

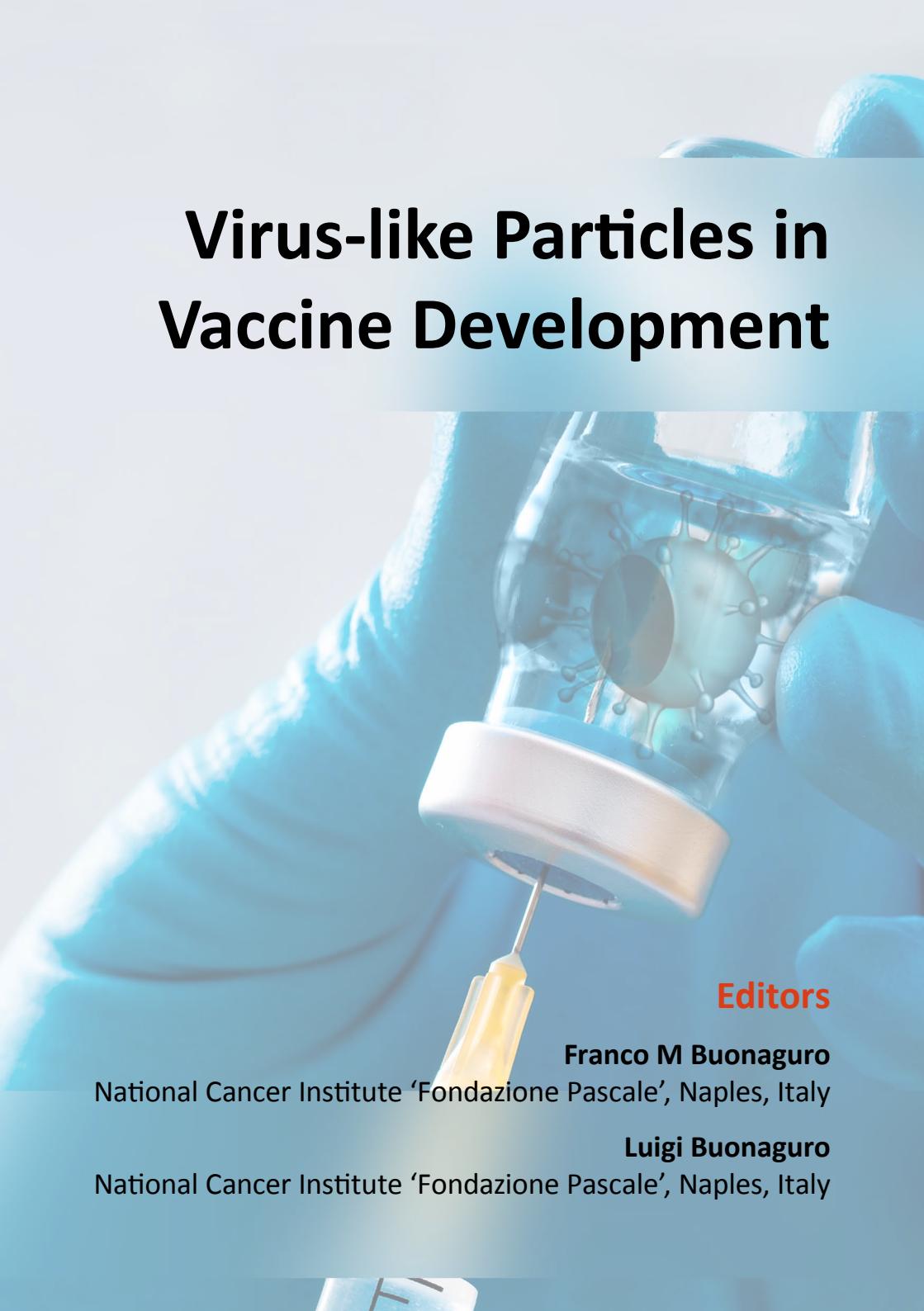
Virus-like Particles in Vaccine Development



Future
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Franco M Buonaguro
& Luigi Buonaguro

Virus-like Particles in Vaccine Development



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Foreword

Virus-like particles in vaccine development

Franco M Buonaguro & Luigi Buonaguro

Most of the successful vaccines against infectious diseases are based on live-attenuated or inactivated pathogens that have, however, shown some adverse effects, raising concerns regarding their safety. In order to address such concerns, several alternative vaccine strategies have been and are constantly developed aiming at increasing the safety without significant loss of immunogenicity.

Among such a broad array of strategies, virus-like particles (VLPs) offer several valuable features and represent a very appealing model. After the first demonstration of the polyomavirus major capsid protein, VP1, self-assembling into VLPs in the absence of viral genetic material [1], VLPs have been produced from a broad spectrum of enveloped and nonenveloped viruses. Several VLP-based vaccine candidates are in clinical trials or undergoing preclinical evaluation, while many others are still in the early stages of development. Manufacturing complexity as well as regulatory concerns have delayed the approval for marketing of VLP-based vaccines.

More recently, however, prophylactic vaccines based on VLP technology have been approved and are commercialized – that is, by GlaxoSmithKline's (NC, USA) Enerix® (hepatitis B virus) and Cervarix® (human papillomavirus) and Merck and Co.'s (NJ, USA) Recombivax HB® (hepatitis B virus) and Gardasil® (human papillomavirus). This has dramatically boosted the overall VLP-based vaccine field, which is currently considered to be the best

alternative vaccine approach for specific viral infections, which may require a large and timely manufacturing readiness (i.e., influenza virus). In this framework, this book contains a series of timely in-depth chapters written by leaders in the field covering a range of current topics in VLP-based vaccine development and clinical assessment for several human infectious diseases.

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Reference

- 1 Brady JN, Consigli RA. Chromatographic separation of the polyoma virus proteins and renaturation of the isolated VP1 major capsid protein. *J. Virol.* 27(2), 436–442 (1978).

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Chapter

1

Developments in virus-like particle-based vaccines for HIV

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Luigi Buonaguro, Maria Tagliamonte, Maria Luisa Visciano, Maria Lina Tornesello & Franco M Buonaguro

Virus-like particles (VLPs) hold great promise for the development of effective and affordable vaccines. VLPs, indeed, are suitable for presentation and efficient delivery to antigen-presenting cells of linear as well as conformational antigens. This will ultimately result in a cross-presentation with both MHC class I and II molecules to prime CD4⁺ T-helper cells as well as CD8⁺ cytotoxic T cells. This chapter provides an update on the development and use of VLPs as vaccine approaches for HIV.

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The development of an effective, safe, and affordable HIV/AIDS vaccine remains a crucial goal for both industrialized and developing countries. Combined antiretroviral therapy (ART) has proven to be extremely effective in reducing both HIV-related morbidity and mortality worldwide [1]; however, it fails to eradicate the infection and might select for resistant viral populations, which represents a severe limitation in the overall efficacy of drug treatment [2]. Moreover, long-term adverse effects are frequently observed in ART-treated HIV-infected individuals, which may conceivably become even more pronounced with the increasing age of HIV-infected populations [3]. Furthermore, in spite of an increasing number of HIV-infected individuals in low- and middle-income countries now having access to ART, highly skilled clinical and laboratory settings still need to be implemented for the best use of ART, in terms of long-term outcomes and cost-effectiveness [4].

In this context, the development of a safe and effective HIV-1 vaccine remains the best solution for the ultimate control of the worldwide AIDS pandemic. However, HIV-1 vaccine development efforts have not yet proven successful, given that the unique characteristics of HIV and its infection represent unprecedented challenges [5].

The role of the two arms of the adaptive immune system in the containment of HIV infection is still disputed, with proof supporting both the humoral [6] and/or the cellular [7] immune response. It is, therefore, a common belief that an optimal anti-HIV vaccine needs to engage both arms of the adaptive immunity to prevent infection and contain disease progression upon infection [8].

Since 1987, the year of the first Phase I trial of an HIV vaccine [9], more than 30 candidate vaccines have been tested, alone or in combination, in over 230 Phase I/II/III clinical trials in both industrialized and developing countries (**Table 1.1**) [10]. Throughout the years, in the quest of an effective protective HIV vaccine, several immunization strategies have been developed, which may be grouped in three ‘waves’. The first ‘wave’ of candidate vaccines aimed at inducing neutralizing antibodies, typically using recombinant soluble proteins. The second ‘wave’ focused on stimulation of CD8⁺ T-cell responses, typically using viral vectors. The current ‘wave’ of HIV vaccine research is aimed at optimizing both humoral and cell-mediated immune responses by heterologous prime-boost strategies [10]. All of these different vaccine strategies developed and tested along the years show individual features of efficacy and safety. Only very few concepts have reached the Phase IIb/III stage, but none has provided satisfactory outcome [11].

Table 1.1. List of completed or ongoing clinical trials for HIV vaccine.

Strategy	Trials (n)	Phase
Bacterium plus glycoprotein 120	1	I
DNA	67	I-IIb
Protein	87	I-III
Viral vector (adeno)	35	I-IIb
Viral vector (adeno-associated virus)	2	I-II
Viral vector (alphavirus)	2	I
Viral vector (pox)	87	I-III
Viral vector (replicating)	1	I
Virus-like particles	1	I

The sum largely exceeds the total number of conducted trials, because the same vaccine strategy may have been used also in prime-boost combinations with other strategies.

Data taken from [101].

This chapter aims to describe the characteristics of **HIV virus-like particles (VLPs)** and preclinical as well as clinical results obtained with such a HIV vaccine approach.

Virus-like particles

In the quest of novel, effective HIV vaccine strategies, VLPs offer several valuable features. VLPs are made of viral capsid proteins which self-assemble into particulate structures closely resembling the natural virus from which they are derived. VLPs are replication- as well as infection-incompetent, and can be employed as delivery systems for antigenic structures as well as DNA molecules [12].

VLPs have been produced from enveloped and nonenveloped viruses [12]. The latter can be generated with single or multiple capsid proteins without a surrounding cell membrane. Examples of such VLPs are those derived from the expression of the major capsid protein of papillomaviruses, parvoviruses, polyomaviruses and RNA bacteriophages [13–16] or multiple interacting capsid proteins of the Reoviridae family [17].

Alternatively, enveloped VLPs are further surrounded by a cell membrane and have been generated for hepatitis B and C virus, influenza A and retroviruses, including HIV-1 [18–22]. The cell membrane coating enveloped VLPs makes possible the presentation of full-length monomeric or multimeric



Virus-like particles (VLPs): molecular structures formed by the self-assembly of capsid proteins from many viruses. VLPs do not contain infectious genetic material.

HIV-VLPs: enveloped VLPs made of the protein Gag of HIV.

conformational proteins anchored on the particle's surface through transmembrane domains [21,23].

Immunogenicity of VLPs

VLPs mimic the native viruses and display an array of pathogen-associated molecular patterns to the innate immunity, leading to subsequent activation of adaptive and inflammatory responses [24].

Most of the immunogenic efficacy of VLPs is related to their morphological properties (size and shape), resulting in efficient uptake by the antigen-presenting cells (APCs), particularly dendritic cells (DCs), which undergo subsequent maturation and migration to lymph nodes for induction of the adaptive T-cell response [25,26]. Moreover, unlike other exogenous antigens, which cannot reach the MHC class I pathway [27,28], antigens delivered by VLPs are cross-presented in association with both MHC class I and class II in the absence of infection or intracellular replication [29,30], eliciting effective CD4⁺ T-helper and CD8⁺ cytotoxic T-lymphocyte responses [31–33].

