Q β . AngQb was designed to induce antibodies with specificity for the carboxy-terminus of Ang II and thus bind Ang II rather than Ang I or Ang-N.

Preclinical efficacy for AngQb was established using spontaneous hypertensive rats surgically implanted with telemetric devices to enable the continuous monitoring of blood pressure (Ambuhl et al., 2007). The study was conducted for 11 weeks during which time the group mean systolic blood pressure of animals treated with AngQb was reduced by up to 15 mm Hg lower in the treated animals compared to control animals immunized with Q β VLP alone ($p < 0.001$). This significant and consistent reduction in blood pressure occurred between days 57 and 79 and coincided with the development of high Ang-specific antibody titers. The high affinities (3–11 nM) and concentration (300 nM) of antibodies raised by AngQb were calculated to be sufficient to bind 96–99% of systemic Ang II. Furthermore, the finding that levels of both renin and total Ang II (predominantly antibody bound) increased provided support for a mechanism of action based on sequestration of Ang II by antibodies induced by the vaccine which would lead to a reactive rise in renin.

A phase I trial conducted in healthy volunteers receiving a single subcutaneous dose of AngQb formulated with Alhydrogel demonstrated a strong antibody response with a 100% response rate; the first time a VLP-conjugate vaccine was reported to break B-cell unresponsiveness in humans (Ambuhl et al., 2007). Most importantly, AngQb was safe and well tolerated and the anti-Ang II antibody titers declined with time, a key safety parameter for immunotherapeutic vaccines inducing auto-antibodies.

A multicenter, double-blind, randomized, placebo-controlled Phase IIa trial was subsequently conducted in 72 mild to moderate hypertensive patients (Tissot et al., 2008). Subjects received three subcutaneous injections of placebo, 100 μ g or 300 μ g CYT006-AngQb combined with Alhydrogel at weeks 0, 4 and 12. Twenty-four hour ambulatory blood pressure and plasma renin were measured before treatment and at week 14. Analyses of the study showed that AngQb was again safe and well tolerated; the majority of side effects were mild, transient injection site reactions. The anti-Ang II antibody response was reversible after repeated doses of the vaccine with a half-life of approximately 4 months. Importantly, at week 14 a significant reduction, from baseline, in mean ambulatory day-time systolic/diastolic blood pressure of -9.0/-4.0 mm Hg compared to placebo ($p = 0.015$ and $p = 0.064$, respectively) was observed. Furthermore, plasma renin was increased from baseline by 24% ($p = 0.02$).

Interestingly, the early morning blood pressure surge, which occurs when the RAAS is stimulated, was reduced between 05:00 and 08:00 h compared to placebo ($p = 0.012$ and $p = 0.035$, respectively) in the 300 μ g group, with a change at 08:00 h of -25/-13 mm Hg ($p < 0.0001$ and $p = 0.0035$, respectively).

The potency of VLP vaccines has permitted clinical proof of concept to be achieved and provided a promising new and advantageous approach for the treatment of hypertension. Further clinical development of this vaccine is currently being undertaken.

VLP vaccines for Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by the presence of cerebral β -amyloid plaques, neurofibrillary tangles and neuronal loss. Until recently, therapeutic options for treatment of AD principally focused on modifying the systems involved with neurological transmission, such as the cholinergic system. These symptomatic treatments have little or no effect on the underlying disease process. A hallmark of AD is cerebral β -amyloid plaques composed of various forms of the A β peptide (39–43 amino acid) derived from extracellular region of the transmembrane amyloid precursor protein (APP). A β peptide is toxic to neurons and thought to play a central role in AD pathogenesis. Consequently, several potential disease modifying strategies aimed at reducing the amyloid burden are under investigation.

Preclinical proof of concept for an immunotherapeutic approach involving active vaccination was obtained using PDAPP transgenic mice immunized with aggregated human A β_{1-42} in combination with CFA, IFA and phosphate-buffered saline (PBS) (Schenk et al., 1999). These seminal experiments showed that immunization of young PDAPP mice prevented development of β -amyloid plaque, neuritic dystrophy and astrogliosis and prevented behavioral defects. Unfortunately, a subsequent phase IIa clinical trial using fibrillar A β_{1-42} in combination with the adjuvant QS21 (AN-1792) was stopped due to the development of 18 cases of meningoencephalitis amongst the 300 patients on active treatment (Gilman et al., 2005). This inflammatory response in humans was most likely the result of a T-cell mediated event rather than a humoral one (Gelinas et al., 2004; Gilman et al., 2005). T-cell epitopes have been mapped to the middle and carboxy terminal regions of the A β peptide. This notion was supported by autopsy studies from two affected patients, which demonstrated a T-cell mediated autoimmune response presumably directed against A β .