VLPs are generally more immunogenic than subunit or recombinant protein immunogens, stimulating the humoral as well as cellular immunity. Indeed, as the native virus, VLPs display organized arrays of conformational antigens that activate B-cell receptors and T-cell-independent IgM responses [34]. This property gives a significant advantage compared with soluble antigens delivered in a monomeric status.

In this context, HIV-1 precursor of p55 Gag protein (Pr55^{Gag}) VLPs have been shown by our group to induce maturation and activation of myeloid-derived DCs or CD14⁺ uncultured peripheral blood mononuclear cells, with a transcriptional profile characterized by activation of genes with functions associated with antigen presentation and myeloid-derived DC migration [25,26,35,36].

However, VLPs may induce poor T-cell responses when administered alone, lacking additional stimuli (e.g., type 1 interferons and proinflammatory cytokines) to the innate immune system and in particular to APCs [37]. In this regard, coadministration of VLPs with stimuli of the innate immune system will result in strong cytotoxic T lymphocyte responses [38]. Alternatively, VLPs have been developed to co-deliver Toll-like receptor ligands and T-cell epitopes to DCs, and numerous ligands for Toll-like receptors have been tested [39,40]. DNA containing CpG motifs, a ligand for Toll-like receptor 9, were by far the most potent adjuvants in inducing robust T-cell responses [39,41]. Packaging the potentiators of innate immunity within VLPs, besides improving the immune response, protects them from degradation and improves their pharmacokinetic profile,

reducing nonspecific binding to serum proteins. Furthermore, delivery of these molecules to target APCs limits systemic distribution and reduces side effects, such as splenomegaly [39] and autoimmune disorders [42].

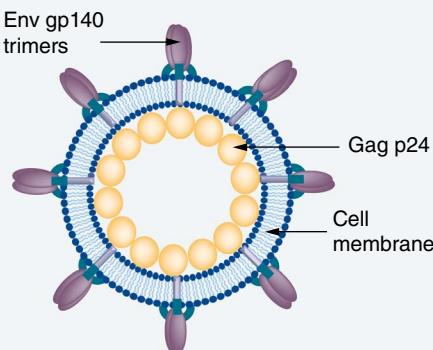
VLP-based vaccine for HIV

HIV-1 or simian immunodeficiency virus (SIV) Gag polyprotein precursors have been shown to assemble in 100–120-nm VLPs [21]. The HIV-1 Pr55^{gag}-based VLP model has been developed to deliver epitopes or whole proteins (i.e., HIV envelope protein), with induction of both arms of the immune response. HIV-VLPs have been produced in different expression systems [43,44]; however, the baculovirus-based transient protein expression vector system is the most widely used for several reasons. The most important reasons are the large production of correctly folded recombinant proteins and the narrow host range of the baculovirus, with limited or absent threat to vaccinated individuals [45].

Several alternative approaches have been described to develop HIV-VLPs expressing HIV epitopes relevant for induction of humoral and cellular immunity. In particular, a Gag reverse transcriptase fusion VLP has been shown to effectively boost CD8⁺ T-cell-specific responses [46]. HIV-1 envelope V3 epitopes have been swapped into the Pr55^{gag} sequence [47] or fused to the 3' end of the Gag open reading frame [48], resulting in the induction of a modest immune response against the HIV-1 epitopes [48]. The entire glycoprotein (gp)120 molecule has been expressed on the Pr55^{gag}-VLPs as conformational antigen in oligomeric status [21] and, more recently, trimeric forms of whole HIV-1 envelopes have been expressed on the surface of Pr55^{gag}-VLPs [23,49]. All of these approaches induce relevant envelope (Env)-specific humoral and cellular immune responses (**Figure 1.1**).

HIV-VLPs developed in our laboratory are based on a Gag protein from a HIV-1 B subtype and an Env protein from a HIV-1 A subtype [21], and have been shown to elicit HIV-1-specific CD4⁺ and CD8⁺ T-cell responses, as well as cross-clade neutralizing antibodies in immunized BALB/c mice [31]. Moreover, systemic and mucosal immune responses have been elicited by intraperitoneal and

Figure 1.1. Enveloped Gag HIV virus-like particles.



Schematic representation of the Gag HIV virus-like particles surrounded by membrane, acquired during the budding from expressing cells, from which are protruding Env gp140 trimers.
Env: Envelope; gp140: Glycoprotein 140.

intranasal administration of the HIV-1 A subtype in mice, with or without adjuvants [32,33].

Several preclinical studies in different animal models have been performed with HIV Pr55^{gag}-based VLP expressing either specific HIV-relevant epitopes (i.e., V3) [47,50] or the whole HIV glycoprotein [31,32,51]. All of the studies showed the induction of significant antibody responses to the HIV antigens displayed by the HIV-VLPs with a broad range of neutralization activity in *ex vivo* assays; only a few of them also evaluated cellular immunity [31,33]. Moreover, only a few studies have been performed to assess the induction of the systemic and mucosal immune responses upon mucosal (i.e., intranasal) administration [32,33]. In particular, we have recently shown in nonhuman primates that HIV-VLPs administered by intranasal route do not elicit a measurable immune response but are able to prime the response at the mucosal level after subsequent intramuscular administration [52].

Chimeric enveloped VLPs have been developed using the Gag protein of SIV to present mutated Env glycoproteins of HIV, which have been shown to elicit in mice immune sera with high neutralizing activity [53]. Overall, such data indicate that presentation of HIV-1 envelopes on lentivirus VLPs are an effective strategy for induction of cellular and humoral immune responses. In particular, the trimeric conformation of envelope glycoproteins on the VLPs' surface may provide the appropriate structure in order to elicit broadly neutralizing antibodies, which have been shown to target epitopes preferentially expressed on trimeric envelope protein and spanning conserved regions of the variable loops of the gp120 subunit [54].

Despite such encouraging results, none of these lentivirus VLPs have yet reached human clinical trials, mainly owing to technical and regulatory obstacles. However, recent advancements may open new avenues for the implementation of lentivirus VLPs in human clinical practice. Along this path, we have recently described the establishment of a cell line constitutively producing enveloped Pr55^{gag}-VLPs alone [55] or displaying trimeric HIV gp140 on their surface [49]. This approach shows enhanced expression of membrane-bound glycoproteins and eliminates the potential 'contamination' by copurified baculovirus particles in the baculovirus-based transient protein expression vector system, circumventing potential concerns for regulatory agencies.

In addition, a novel purification approach for VLPs has been developed by Novavax Inc. (MD, USA) to manufacture influenza VLPs, which have been tested in a Phase I/II human clinical trial, showing safety and induction of neutralizing antibodies directed against oligomeric forms of

hemagglutinin [56]. This approach will probably boost the production of HIV-VLPs accepted for human clinical trials.

DNA & live viral vectors for *in vivo* production of HIV-VLPs

HIV-VLPs generated as described in the previous section, as for all other virus-related VLPs, are the result of a controlled production process leading to a fully characterized multiprotein conformational vaccine with the described immunogenic properties (i.e., strong induction of humoral as well as cellular immunity). Alternative strategies of delivering VLPs have been described, using DNA or live viral vectors (i.e., modified vaccinia Ankara [MVA]) carrying several HIV structural genes which, when delivered in cell cultures, show induction of VLP production [57]. However, the same VLP production *in vivo* upon DNA or MVA delivery has not been definitively proven. Moreover, the VLPs possibly produced *in vivo* upon DNA or MVA delivery would have a completely different effect on the immune system compared with standard VLPs administered as multiprotein vaccine (i.e., DNA vs protein immunization).

Nevertheless, the combination of DNA and MVA, as preventive vaccine, has shown elicitation of strong T-cell as well as antibody responses in nonhuman primates, with control of viremia upon live virus challenge [58]. Alternatively, a plasmid DNA expressing several copies of HIV structural and regulatory genes has been tested as a therapeutic approach in SIV251-infected macaques, showing suppression of viremia [59]. Both concepts have been moved to Phase I human clinical trial. The DNA–MVA combination has shown safety in HIV-seronegative vaccinees and induction of CD4⁺ and CD8⁺ responses as well as Env-specific antibodies with neutralizing activity [60]. The plasmid DNA has been tested in the DermaVir (Genetic Immunity, VA, USA; Budapest, Hungary) formulation, showing safety in HIV-positive subjects, with very mild and transient side effects, and a boosting of the specific HIV response characterized by CD4⁺ and CD8⁺ T cells expressing IFN-γ and IL-2 [61].

Beyond a VLP-based vaccine for HIV

Although VLPs represent a highly attractive vaccine strategy for HIV, gp140 antigen presentation on enveloped VLPs reproduces the low-density distribution observed on the authentic virions.

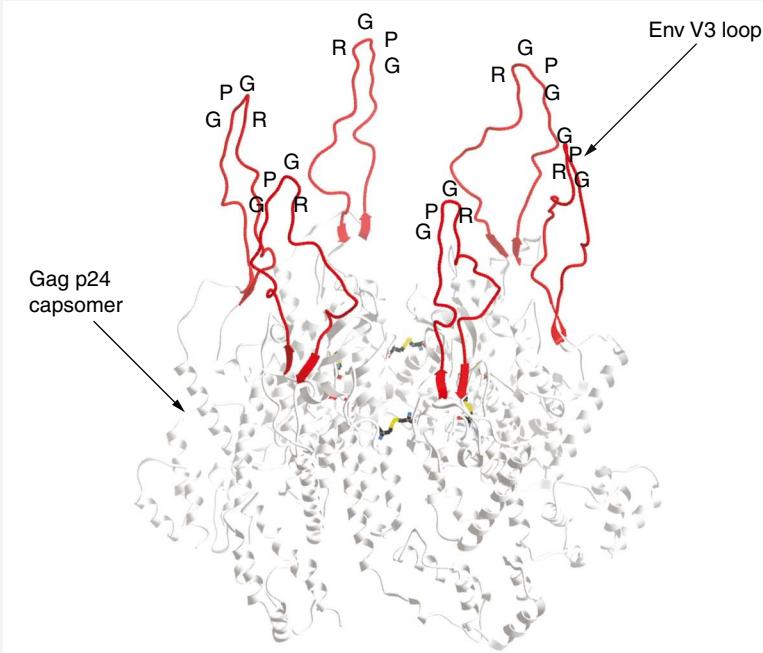
Nonenveloped capsomer vaccines based on assembled chimeric HIV p24 Gag core protein can overcome such a limitation. In this context, we have recently shown that the HIV p24 capsid protein is a highly attractive molecule to be used as particulate protein scaffold for presenting dense repetitive arrays of minimal structural and antigenic HIV Env epitopes aiming at eliciting broadly neutralizing antibodies. Biocomputational analysis has shown, as proof of

concept, that the full Env V3 loop can be grafted on the HIV p24 protein, preserving both the scaffold hexamer structures and the V3 epitope antibody-bound conformation (**Figure 1.2**) [62,63]. Moreover, the conformational V3 loop presented on p24 scaffold is recognized by a panel of anti-V3 monoclonal antibodies [64], and *in vivo* immunogenicity studies are currently ongoing to verify the elicitation of broadly neutralizing antibodies.

Conclusion

VLPs represent a powerful tool for vaccine development, including for HIV, representing the closest strategy to the native viruses for displaying and delivering conformational epitopes, with improved induction of antibodies and immune response in its entirety. Moreover, the lack of genetic material makes VLPs the ‘safer counterpart’ of live-attenuated or killed viral vaccines which, indeed, may induce limited but possible undesired effects.

Figure 1.2. Gag p24 capsomeres as scaffold for Env epitopes.



Schematic representation of the Gag p24 proteins assembled into nonenveloped capsomeres made of six molecules (hexamers). Each of the assembled p24 proteins presents a conformational Env V3 loop for inducing neutralizing antibodies. G, P and R represent glycine, proline and arginine, respectively.

Despite several VLP-based vaccines for HIV having been tested preclinically, only one has been evaluated in a human Phase I clinical trial almost 20 years ago, based on a chimeric p17/p24:Ty VLP, not a Gag-based VLP, and displayed Gag epitopes instead of Env epitopes [65]. The difficulty in testing VLP-based HIV vaccines in human clinical trials is mainly due to technical and regulatory difficulties, which have significantly slowed the feasibility of translating the VLPs from research to development and marketing. In particular, enveloped VLPs represent a major technical challenge because the complexity of the biological system (i.e., transduction of ‘contaminant’ cellular genetic material) is more difficult to be resolved without loss of immunological effectiveness, and the optimal density of surface glycoproteins is yet to be achieved. We strongly believe that VLP production by stable transgenic cell lines, instead of transiently infected/transfected cells, will represent a valid strategy to address both aspects. Moreover, improved purification strategies for biopharmaceutical applications of enveloped VLPs (i.e., influenza VLPs) [66] would foster the possibility of producing HIV-VLPs for human clinical trials.

Alternatively, the nonenveloped capsomer vaccines based on assembled chimeric HIV p24 Gag core protein, presenting dense repetitive arrays of minimal structural and antigenic HIV Env epitopes, may represent a very efficient strategy to completely overcome all such hurdles.

Overall, HIV-VLP vaccines have shown to be safe and immunologically effective by different routes of administration with or without adjuvants. Such immunogenicity derives from the characteristic of incorporating key immunogenic properties of viruses into a single entity, and the ability to activate key cells for the initiation of effective adaptive immune response.

Future perspective

The exploitation of VLPs as a vaccine approach in humans is likely to expand significantly in the next 5 years to several virus-related diseases. To this end, the marketing of nonenveloped VLPs, produced in baculovirus expression system, as a preventive vaccine for the human papillomavirus has represented a major step forward. Similarly, the human clinical trials performed using VLPs for influenza, respiratory syncytia virus and rotavirus will provide essential information on the manufacturing optimization, safety and immunogenicity on VLP-based vaccines.

Inevitably, these advancements will foster the exploitation of VLP technology for HIV. Indeed, the unique properties of VLPs (closely mimicking the antigenic structure of the native virus, presenting conformational structures, delivering to APCs and effectively eliciting a humoral immune

response) make such a vaccination strategy a formidable tool for HIV, also in a heterologous prime–boost schedule. Obviously, the efficacy in terms of protection from acquiring HIV will depend on the identification of the appropriate immune target able to elicit antibodies with a broad neutralization activity, which represents so far an unprecedented scientific challenge.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.



Summary

- Virus-like particles (VLPs) closely mimic the native virus that they are derived from, but they are safer being replication- as well as infection-incompetent.
- They display on the surface an array of conformational antigens, which efficiently crosslink B-cell receptors to induce both T-cell-independent and T-cell-dependent B-cell responses.
- They are efficiently internalized by antigen-presenting cells and cross-presented in association with both MHC class I and class II molecules.
- HIV-VLPs have been shown in several preclinical studies to be highly efficient in inducing effective and memory humoral as well as cellular immune responses.
- A single human Phase I clinical trial has been conducted, almost 20 years ago, based on a chimeric p17/p24:Ty VLP displaying HIV Gag and not Env epitopes.
- Further technical challenges need to be overcome to produce enveloped VLPs, including HIV-VLPs, which may meet all of the production and regulatory requirements.
- Alternative strategies to produce VLPs *in vivo* have been described, although final proof of VLP production upon delivery of DNA or modified vaccinia Ankara has not yet been provided.
- New strategies, beyond HIV-VLPs, may help to foster development of vaccine molecules for human clinical trials.
- VLP technology has potential to play a relevant role in the HIV vaccinology field of the coming years.

References

- 1 Volberding PA, Deeks SG. Antiretroviral therapy and management of HIV infection. *Lancet* 376(9734), 49–62 (2010).
- 2 Paredes R, Clotet B. Clinical management of HIV-1 resistance. *Antiviral Res.* 85(1), 245–265 (2010).
- 3 Maagaard A, Kvale D. Long term adverse effects related to nucleoside reverse transcriptase inhibitors: clinical impact of mitochondrial toxicity. *Scand. J. Infect. Dis.* 41(11–12), 808–817 (2009).
- 4 Bartlett JA, Shao JF. Successes, challenges, and limitations of current antiretroviral therapy in low-income and middle-income countries. *Lancet Infect. Dis.* 9(10), 637–649 (2009).
- 5 Picker LJ, Hansen SG, Lifson JD. New paradigms for HIV/AIDS vaccine development. *Annu. Rev. Med.* 63, 95–111 (2012).
- 6 Mascola J, Lewis MG, Stiegler G et al. Protection of macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. *J. Virol.* 73(5), 4009–4018 (1999).
- 7 Betts MR, Nason MC, West SM et al. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8⁺ T cells. *Blood* 107(12), 4781–4789 (2006).
- 8 Fauci AS, Johnston MI, Dieffenbach CW et al. HIV vaccine research: the way forward. *Science* 321(5888), 530–532 (2008).
- 9 Ezzell C. Trials of vaccine against AIDS to begin in humans. *Nature* 328(6133), 747–(1987).
- 10 Lu S. Heterologous prime-boost vaccination. *Curr. Opin. Immunol.* 21(3), 346–351 (2009).
- 11 Ross AL, Brave A, Scarlatti G, Manrique A, Buonaguro L. Progress towards development of an HIV vaccine: report of the AIDS Vaccine 2009 Conference. *Lancet Infect. Dis.* 10(5), 305–316 (2010).
- 12 Buonaguro L, Tornesello ML, Buonaguro FM. Virus-like particles as particulate vaccines. *Curr. HIV Res.* 8(4), 299–399 (2010).
- 13 Kirnbauer R, Booy F, Cheng N, Lowy DR, Schiller JT. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. *Proc. Natl Acad. Sci. USA* 89(24), 12180–12184 (1992).
- 14 Brautigam S, Snezhkov E, Bishop DH. Formation of poliovirus-like particles by recombinant baculoviruses expressing the individual VPO, VP3, and VP1 proteins by comparison to particles derived from the expressed poliovirus polyprotein. *Virology* 192(2), 512–524 (1993).
- 15 Kozlovska TM, Cielens I, Dreilinna D et al. Recombinant RNA phage Q beta capsid particles synthesized and self-assembled in *Escherichia coli*. *Gene* 137(1), 133–137 (1993).
- 16 Peabody DS, Manifold-Wheeler B, Medford A, Jordan SK, do Carmo CJ, Chackerian B. Immunogenic display of diverse peptides on virus-like particles of RNA phage MS2. *J. Mol. Biol.* 380(1), 252–263 (2008).
- 17 French TJ, Roy P. Synthesis of bluetongue virus (BTV) core like particles by a recombinant baculovirus expressing the two major structural core proteins of BTV. *J. Virol.* 64(4), 1530–1536 (1990).
- 18 Baumert TF, Ito S, Wong DT, Liang TJ. Hepatitis C virus structural proteins assemble into viruslike particles in insect cells. *J. Virol.* 72(5), 3827–336 (1998).
- 19 McAleer WJ, Buynak EB, Maigetter RZ, Wampler DE, Miller WJ, Hilleman MR. Human hepatitis B vaccine from recombinant yeast. *Nature* 307(5947), 178–180 (1984).
- 20 Kang SM, Song JM, Quan FS, Compans RW. Influenza vaccines based on virus-like particles. *Virus Res.* 143(2), 140–146 (2009).
- 21 Buonaguro L, Buonaguro FM, Tornesello ML et al. High efficient production of Pr55^{gag} virus-like particles expressing multiple HIV-1 epitopes, including a gp120 protein derived from an Ugandan HIV-1 isolate of subtype A. *Antiviral Res.* 49(1), 35–47 (2001).
- 22 Gheysen D, Jacobs E, de Foresta F et al. Assembly and release of HIV-1 precursor Pr55^{gag} virus-like particles from recombinant baculovirus-infected insect cells. *Cell* 59(1), 103–112 (1989).

- 23 Crooks ET, Moore PL, Franti M et al. A comparative immunogenicity study of HIV-1 virus-like particles bearing various forms of envelope proteins, particles bearing no envelope and soluble monomeric gp120. *Virology* 366(2), 245–262 (2007).
- 24 Moron VG, Rueda P, Sedlik C, Leclerc C. *In vivo*, dendritic cells can cross-present virus-like particles using an endosome-to-cytosol pathway. *J. Immunol.* 171(5), 2242–2250 (2003).
- 25 Buonaguro L, Tornesello ML, Tagliamonte M et al. Baculovirus-derived human immunodeficiency virus type 1 virus-like particles activate dendritic cells and induce *ex vivo* T-cell responses. *J. Virol.* 80(18), 9134–9143 (2006).
- 26 Buonaguro L, Tornesello ML, Gallo RC, Marincola FM, Lewis GK, Buonaguro FM. Th2 polarization in peripheral blood mononuclear cells from human immunodeficiency virus (HIV)-infected subjects, as activated by HIV virus-like particles. *J. Virol.* 83(1), 304–313 (2009).
- 27 Bachmann MF, Kundig TM, Kalberer CP, Hengartner H, Zinkernagel RM. Formalin inactivation of vesicular stomatitis virus impairs T-cell-but not T-help-independent B-cell responses. *J. Virol.* 67(7), 3917–3922 (1993).
- 28 Braciale TJ, Morrison LA, Sweetser MT, Sambrook J, Gething MJ, Braciale VL. Antigen presentation pathways to class I and class II MHC-restricted T lymphocytes. *Immunol. Rev.* 98, 95–114 (1987).
- 29 Bachmann MF, Lutz MB, Layton GT et al. Dendritic cells process exogenous viral proteins and virus-like particles for class I presentation to CD8⁺ cytotoxic T lymphocytes. *Eur. J. Immunol.* 26(11), 2595–2600 (1996).
- 30 Ruedl C, Schwarz K, Jegerlehner A, Storni T, Manolova V, Bachmann MF. Virus-like particles as carriers for T-cell epitopes: limited inhibition of T-cell priming by carrier-specific antibodies. *J. Virol.* 79(2), 717–724 (2005).
- 31 Buonaguro L, Racioppi L, Tornesello ML et al. Induction of neutralizing antibodies and CTLs in Balb/c mice immunized with virus-like particles presenting a gp120 molecule from a HIV-1 isolate of clade A (HIV-VLPAs). *Antiviral Res.* 54(3), 189–201 (2002).
- 32 Buonaguro L, Visciano ML, Tornesello ML, Tagliamonte M, Biryahwaho B, Buonaguro FM. Induction of systemic and mucosal cross-clade neutralizing antibodies in BALB/c mice immunized with human immunodeficiency virus type 1 clade A virus-like particles administered by different routes of inoculation. *J. Virol.* 79(11), 7059–7067 (2005).
- 33 Buonaguro L, Devito C, Tornesello ML et al. DNA-VLP prime–boost intra-nasal immunization induces cellular and humoral anti-HIV-1 systemic and mucosal immunity with cross-clade neutralizing activity. *Vaccine* 25(32), 5968–5977 (2007).
- 34 Bachmann MF, Rohrer UH, Kundig TM, Burki K, Hengartner H, Zinkernagel RM. The influence of antigen organization on B cell responsiveness. *Science* 262(5138), 1448–1451 (1993).
- 35 Aricò E, Wang E, Tornesello ML et al. Immature monocyte derived dendritic cells gene expression profile in response to virus-like particles stimulation. *J. Transl. Med.* 3, 45 (2005).
- 36 Monaco A, Marincola FM, Sabatino M et al. Molecular immune signatures of HIV-1 vaccines in human PBMCS. *FEBS Lett.* 583(18), 3004–3008 (2009).
- 37 Takeuchi O, Akira S. Recognition of viruses by innate immunity. *Immunol. Rev.* 220, 214–224 (2007).
- 38 Storni T, Lechner F, Erdmann I et al. Critical role for activation of antigen-presenting cells in priming of cytotoxic T cell responses after vaccination with virus-like particles. *J. Immunol.* 168(6), 2880–2886 (2002).
- 39 Storni T, Ruedl C, Schwarz K, Schwendener RA, Renner WA, Bachmann MF. Nonmethylated CG motifs packaged into virus-like particles induce protective cytotoxic T cell responses in the absence of systemic side effects. *J. Immunol.* 172(3), 1777–1785 (2004).
- 40 Wang BZ, Quan FS, Kang SM, Bozja J, Skountzou I, Compans RW. Incorporation of membrane-anchored flagellin into influenza virus-like particles enhances the breadth of immune responses. *J. Virol.* 82(23), 11813–11823 (2008).
- 41 Schwarz K, Storni T, Manolova V et al. Role of