Despite the safety concerns, this study provided an indication that active immunization targeting A β may provide clinical benefit to patients suffering from AD. Almost 20% of the treated subjects were judged to be antibody responders. While no significant improvement in the cognitive and functional measure tests used as the primary endpoints were reported, a nine component neurophysiological test battery z-score analysis showed less worsening of performance in antibody responders, and cerebrospinal fluid (CSF) Tau was decreased. Additionally,

several post-mortem case reports from patients immunized with AN-1792 showed evidence of plaque removal suggestive of the fact that the immune response generated against the peptide elicited clearance of A β plaques in these patients (Nicoll et al., 2003; Masliah et al., 2005).

Hence, impetus and direction has been provided to a second generation of vaccines which incorporate improved design elements aimed at inducing high-titer anti-A β antibodies in the absence of inflammatory auto reactive T-cell responses. VLPs represent an ideal platform for implementing these requirements. Several VLP-based vaccines have to date been tested in preclinical settings. To avoid A β -specific T-cell responses vaccines have been designed to exclude regions of the A β peptide harboring known T-cell epitopes. Typically, the target antigen is a short peptide containing sequences from the NH₂ terminus which incorporate the B-cell epitope which elicits antibodies that can bind free and/or plaque bound A β peptide.

Zamora and colleagues (2006) genetically fused A β ₁₋₉ into the hyper-variable region of the L1 major capsid protein of bovine Papillomavirus type 1 (A β -VLP). Vaccination of mice and rabbits, the latter in the presence of CFA and IFA, induced high titer antibody responses against A β that prevented *in vitro* assembly of A β ₁₋₄₂ into neurotoxic fibrils. APP/presenilin TG mice immunized with A β -VLP showed reduced A β deposits in the brain. Conjugate VLP vaccines comprising short A β peptide covalently coupled to VLPs derived from either human papillomavirus or Q β bacteriophage-elicited anti-A β antibody responses at low doses and without the use of adjuvants and induced weak or negligible T-cell responses against A β (Chackerian et al., 2006).

The most comprehensive development of a VLP-based vaccine for treating AD has been undertaken by Novartis Pharmaceuticals using technology licensed from Cytos Biotechnology AG. Staufenbiel and coworkers (2006) tested a VLP conjugate vaccine comprising A β ₁₋₆ covalently linked to VLPs derived from Q β (CAD106). The B-cell epitope is too short to contain any T-cell epitopes and is thus predicted not to induce T-cell responses. In support of this, immunization of different mouse strains with CAD106 did not induce A β responsive T-cells even in the presence of additional adjuvants. CAD106 generated high antibody responses in mice, rabbits and monkeys without the requirement for added adjuvant. Responder rates of 100% were reported for young and old mice. Anti-A β antibodies induced by CAD106 stained amyloid deposits in human and APP TG mouse brain sections and protected from oligomeric and fibrillar toxicity *in vitro*. Immunization with CAD106 prevented amyloid cortical and vascular plaque deposition in both APP23 and APP24 TG mouse models. Immunization of aged mice with advance amyloid pathology in the brain resulted in reduction of further amyloid deposition. Importantly, no increase in incidence of micro hemorrhages was observed. Novartis is currently performing a multicenter, randomized, double-blind, placebo-controlled, time-lagged, parallel group study in patients with mild to moderate AD to investigate the safety, tolerability and A β -specific antibody response following three subcutaneous injections of CAD106.

Conclusion

The development of VLPs as effective recombinant vaccine platforms has seemingly come of age. Their potent immunogenicity is a result of their ability to incorporate key immunogenic properties of viruses into a single entity. VLPs can certainly be seen as a notable achievement in the quest for the rationale design of recombinant vaccines. The recognition of their potential has spurred intensive development activities during the previous decade. The result of which has seen the marketing of two HPV-VLP vaccines and clinical testing of a dozen other VLP-based vaccines. VLP technology has also helped realize the promise of immunotherapeutic vaccination. While the induction of strong T-cell responses remains a challenge in the process of being met, clinical proof of concept for therapeutic B-cell vaccines aimed at treating chronic diseases, such as hypertension and nicotine addiction, has been attained.

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