- Toll-like receptors in co-stimulating cytotoxic T cell responses. *Eur. J. Immunol.* 33(6), 1465–1470 (2003).
- 42 Sun S, Rao NL, Venable J, Thurmond R, Karlsson L. TLR7/9 antagonists as therapeutics for immune-mediated inflammatory disorders. *Inflamm. Allergy Drug Targets* 6(4), 223–235 (2007).
- 43 Tsunetsugu-Yokota Y, Morikawa Y, Isogai M et al. Yeast-derived human immunodeficiency virus type 1 p55^{gag} virus-like particles activate dendritic cells (DCs) and induce perforin expression in gag-specific CD8⁺ T cells by cross-presentation of DCs. *J. Virol.* 77(19), 10250–10259 (2003).
- 44 Scotti N, Alagna F, Ferraiolo E et al. High-level expression of the HIV-1 Pr55^{gag} polyprotein in transgenic tobacco chloroplasts. *Planta* 229(5), 1109–1122 (2009).
- 45 Carbonell LF, Klowden MJ, Miller LK. Baculovirus-mediated expression of bacterial genes in dipteran and mammalian cells. *J. Virol.* 56(1), 153–160 (1985).
- 46 Halsey RJ, Tanzer FL, Meyers A et al. Chimaeric HIV-1 subtype C Gag molecules with large in-frame C-terminal polypeptide fusions form virus-like particles. *Virus Res.* 133(2), 259–268 (2008).
- 47 Griffiths JC, Harris SJ, Layton GT et al. Hybrid human immunodeficiency virus Gag particles as an antigen carrier system: induction of cytotoxic T-cell and humoral responses by a Gag:V3 fusion. *J. Virol.* 67(6), 3191–3198 (1993).
- 48 Tobin GJ, Li GH, Fong SE, Nagashima K, Gonda MA. Chimeric HIV-1 virus-like particles containing gp120 epitopes as a result of a ribosomal frameshift elicit gag- and SU-specific murine cytotoxic T-lymphocyte activities. *Virology* 236(2), 307–315 (1997).
- 49 Tagliamonte M, Visciano ML, Tornesello ML, De SA, Buonaguro FM, Buonaguro L. HIV-Gag VLPs presenting trimeric HIV-1 gp140 spikes constitutively expressed in stable double transfected insect cell line. *Vaccine* 29(31), 4913–4922 (2011).
- 50 Luo L, Li Y, Cannon PM, Kim S, Kang CY. Chimeric gag-V3 virus-like particles of human immunodeficiency virus induce virus-neutralizing antibodies. *Proc. Natl Acad. Sci. USA* 89(21), 10527–10531 (1992).
- 51 Visciano ML, Diomedè L, Tagliamonte M et al. Generation of HIV-1 virus-like particles expressing different HIV-1 glycoproteins. *Vaccine* 29(31), 4903–4912 (2011).
- 52 Buonaguro L, Tagliamonte M, Visciano ML et al. Immunogenicity of HIV virus-like particles in rhesus macaques by intranasal administration. *Clin. Vaccine Immunol.* 19(6), 970–973 (2012).
- 53 Quan FS, Sailaja G, Skountzou I et al. Immunogenicity of virus-like particles containing modified human immunodeficiency virus envelope proteins. *Vaccine* 25(19), 3841–3850 (2007).
- 54 Scheid JF, Mouquet H, Feldhahn N et al. Broad diversity of neutralizing antibodies isolated from memory B cells in HIV-infected individuals. *Nature* 458(7238), 636–640 (2009).
- 55 Tagliamonte M, Visciano ML, Tornesello ML, De SA, Buonaguro FM, Buonaguro L. Constitutive expression of HIV-VLPs in stably transfected insect cell line for efficient delivery system. *Vaccine* 28(39), 6417–6424 (2010).
- 56 Khurana S, Wu J, Verma N et al. H5N1 virus-like particle vaccine elicits cross-reactive neutralizing antibodies that preferentially bind to the oligomeric form of influenza virus hemagglutinin in humans. *J. Virol.* 85(21), 10945–10954 (2011).
- 57 Somogyi E, Xu J, Gudics A et al. A plasmid DNA immunogen expressing fifteen protein antigens and complex virus-like particles (VLP⁺) mimicking naturally occurring HIV. *Vaccine* 29(4), 744–753 (2011).
- 58 Amara RR, Villinger F, Altman JD et al. Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. *Science* 29, 269–274 (2001).
- 59 Lisziewicz J, Trocchio J, Whitman L et al. DermaVir: a novel topical vaccine for HIV/AIDS. *J. Invest. Dermatol.* 124(1), 160–169 (2005).
- 60 Goepfert PA, Elizaga ML, Sato A et al. Phase 1 safety and immunogenicity testing of DNA and recombinant modified vaccinia Ankara vaccines expressing HIV-1 virus-like particles. *J. Infect. Dis.* 203(5), 610–619 (2011).
- 61 Lisziewicz J, Bakare N, Calarota SA et al. Single DermaVir immunization: dose-dependent

- expansion of precursor/memory T cells against all HIV antigens in HIV-1 infected individuals. *PLoS ONE* 7(5), e35416 (2012).
- 62 Pernillos O, Ganser-Pernillos BK, Kelly BN et al. X-ray structures of the hexameric building block of the HIV capsid. *Cell* 137(7), 1282–1292 (2009).
- 63 Buonaguro L, Tagliamonte M, Tornesello ML, Buonaguro FM. Can HIV p24 be a suitable scaffold for presenting Env antigens? *Clin. Vaccine Immunol.* 18(11), 2003–2004 (2011).
- 64 Tagliamonte M, Marasco D, Ruggiero A et al. HIV p24 as scaffold for presenting conformational HIV Env antigens. *PLoS ONE* 7(8), e43318 (2012).
- 65 Weber J, Cheinsong-Popov R, Callow D et al. Immunogenicity of the yeast recombinant p17/p24-Ty virus-like particles (p24-VLP) in healthy volunteers. *Vaccine* 13(9), 831–834 (1995).
- 66 Vicente T, Mota JP, Peixoto C, Alves PM, Carrondo MJ. Rational design and optimization of downstream processes of virus particles for biopharmaceutical applications: current advances. *Biotechnol. Adv.* 29(6), 869–878 (2011).

Website

- 101 IAVI Report. Database of vaccine candidates in clinical trials.
[www.iavireport.org/
trials-database/pages/
default.aspx](http://www.iavireport.org/trials-database/pages/default.aspx)

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Chapter

2

Virus-like particle vaccines for the prevention of human papillomavirus infection

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Virus-like particles (VLPs) are attractive vaccine platforms, combining safety, ease of production and high-density B- or T-cell epitope display for the optimal induction of potent humoral or cellular immune responses. Indeed, **human papillomavirus** (HPV) vaccines, based on the expression of the major capsid protein L1 in yeast (*Gardasil*®; Merck & Co., NJ, USA) or insect cells (*Cervarix*®; GlaxoSmithKline, London, UK), assembled in VLPs, and formulated in alum or alum and monophosphoryl lipid A, respectively, have been licensed for the prevention of cervical and anogenital HPV disease. Current HPV L1-VLP vaccines provide type-restricted protection, requiring highly multivalent formulations to broaden the coverage to the 12 or more oncogenic HPVs. The lack of screening and high disease burden in developing countries has spurred efforts to develop more affordable, wider protective, second-generation HPV vaccines. In this chapter, the progress and challenges of these efforts, with a focus on how they inform VLP vaccine design, are summarized based on the paper ‘Virus-like particles for the prevention of human

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Virus-like particle: molecular structure formed by the self-assembly of envelope and/or capsid proteins that resembles the actual conformation of the respective native virus, but does not contain any genetic material from the virus and is not infective.

Human papillomavirus: small, nonenveloped, dsDNA virus, with a closed circular genome of approximately 8000 base pairs and comprising a family of more than 120 genotypes, which infects the basal cells of stratified epithelium (skin and mucosa) of patients.

Cervical cancer: a neoplasm arising from a ring in the cervical ostium formed from the squamo-columnar epithelial junction (called the cervical transformation zone), and comprising two major histopathologic types, namely squamous cell carcinoma and adenocarcinoma. Cervical cancer has been causally linked to human papillomavirus (HPV) infection, most frequently the HPV16 and 18 genotypes.

papillomavirus-associated malignancies' by Wang and Roden [1].

The case for VLP vaccine systems

The knowledge that certain infectious agents can trigger cancer has driven the development and licensure of prophylactic vaccines, and the consequent reductions in their associated malignancies. It is estimated that nearly 18% of all cancers worldwide are caused by known infectious agents [2]. Remarkably, HPV is responsible for 5% of cancer worldwide, including 99% of **cervical cancer**, and small subsets of other anogenital cancers (vaginal, vulval, penile and anal) and certain head and neck cancers (predominantly oropharyngeal and

base-of-tongue). The concept of cancer prevention via vaccination was demonstrated within the last 30 years, first with the hepatitis B virus vaccines in the late 1980s and 20 years later with the HPV vaccines Gardasil and Cervarix. These vaccines, which have proven remarkably efficacious in protecting healthy individuals from acquiring new infections, with an excellent safety profile, are all based on recombinant VLPs [3–7]. VLPs have a number of advantages over other vaccine platforms, which are as follows:

- VLPs can be made from the major structural protein(s) of many viruses, which, when expressed alone, are able to self-assemble into highly immunogenic particles, mimicking the morphology, epitope structure and antigenicity of their respective native virion;
- VLPs lack the infectious viral genome and this characteristic makes VLPs safer vaccine candidates compared with their traditional counterparts, such as attenuated or inactive viruses, which could revert back into their infectious form [8], or be problematic in immune-compromised hosts. They can frequently overcome many manufacturing hurdles; viruses are generally prepared in mammalian cell lines, whereas VLPs can be produced in bacteria, yeast or insect cells, greatly facilitating scale-up and reducing safety concerns;
- VLP vaccines are typically highly immunogenic compared with linear peptide vaccines; most VLPs are able to induce very high titers of neutralizing antibodies, often in the absence of adjuvants, and these responses are durable and of high affinity. This reflects the ordered and

closely packed epitope structure, which allows for their presentation to B cells in a manner that effectively crosslinks and activates the B-cell receptor, signaling for potent activation and/or rapid recognition, and uptake by the dendritic cells for the activation of cell-mediated immunity [9–11];

- VLPs can be used as scaffolds for epitopes of heterologous antigens, including against other infectious diseases. While this approach is promising it is still unproven in the clinic, and defining an optimal site for insertion that does not compromise assembly and immunogenicity is unpredictable. Nevertheless, its potential has led many vaccine research groups to continue exploring VLP display platforms using either bacterial, plant or mammalian viruses;
- In many instances VLPs are able to break B-cell tolerance, and this may offer a way to induce antibodies to self antigens, for example to block their deleterious effects by active vaccination rather than using passive therapy with monoclonal antibodies, although active vaccination cannot be stopped like passive antibody therapy [9];
- VLPs are effectively taken up by dendritic cells and can, presumably, enter the cytosol for presentation of epitopes via the MHC class I pathway [10]. This creates an opportunity to attach therapeutic T-cell epitopes to VLPs that elicit potent cellular immune responses to heterologous epitopes, and potentially develop vaccines to treat pre-existing, established infectious diseases or cancers.

An overview of HPV biology

The HPVs are a family of nonenveloped viruses containing a closed circular DNA genome that is approximately 7800 base pairs. The genome encodes six early genes that regulate viral transcription and genome replication (*E1*, *E2*, *E4*, *E5*, *E6* and *E7*), and two late genes that form the viral capsid (*L1* and *L2*). *L1* is the major capsid protein and alone can self-assemble into the viral T=7 icosahedral capsid, comprising 72 pentameric subunits of *L1*. The *L2* capsid protein forms the rest of the capsid and is present at a ratio of up to one *L2* per five *L1* proteins (i.e., 72 *L2* proteins within each HPV virion), although a ratio as low as 1:30 has been described [12,13]. Among the over 100 HPV genotypes, are the 12 so-called ‘high risk’ or ‘oncogenic’ HPV types that have been causally linked to cancers of the cervix and to a subset of cancers of the anus, vagina, vulva, penis and oropharyngeal regions. Over 99% of cervical cancers contain HPV and approximately 50 and 20% contains HPV16 and HPV18, respectively, with the remainder of the HPV genotypes each contributing relatively small

fractions. It is important to recognize that persistent HPV infection is necessary but not sufficient to cause cervical cancer, and most infections are spontaneously resolved by the host's immune system. Therefore, HPV infections are particularly problematic and chronic in immunocompromised patients with HIV or solid-organ transplant recipients, because the infections are more likely to persist.

Current licensed HPV vaccines & their recognized limitations

Gardasil and Cervarix are derived by overexpressing L1 in yeast and insect cells, respectively. L1 is able to self-assemble to form VLPs that are morphologically similar to virions and are able to induce similarly high titers of neutralizing antibodies, as vaccination with native virions, but are noninfectious because they lack the potentially oncogenic viral DNA [14]. Administration of L1-based VLPs allows the immune system to generate antibody titers that are 100-fold greater compared with natural infections [6,15–18]. In addition, passive transfer studies using L1-VLP-vaccinated animal serum into naive animals confers protection [15,19,20]. These studies strongly suggest that the HPV-VLP-induced protection is mediated by humoral immunity, but roles for innate immunity and cell-mediated immunity are yet to be defined.

Current formulations of both licensed HPV L1-VLP vaccines are based on two oncogenic HPV genotypes, HPV16 and HPV18, which account for approximately 70% of all cervical cancers cases. Gardasil also targets HPV6 and HPV11, which are the two most common genotypes, causing benign genital warts (also called *Condyloma acuminata*). Vaccinated individuals, however, have, to a varied extent, protection against the remaining 12 or so oncogenic HPV genotypes that collectively cause approximately 30% of all cervical cancer cases, and this is a recognized limitation of these current vaccines [12,21–23]. Consequently, Merck currently has a nonavalent vaccine in advanced clinical trials that will likely broaden the efficacy to the other oncogenic HPV types, while still targeting HPV6 and HPV11. Another currently recognized limitation of L1-based vaccines is that the manufacture of highly multivalent vaccines is complex and may, therefore, be difficult to produce at low cost (**Table 2.1**) [24]. Finally, the currently licensed vaccines have not been shown to have a therapeutic effect against existing infections.

Extending HPV vaccine coverage: HPV L2 as an alternative candidate

The minor capsid protein L2 is necessary for PV infection and is also a prophylactic vaccine antigen [25,26]. Several studies using rabbit and bovine papillomavirus models showed that vaccination with the cognate L2 protein

Table 2.1. Recognized limitations of current licensed human papillomavirus vaccines Cervarix® (GlaxoSmithKline, London, UK) and Gardasil® (Merck & Co., NJ, USA).

Limitation	Impact	Solution & alternatives
Cost of manufacturing, trials and IP leads to high vaccine price	Developing countries, who do not have the economic means to afford the vaccine, continue to bear the burden of cervical cancer	Investigate manufacturing generic L1-VLP vaccines in cheaper systems, such as plant or bacterial expression systems
Type restriction	Vaccine does not protect against all HPV types causing cervical cancer. Screening via Papanicolaou smear test, an invasive procedure, must continue for HPV-vaccinated women.	More HPV VLP types to create a highly multivalent HPV vaccine. Alternative vaccine targeting the broadly protective antigen L2
No therapeutic value	No effect on individuals with pre-existing HPV infection	Alternative vaccine constructs displaying <i>E6</i> and <i>E7</i> . Optimize immunization schedule and route of administration

HPV: Human papillomavirus; IP: Intellectual property; VLP: Virus-like particle.

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prepared by recombinant expression in bacteria confers immunity against experimental papillomavirus challenge at either an in-skin or mucosal site [19,27–30]. Further studies showed that there are several broadly cross-neutralizing epitopes on the N-terminus of L2, within amino acids 11–88, which suggest the potential for broad protection from a single antigen [27,31–35]. Animal studies showing cross-neutralization of a broad range of HPV genotypes by L2 antisera, and protection against a diversity of HPV genotypes upon vaccination with a single L2 epitope further suggests the possibility of a simple pan-HPV prophylactic vaccine derived from L2. In addition, the ability for production of a single antigen in bacteria suggests lower manufacturing and production costs, thus promising potentially better distribution. However, due to its weak immunogenicity compared with L1-VLP, a key challenge in L2-based vaccine development is effective display of these broadly crossneutralizing L2 epitopes to the immune system, to trigger high-titer, high-avidity and long-lived antibody responses.

Methods to improve L2-based VLP vaccines

One potential method to improve L2 immunogenicity in the context of a VLP vaccine is to increase the density of the surface display of L2. Indeed, several groups have produced chimeric HPV VLPs by inserting L2-neutralizing epitopes into the immunodominant L1-neutralizing epitopes of HPV L1-VLPs or bovine papillomavirus type 1 L1-VLPs [36–38]. Display in L1 capsomers (made of five L1 proteins and the building block for VLPs) could

also be another strategy. Garcea and colleagues showed that by deleting the ninth and 26th amino acids from the N and C terminuses of L1, respectively, L1 readily forms capsomeres (made of five L1 proteins) instead of full VLPs [39]. Like the licenced L1-VLP vaccines, L1 capsomeres retain the ability to induce neutralizing antibodies, although at somewhat lower antibody titers.

Jagu and colleagues have also previously developed a broadly protective L2 peptide vaccine candidate ($L2_{11-88}X5$) by fusing five different HPV L2 regions 11–88 (HPV1, HPV5, HPV6, HPV16 and HPV18) and expressing this multimer in bacteria [40]. While broadly protective against numerous HPV genotypes *in vivo*, the *in vitro* neutralization titer data of $L2_{11-88}X5$ are still lower than L1-VLP vaccines [40]. As HPV VLPs can directly activate dendritic cells, it is possible that they could act as an adjuvant. Therefore, another possible approach to augment the L2 antibody titers, and broaden the vaccine coverage at the same time, would be to combine the $L2_{11-88}X5$ with currently licensed HPV vaccines to extend the protective coverage. Subsequent studies have, however, shown that when mixing L1-VLPs or L1 capsomeres with L2 peptide vaccines, the L1- and L2-specific responses essentially act independently [41], and that the L2 epitopes most likely need to be displayed directly on VLP, rather than mixed with them, to boost the response. In addition, to prevent immune subdominance of L1 over L2 responses, considerations must also be made on displaying the L2 epitopes in the immunodominant epitopes of the L1 capsomer or capsid structure. Alternatively, to circumvent any issues relating to immunodominance, several other studies have also attempted using non-HPV VLPs as platforms for L2-based VLP vaccines as another possible strategy (**Table 2.2**).

Therapeutic VLP-based HPV vaccines

A third recognized limitation of the current medically licensed HPV vaccines is that they are entirely prophylactic and offer no therapeutic value to individuals already infected with HPV. As HPV E6 and E7 are expressed in all HPV-infected cells, and are upregulated in cancer cells, they are generally considered the logical targets for therapeutic vaccination compared with L1 or L2. E6 and E7 have already been targeted in numerous HPV therapeutic vaccination strategies, including peptide, protein, live vector, DNA and whole-cell-based approaches. Due to space limitations, the discussion will be focused on the efforts using VLP-based therapeutic vaccine approaches. For other therapeutic strategies, the reader is directed to [42,43].

HPV E6 and E7 peptide sequences contain several T-cell epitopes that can elicit an antitumor cytotoxic T-lymphocyte response [43–45]. However,

Table 2.2. Summary of virus-like particle-based prophylactic human papillomavirus vaccines.

Second-generation prophylactic HPV VLP candidate vaccines based on HPV L2	Potential or current advantages over licensed HPV L1-VLP vaccines	Potential or current disadvantages	Ref.
L1-VLPs expressing L2 epitopes	Broad-spectrum protection	L2 antibody-neutralizing titers detected were low and uses the same expression system as current L1-VLPs (Cervarix® [GlaxoSmithKline, London, UK] or Gardasil® [Merck & Co., NJ, USA]), which does not bring down cost of potential vaccine	[36–38]
L1 capsomeres	Bacterial expression system lowers the cost of manufacturing	Lower antibody avidity compared with L1-VLPs and type-restricted protection, but can be overcome by creating a variety of capsomeres or combinatorial vaccination methods with L2	[39,41,50]
Plant-VLPs (e.g., tobacco mosaic virus)	Broad-spectrum protection and plant expression system lowers the cost of manufacturing	Studies using this method showed L2 antibody-neutralizing titers that were low and no issues of bacterial endotoxin contamination	[51,52]
Bacteriophage VLPs	Broad-spectrum protection, bacterial expression system lowers the cost of manufacturing and no adjuvant needed	Endotoxin contamination	[53,54]
Non-HPV mammalian virus VLPs (e.g., AAVLP)	Broad-spectrum protection	Requires adjuvant and pre-existing neutralizing antibodies against the AAV or other carrier	[55]

AAV: Adeno-associated virus; AAVLP: Adeno-associated virus-like particle; HPV: Human papillomavirus; VLP: Virus-like particle.

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typically the T-cell responses induced by natural infection are very low or undetectable, possibly reflecting the localized nature of the infection and viral immune evasion mechanisms, and indicating the potential for vaccination to improve E6/E7-specific immune responses. HPV VLPs are able to directly activate dendritic cells and, despite being an exogenous antigen, they can provide MHC class I presentation by pseudoinfecting cells [10,46]. Therefore, in addition to inducing high antibody titers, VLPs have features that can induce cell-mediated immunity.

Utilizing the advantages of VLPs, numerous groups have attempted to improve E6/E7 T-cell responses by forming chimeric HPV VLPs (cVLPs) in which the T-cell epitopes of HPV E6, E7 or both are fused onto the C-terminus of either HPV L1-VLP or L1/L2-VLPs. Fusion of the E7 protein to the C-terminus of L1 in VLPs provides a strong, specific cytotoxic T-lymphocyte response. However, there are significant constraints to the size of the E7 polypeptide fused to L1 before it results in impaired cVLP production. Conversely, fusing E7 to L2 in L1/L2 E7-cVLPs is more tolerant to larger fusions without disrupting assembly, and it was noted that L2 is required for viral infection, but not class I antigen presentation of the E7 epitopes included in the cVLPs [47]. While both approaches provide *in vivo* tumor protection in mouse models, more studies in more stringent therapeutic models are warranted to better assess their potential.

The first Phase I clinical trial of cVLP was performed in 2007. HPV16 L1E7-cVLP was administered four-times to patients with HPV16-positive, high-grade cervical intraepithelial neoplasia (cervical intraepithelial neoplasia 2/3) [48]. The HPV16 L1E7-cVLPs were well tolerated and able to induce cellular immune responses in vaccinated patients. Unfortunately, in terms of clinical efficacy, which was measured via 50% lesion size reduction and disappearance of HPV16 DNA, there was no significant difference in either parameter between the placebo and vaccinated groups. One possible reason for the poor clinical efficacy of the HPV16 L1E7-cVLP could be pre-existing neutralizing antibodies against HPV16 L1 (triggered by natural infection) that would limit the effectiveness of the cVLP vaccination, as shown in cVLP-vaccinated mice by Da Silva and colleagues [49]. Alternatively, the induced-therapeutic HPV immunity was not appropriately targeted to the lesion, or the local suppressive environment prevented viral clearance.

Conclusion & future perspective

As HPV is currently responsible for 5% of all cancer cases worldwide, the licensure and implementation of many national immunization campaigns for the prophylactic L1-VLP vaccines will be of benefit to human health as they complement existing cervical cancer prevention procedures, such as Papanicolaou screening tests and the emerging implementation of HPV DNA screening. Nevertheless, much work remains, such as ensuring global access to HPV vaccination and broadening coverage to all oncogenic HPV genotypes. Information about the key mechanisms that have made these HPV vaccines so effective should also be studied in detail, as this would drive the rational development of VLP vaccines to targets other than HPV. HPV VLPs are also particularly interesting in their ability to directly activate dendritic and other antigen-presenting cells. This highlights their potential as platforms for

presenting heterologous epitopes, including viral and tumor antigens via the MHC class I pathway to generate therapeutic immune responses. However, with only a single existing human clinical trial using such HPV L1-E7-cVLPs, these studies remain in their infancy and should be continued. However, it is clear that in addition to triggering robust E6 and/or E7 antigen-specific T-cell responses, considerations of targeting cytotoxic T-cell responses to disease sites and overcoming local tolerogenic factors are likely to influence the success of therapeutic HPV vaccines based on cVLP vaccines.

Financial & competing interests disclosure

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Summary

- Cervical cancer and other human papillomavirus (HPV)-associated malignancies are preventable through HPV virus-like particle (VLP) vaccination and cytologic screening.
- Global implementation of HPV vaccination is required to realize a dramatic reduction in rates of HPV-associated cancers, but there are numerous practical implementation barriers to achieving this goal.
- Development of low-cost vaccines and overcoming logistical barriers (e.g., via heat stability, fewer doses, not requiring needles and local manufacture to facilitate delivery) are critical.
- Mechanistic study of the human immune response to HPV VLPs, especially early innate events and the induction of immune memory, will inform rational design of vaccines to other agents.
- Robust and durable protection must be extended to all oncogenic HPV types by developing highly multivalent L1-VLP formulations, or possibly by use of L2, to mitigate the need and cost for screening.
- While VLPs have promise for the display of heterologous linear epitopes to induce robust titers of high-avidity and long-lived antibodies, the outcomes have been variable and unpredictable, suggesting we need a better understanding of their presentation of epitopes to the immune system.
- HPV infection and the associated diseases remain very common despite licensed vaccines, and the need for a therapeutic vaccine remains.
- Recombinant VLPs can effectively deliver heterologous epitopes to MHC class I pathways for presentation and induction of a robust cellular immune response.
- Consideration of targeting cellular immunity to the disease site and approaches to modulate the lesion microenvironment is important in developing therapeutic vaccines.

References

- 1 Wang JW, Roden RBS. Virus-like particles for the prevention of human papillomavirus-associated malignancies. *Expert Rev. Vaccines* 12(2), 129–141 (2013).
- 2 Zur HH. Viruses in human cancers. *Science* 254(5035), 1167–1173 (1991).
- 3 Day PM, Gambhiria R, Roden RB, Lowy DR, Schiller JT. Mechanisms of human papillomavirus type 16 neutralization by L2 cross-neutralizing and L1 type-specific antibodies. *J. Virol.* 82(9), 4638–4646 (2008).
- 4 Romanowski B, de Borba PC *et al.*; GlaxoSmithKline Vaccine HPVS. Sustained efficacy and immunogenicity of the human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine: analysis of a randomised placebo-controlled trial up to 6.4 years. *Lancet* 374(9706), 1975–1985 (2009).
- 5 Harper DM, Franco EL, Wheeler CM *et al.* Sustained efficacy up to 4.5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18, follow-up from a randomised control trial. *Lancet* 367(9518), 1247–1255 (2006).
- 6 Mao C, Koutsy LA, Ault KA *et al.* Efficacy of human papillomavirus-16 vaccine to prevent cervical intraepithelial neoplasia: a randomized controlled trial. *Obstet. Gynecol.* 107(1), 18–27 (2006).
- 7 Shih HH, Chang MH, Hsu HY, Lee PI, Ni YH, Chen DS. Long term immune response of universal hepatitis B vaccination in infancy: a community-based study in Taiwan. *Pediatr. Infect. Dis. J.* 18(5), 427–432 (1999).
- 8 Roldao A, Mellado MC, Castilho LR, Carrondo MJ, Alves PM. Virus-like particles in vaccine development. *Expert Rev. Vaccines* 9(10), 1149–1176 (2010).
- 9 Chackerian B, Lenz P, Lowy DR, Schiller JT. Determinants of autoantibody induction by conjugated papillomavirus virus-like particles. *J. Immunol.* 169(11), 6120–6126 (2002).
- 10 Lenz P, Day PM, Pang YY *et al.* Papillomavirus-like particles induce acute activation of dendritic cells. *J. Immunol.* 166(9), 5346–5355 (2001).
- 11 Lenz P, Thompson CD, Day PM, Bacot SM, Lowy DR, Schiller JT. Interaction of papillomavirus virus-like particles with human myeloid antigen-presenting cells. *Clin. Immunol.* 106(3), 231–237 (2003).
- 12 Buck CB, Trus BL. The papillomavirus virion: a machine built to hide molecular Achilles' heels. *Adv. Exp. Med. Biol.* 726, 403–422 (2012).
- 13 Chen XS, Garcea RL, Goldberg I, Casini G, Harrison SC. Structure of small virus-like particles assembled from the L1 protein of human papillomavirus 16. *Mol. Cell* 5(3), 557–567 (2000).
- 14 McNeil C. Who invented the VLP cervical cancer vaccines? *J. Natl Cancer Inst.* 98(7), 433 (2006).
- 15 Einstein MH, Baron M, Levin MJ *et al.* Comparison of the immunogenicity and safety of Cervarix® and Gardasil® human papillomavirus (HPV) cervical cancer vaccines in healthy women aged 18–45 years. *Hum. Vaccin.* 5(10), 705–719 (2009).
- 16 Koutsy LA, Ault KA, Wheeler CM *et al.* A controlled trial of a human papillomavirus type 16 vaccine. *N. Engl. J. Med.* 347(21), 1645–1651 (2002).
- 17 Poland GA, Jacobson RM, Koutsy LA *et al.* Immunogenicity and reactogenicity of a novel vaccine for human papillomavirus 16: a 2-year randomized controlled clinical trial. *Mayo Clin. Proc.* 80(5), 601–610 (2005).
- 18 Villa LL, Costa RL, Petta CA *et al.* Prophylactic quadrivalent human papillomavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in young women: a randomised double-blind placebo-controlled multicentre Phase II efficacy trial. *Lancet Oncol.* 6(5), 271–278 (2005).
- 19 Breitburd F, Kirnbauer R, Hubbert NL *et al.* Immunization with viruslike particles from cottontail rabbit papillomavirus (CRPV) can protect against experimental CRPV infection. *J. Virol.* 3959–3963 (1995).
- 20 Day PM, Kines RC, Thompson CD *et al.* In vivo mechanisms of vaccine-induced protection against HPV infection. *Cell Host Microbe* 8(3), 260–270 (2010).
- 21 Roden R, Wu TC. How will HPV vaccines affect cervical cancer? *Nat. Rev. Cancer* 6(10), 753–763 (2006).

- 22 Roden R, Monie A, Wu TC. The impact of preventive HPV vaccination. *Discov. Med.* 6(35), 175–181 (2006).
- 23 Schiller JT, Day PM, Kines RC. Current understanding of the mechanism of HPV infection. *Gynecol. Oncol.* 118(1 Suppl.), S12–S17 (2010).
- 24 Lowy DR, Solomon D, Hildesheim A, Schiller JT, Schiffman M. Human papillomavirus infection and the primary and secondary prevention of cervical cancer. *Cancer* 113(7 Suppl.), 1980–1993 (2008).
- 25 Richards RM, Lowy DR, Schiller JT, Day PM. Cleavage of the papillomavirus minor capsid protein, L2, at a fur in consensus site is necessary for infection. *Proc. Natl Acad. Sci. USA* 105(3), 1522–1527 (2006).
- 26 Fay A, Yutzy WH, Roden RB, Moroianu J. The positively charged termini of L2 minor capsid protein required for bovine papillomavirus infection function separately in nuclear import and DNA binding. *J. Virol.* 78(24), 13447–13454 (2004).
- 27 Campo MS, O’Neil BW, Grindlay GJ, Curtis F, Knowles G, Chandrachud L. A peptide encoding a B-cell epitope from the N-terminus of the capsid protein L2 of bovine papillomavirus-4 prevents disease. *Virology* 234(2), 261–266 (1997).
- 28 Christensen ND, Kreider JW, Kan NC, Di Angelo SL. The open reading frame L2 of cottontail rabbit papillomavirus contains antibody-inducing neutralizing epitopes. *Virology* 181(2), 572–579 (1991).
- 29 Lin YL, Borenstein LA, Selvakumar R, Ahmed R, Wettstein FO. Effective vaccination against papilloma development by immunization with L1 or L2 structural protein of cottontail rabbit papillomavirus. *Virology* 187(2), 612–619 (1992).
- 30 Embers ME, Budgeon LR, Pickel M, Christensen ND. Protective immunity to rabbit oral and cutaneous papillomaviruses by immunization with short peptides of L2, the minor capsid protein. *J. Virol.* 76(19), 9798–9805 (2002).
- 31 Chandrachud LM, Grindlay GJ, McGarvie GM et al. Vaccination of cattle with the N-terminus of L2 is necessary and sufficient for preventing infection by bovine papillomavirus-4. *Virology* 211(1), 204–208 (1995).
- 32 Gambhira R, Jagu S, Karanam B et al. Protection of rabbits against challenge with rabbit papillomaviruses by immunization with the N terminus of human papillomavirus type 16 minor capsid antigen L2. *J. Virol.* 81(21), 11585–11592 (2007).
- 33 Gambhira R, Karanam B, Jagu S et al. A protective and broadly cross-neutralizing epitope of human papillomavirus L2. *J. Virol.* 81(24), 13927–13931 (2007).
- 34 Kawana K, Kawana Y, Yoshikawa H, Taketani Y, Yoshiike K, Kanda T. Nasal immunization of mice with peptide having a cross-neutralization epitope on minor capsid protein L2 of human papillomavirus type 16 elicit systemic and mucosal antibodies. *Vaccine* 19(11–12), 1496–1502 (2001).
- 35 Rubio I, Seitz H, Canali E et al. The N-terminal region of the human papillomavirus L2 protein contains overlapping binding sites for neutralizing, cross-neutralizing and non-neutralizing antibodies. *Virology* 409(2), 348–359 (2011).
- 36 Slupetzky K, Gambhira R, Culp TD et al. A papillomavirus-like particle (VLP) vaccine displaying HPV16 L2 epitopes induces cross-neutralizing antibodies to HPV11. *Vaccine* 25(11), 2001–2010 (2007).
- 37 Schellenbacher C, Roden R, Kirnbauer R. Chimeric L1–L2 virus-like particles as potential broad-spectrum human papillomavirus vaccines. *J. Virol.* 83(19), 10085–10095 (2009).
- 38 Varsani A, Williamson AL, de Villiers D, Becker I, Christensen ND, Rybicki EP. Chimeric human papillomavirus type 16 (HPV-16) L1 particles presenting the common neutralizing epitope for the L2 minor capsid protein of HPV-6 and HPV-16. *J. Virol.* 77(15), 8386–8393 (2003).
- 39 Rose RC, White WI, Li M, Suzich JA, Lane C, Garcea RL. Human papillomavirus type 11 recombinant L1 capsomeres induce virus-neutralizing antibodies. *J. Virol.* 72(7), 6151–6154 (1998).
- 40 Jagu S, Karanam B, Gambhira R et al. Concatenated multitype L2 fusion proteins as candidate prophylactic pan-human papillomavirus vaccines.

- J. Natl Cancer Inst.* 101(11), 782–792 (2009).
- 41 Jagu S, Kwak K, Garcea RL, Roden RB. Vaccination with multimeric L2 fusion protein and L1 VLP or capsomeres to broaden protection against HPV infection. *Vaccine* 28(28), 4478–4486 (2010).
- 42 Lin K, Doolan K, Hung CF, Wu TC. Perspectives for preventive and therapeutic HPV vaccines. *J. Formos. Med. Assoc.* 109(1), 4–24 (2010).
- 43 Hung CF, Ma B, Monie A, Tsen SW, Wu TC. Therapeutic human papillomavirus vaccines: current clinical trials and future directions. *Expert Opin. Biol. Ther.* 8(4), 421–439 (2008).
- 44 Wu CY, Monie A, Pang X, Hung CF, Wu TC. Improving therapeutic HPV peptide-based vaccine potency by enhancing CD4⁺ T help and dendritic cell activation. *J. Biomed. Sci.* 17, 88 (2010).
- 45 Barrios K, Celis E. TriVax-HPV: an improved peptide-based therapeutic vaccination strategy against human papillomavirus-induced cancers. *Cancer Immunol. Immunother.* 61(8), 1307–1317 (2012).
- 46 Rudolf MP, Fausch SC, Da Silva DM, Kast WM. Human dendritic cells are activated by chimeric human papillomavirus type-16 virus-like particles and induce epitope-specific human T cell responses *in vitro*. *J. Immunol.* 166(10), 5917–5924 (2001).
- 47 Wakabayashi MT, Da Silva DM, Potkui RK, Kast WM. Comparison of human papillomavirus type 16 L1 chimeric virus-like particles versus L1/L2 chimeric virus-like particles in tumor prevention. *Intervirology* 45(4–6), 300–307 (2002).
- 48 Kaufmann AM, Nieland JD, Jochmus I et al. Vaccination trial with HPV16 L1E7 chimeric virus-like particles in women suffering from high grade cervical intraepithelial neoplasia (CIN 2/3). *Int. J. Cancer* 121(12), 2794–2800 (2007).
- 49 Da Silva DM, Pastrana DV, Schiller JT, Kast WM. Effect of preexisting neutralizing antibodies on the anti-tumor immune response induced by chimeric human papillomavirus virus-like particle vaccines. *Virology* 290(2), 350–360 (2001).
- 50 Bian T, Wang Y, Lu Z et al. Human papillomavirus type 16 L1E7 chimeric capsomeres have prophylactic and therapeutic efficacy against papillomavirus in mice. *Mol. Cancer Ther.* 7(5), 1329–1335 (2008).
- 51 Cerovska N, Hoffmeisterova H, Moravec T et al. Transient expression of human papillomavirus type 16 L2 epitope fused to N- and C-terminus of coat protein of Potato virus X in plants. *J. Biosci.* 37(1), 125–133 (2012).
- 52 Paz De la Rosa G, Monroy-Garcia A, Mora-Garcia Mde L et al. An HPV 16 L1-based chimeric human papilloma virus-like particles containing a string of epitopes produced in plants is able to elicit humoral and cytotoxic T-cell activity in mice. *Virol. J.* 6, 2 (2009).
- 53 Caldeira Jdo C, Medford A, Kines RC et al. Immunogenic display of diverse peptides, including a broadly cross-type neutralizing human papillomavirus L2 epitope, on virus-like particles of the RNA bacteriophage PP7. *Vaccine* 28(27), 4384–4393 (2010).
- 54 Tumban E, Peabody J, Peabody DS, Chackerian B. A pan-HPV vaccine based on bacteriophage PP7 VLPs displaying broadly cross-neutralizing epitopes from the HPV minor capsid protein, L2. *PLoS ONE* 6(8), e23310 (2011).
- 55 Nieto K, Weghofer M, Sehr P et al. Development of AAVLP(HPV16/31L2) particles as broadly protective HPV vaccine candidate. *PLOS ONE* 7(6), e39741 (2012).

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Virus-like particle-based vaccines against hepatitis C virus infection

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Bertrand Bellier &
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Hepatitis C virus (HCV) infection is a worldwide health problem and much effort is being made to develop novel therapies. New vaccines are designed for preventive and therapeutic use through induction of robust immunity, including neutralizing antibodies and T-cell-mediated immunity. Novel future vaccine approaches include virus-like-particle (VLP)-based vaccines that have been successfully employed to prevent infections by hepatitis B virus or human papillomavirus. The HCV-derived VLP approach simplifies the delivery of neutralizing antibody- and core-specific T-cell epitopes in a highly immunogenic single construct resembling mature HCV virions. The size, particulate nature and dense, repetitive structure of VLPs are the basis for their innate immunogenicity. Consequently, VLPs have also been exploited as antigenic platforms. Association of HCV antigens with heterologous structural viral proteins able to form recombinant VLPs (e.g., murine leukemia virus Gag, surface antigen of hepatitis B virus, hepatitis B core antigen and papaya mosaic virus coar protein) is also a promising approach for induction of HCV-specific immune responses. Here, the authors report the different VLP-based vaccine approaches that are currently under development.

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Aa Hepatitis C virus (HCV): infects more than 130 million people globally and is now the leading cause of liver transplantation worldwide. A total of 20% of acute HCV infections in humans will clear spontaneously – clearance is associated with some degree of protective immunity. A major challenge for vaccine development is the high genetic variability of the virus.

The hepatitis C virus (HCV) is a major pathogen known to cause chronic liver disease, which may progress to cirrhosis and hepatocellular carcinoma. Chronic HCV infection is currently a major public health problem, affecting approximately 3% of the world's population (130–170 million individuals) and causes an estimated 476,000

deaths per year [1]. The chronic hepatitis C treatment efficacy is limited, with a sustained virological response rate of only 55%, with the combination of pegylated IFN- α and ribavirin. Although newly approved antivirals are available, only a small portion of patients with chronic hepatitis C can currently be cured in most real-world settings. The development of a prophylactic vaccine against HCV is therefore a major medical priority. However, the development of such a vaccine is very challenging. The multiple HCV genotypes, the limited availability of animal models and the complex nature of the immunological response to HCV represent the main drawbacks [2]. Although the correlates of protective immunity are not completely understood, it is now admitted that an effective vaccine against HCV will need to induce both cellular and humoral immune responses, including neutralizing antibodies (NAbs) and address viral heterogeneity to prevent immune escape [3]. As a result, the vaccine formulation must incorporate antigens from HCV structural proteins (e.g., E1 and E2) in their correct 3D conformations to induce NAbs, together with T-cell epitopes from HCV nonstructural proteins (e.g., NS3, NS4 and NS5), to elicit strong cellular responses [4,5].

The potential dangers associated with using attenuated HCV viral particles as a vaccine have led to the formulation and delivery of HCV antigens in the form of modern vaccine modalities. Several different strategies, including recombinant protein (subunit vaccine), synthetic peptides, DNA and live

vector-based vaccines, as well as prime-boost strategies combining different vaccines, have been investigated for HCV vaccine with variable success (review in [4,5]), with some in Phase I or I/II trials (review in [6,7]). Alternatively, virus-like particle (VLP)-based vaccines have been developed to improve the delivery system for HCV NAb- and T-cell epitopes. This approach is justified by previous reports demonstrating that VLPs have an intrinsic advantage over soluble antigens, which have been shown to



The size, particulate nature and dense, repetitive structure of virus-like particles (VLPs) are the basis for VLPs' innate immunogenicity and their efficacy in inducing neutralizing antibody (NAb) and T-cell responses. VLPs are considered as promising HCV vaccine candidates.

The greatest challenge to NAb-based vaccines is the generation of heterologous protection that can neutralize more than one genotype of HCV. Conformational epitopes of HCV envelope proteins may be utilized for eliciting NAbs. Broad and strong T-cell responses may be induced to neutralize the HCV infection.

fail in several vaccine approaches due to their weak immunogenicity or instability. The size, particulate nature and dense, repetitive structure of VLPs are the basis for their innate immunogenicity [8]. The VLPs represent a molecular display system that can be used to induce potent and broad immune responses against the antigens displayed in an appropriate conformation. Consequently, VLPs have also been exploited as platforms to induce immune responses against heterologous virus-derived antigens [9]. Here, the authors review the different HCV-specific vaccine strategies using homologous (HCV-VLPs) or heterologous (recombinant VLPs displaying HCV antigens) VLPs (**Table 3.1**) and genetic vaccines expressing these two types of VLPs (**Table 3.2**).

HCV-derived VLPs

It was reported that HCV structural proteins (the core protein and the two envelope glycoproteins, E1 and E2) expressed in recombinant

Table 3.1. Homologous and recombinant virus-like particle vaccines.

Viral particle core	Structure	Antigen insertion	HCV antigens	Immunogenicity studies	Ref.
Hepatitis C virus core	Spherical shape D = 30–80 nm 90 copies of E1E2		Core, E1, E2	Mice, macaques and chimpanzees	[13–15,41,42]
HBc	Spherical shape D = 30–34 nm 120 copies of HBc	C-term. HBc (internal)	Core (98aa) NS3 (155aa) Core (60aa) HBV-S1 (27aa)	Mice	[17]
		N-term. S (external)	E1E2, E1	Mice	[18]
Hepatitis B virus small surface Ag	Spherical shape D = 22 nm 100 copies of S	aa127-S(external) N-term. S (external)	HVR-1 E2 (36aa)	Mice	[21,22,43]
Papaya mosaic virus CP	Rod shape L = 60–100 nm, D = 14 nm 150–250 copies of CP	C-term. CP (external)	E2 (aa411–613)	Rabbits	[24]
MLV capsid (Mo-MLV Gag)	Spherical shape D = 90–100 nm 2000 copies of Gag	Envelope (external) C-term. Gag (internal)	E1E2, E1 NS3 (442 aa)	Mice and macaques Mice	[30,32,35]

aa: Amino acid; Ag: Antigen; C-term: C-terminus; CP: Coat protein; D: Diameter; HBc: Hepatitis B virus core; HVR-1: Hypervariable region 1; L: Length; MLV: Murine leukemia virus; S: Small.

Table 3.2. Genetic vaccines expressing hepatitis C virus recombinant virus-like particle vaccines.

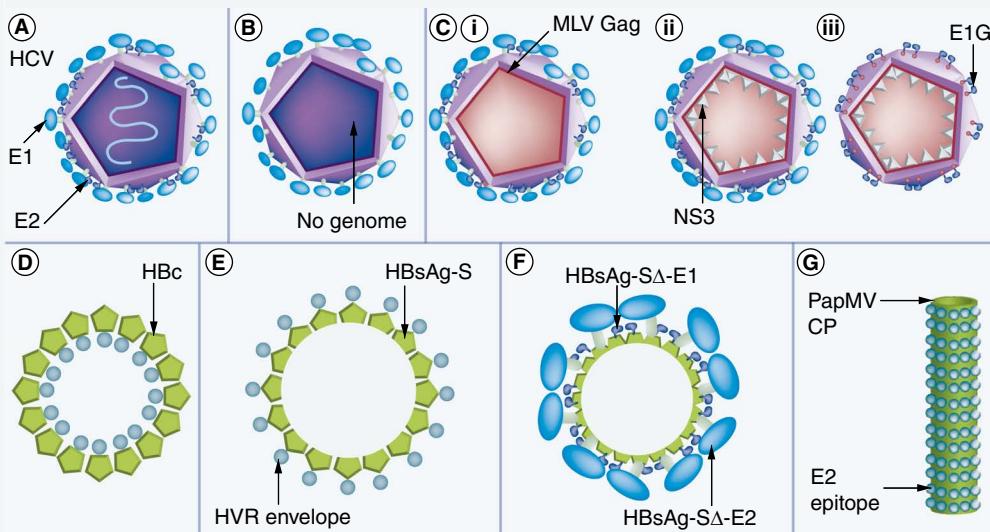
Vector	VLP	Structural components	HCV antigens	Immunogenicity studies	Ref.
rVSV	HCV	Core	Core, E1, E2	Mice	[39]
Plasmid DNA	HCV	Core	Core, E1, E2	Mice, rabbits and macaques	[14,44]
	retroVLP	Mo-MLV Gag	E1, E2 NS3	Mice Mice	[30,35] [35]

HCV: Hepatitis C virus; MLV: Murine leukemia virus; retroVLP: Retrovirus-derived virus-like protein;

rVSV: Recombinant vesicular stomatitis virus; VLP: Virus-like protein.

baculovirus-infected insect cells assemble into virus-like structures (**Figure 3.1B**). These HCV-VLPs have similar biophysical, ultrastructural and antigenic properties to those of the putative virions [10], and are highly immunoreactive when incubated with purified Abs from the serum of infected patients or with various monoclonal Abs recognizing conformational determinants, suggesting that they contain correctly assembled HCV structural proteins [10,11]. Although a proper conformation of envelope glycoproteins E1E2 in HCV-VLPs is assumed, modifications of envelope proteins have been reported relating to different post-translational processing of proteins in insect and mammalian expression systems [12].

Immunogenicity of HCV-VLPs was tested in different animal models and gave encouraging results for HCV vaccine design. It was reported that HCV-VLPs induce a high-titer Ab response that is broadly directed against core and E2 proteins in both mice and rabbits [10]. However, anti-E1 Abs were not detected in the immunized animals. Furthermore, the induction of NAb by HCV-VLPs has not yet been demonstrated. One of the major advantages of VLPs is also their ability to induce cytotoxic T-lymphocyte (CTL) responses, in contrast with recombinant protein vaccines. It was shown that HCV-VLPs induce effective CTL responses against E2 and core proteins in mice [13,14]. The efficacy of HCV-VLPs in inducing immune responses is strongly dependent on particle formation since immunization with heat-denatured particles resulted in lower antigen-specific cellular and humoral immune responses [13]. The protective immunity induced by HCV-VLPs was first established in a surrogate challenge model with recombinant HCV-vaccinia, due to the lack of a convenient animal model for HCV infection [14]. HCV-VLP vaccine efficacy was confirmed in a chimpanzee model, the only established animal model susceptible to HCV infection. Chimpanzees immunized with HCV-VLPs in the presence or not of adjuvants developed HCV-specific CD4⁺ and CD8⁺ T-cell responses and quickly

Figure 3.1. Virus-like particles used for hepatitis C virus vaccine design.

(A) Wild-type HCV is a small (55–65 nm) enveloped virus displaying E1E2 heterodimers and contains in the capsid (core) a positive-sense RNA genome (ssRNA). **(B)** HCV-derived virus-like particles (VLPs) formed with HCV core, E1 and E2 structural proteins resemble the putative HCV virions, but contain no viral genome. **(C)** Retrovirus-based VLPs contain the dense MLV Gag retroviral nucleocapsid surrounded by a lipid bilayer, as regular 100-nm spherical particles, and pseudotyped with **(C, i)** wild-type E1E2 glycoproteins or **(C, iii)** chimeric E1 protein (E1G) alone. **(C, ii)** Recombinant retrovirus-based VLPs displaying NS3-derived antigens inside the particles exemplify the flexibility of the retrovirus-based platform. **(D)** Recombinant HBC proteins fused with HCV-derived antigens (core or NS3 epitopes) form 30-nm spherical recombinant VLPs. **(E)** Recombinant HBsAg-S, which self-assemble into highly organized 22-nm pseudoparticles, are used to display HCV antigens (HVR1 E2 epitopes). **(F)** Chimeric HBV-HCV envelope proteins in which the N-terminal transmembrane domain of S was replaced (HBsAg-SD) with the transmembrane domain of the HCV envelope proteins (HBsAg-SD-E1 and HBsAg-SD-E2) coassemble with wild-type HBsAg-S into VLPs. **(G)** Recombinant CP derived from PapMV self-assemble into nanoparticles ranging from 60 to 100 nm in length and display E2-HCV derived epitopes.

CP: Coat protein; HBsAg-S: Small surface antigen of HBV; HBC: Hepatitis B virus core; HCV: Hepatitis C virus; HVR: Hypervariable region; MLV: Murine leukemia virus; PapMV: Papaya mosaic virus.

controlled HCV infection [15]. These results highlight the potential success of HCV-VLPs as vaccine.

Recombinant VLPs displaying HCV antigens

VLP technologies possess obvious advantages for the generation of safe and efficacious vaccines, and thus VLPs have been exploited as platforms to induce CTL or Ab responses against pathogen- and tumor cell-derived antigens. Chimeric VLPs can be generated by conjugating or fusing target antigens to viral structural proteins that self assemble into VLPs.

Recombinant hepatitis B virus core-based VLPs

The hepatitis B virus (HBV) core (HBc) protein was first reported as a promising recombinant VLP (**Figure 3.1D**). It was demonstrated that foreign sequences of up to approximately 40 amino acid residues at the N terminus, 50 or 100 amino acids in the central immunodominant HBc and HBe regions 1 epitope region of HBc antigen, and up to 100 or even more residues at the C terminus did not interfere with particle formation [16]. Retention of self-assembly competence is of the utmost importance for the construction of vaccine candidates. Chimeric HBc/HCV were first developed for diagnosis and later tested as vaccine candidates (**Table 3.1**). Multivalent chimeric HBc-based VLP vaccines that carried highly conserved HCV core and NS3 antigens have been designed [17,18]. However, immunization with the chimeric HBc/HCV particles led to relatively low immune responses to HCV epitopes [17,18], highlighting the influence of antigens position within HBc antigen to their immunogenicity [19].

Recombinant HBV small-surface antigen-based VLPs

The small surface antigen of HBV (HBsAg-S) that is currently used in commercial vaccines against hepatitis B self-assembles into highly organized 22-nm pseudoparticles. These HBV surface-derived VLPs contain approximately 100 HBsAg-S molecules [20] and offer a unique opportunity to display multiple copies of foreign epitopes. HCV-specific epitopes derived from the hypervariable region 1 of E2 were selected and inserted into HBsAg-S (**Figure 3.1E & Table 3.1**). Hypervariable region 1-specific Ab responses were efficiently induced after vaccination [21,22], demonstrating that HBsAg VLPs constitute a suitable platform as an epitope delivery system for defined neutralizing epitopes.

Alternatively, an original strategy was used to incorporate the full-length HCV envelope proteins into HBsAg VLPs (**Figure 3.1F**). Chimeric HBV–HCV envelope proteins have been designed in which the N-terminal transmembrane domain of the HBsAg-S protein was replaced by the transmembrane domain of HCV-E1 or E2, to generate fusion proteins containing the entire HCV-E1 or -E2 sequence that are efficiently coassembled with HBsAg-S into particles [23], in an appropriate conformation. The chimeric HBV–HCV-VLPs were used to immunize rabbits (**Table 3.1**), and high titers of Abs capable of neutralizing various HCV genotypes and HBV were reported after vaccination [24], providing further support for the development of a bivalent HBV–HCV vaccine [24].

Recombinant papaya mosaic virus coat protein-based VLPs

Numerous plant viruses possess a complex and repetitive organization that can be reproduced after expression of structural proteins with

self-assembling properties. The resulting VLPs have been successfully used as vaccine platforms to present peptides of interest. Proof of concept was established with Johnson grass mosaic virus coat protein (CP)-based VLPs used as a recombinant vaccine platform and conferring protection against Japanese encephalitis virus in a mouse model [25]. Similarly, papaya mosaic virus (PapMV) CP can self-assemble into VLPs. The nanoparticles ranging from 60 to 100 nm in length are formed with several hundred CP subunits organized in a repetitive manner [26]. PapMV VLPs were proposed as a recombinant peptide vaccine platform using chimeric protein in which HCV-E2-derived peptide was fused to the C-terminal PapMV CP (**Figure 3.1G**). It has been shown that the recombinant multimeric PapMV vaccine platform triggers strong and long-lasting immune responses against the C-terminal fused HCV epitope after mouse immunization [27,28]. Notably, the same platform lost its immunogenic properties when injected as a monomeric protein, validating the finding that a repetitive organization is one of the key properties of molecules triggering an immune response.

Altogether, while the HBc, HBV surface and PapMV vaccine platforms are clearly versatile, and induce antigen-specific immune responses, it was also shown that the fusion of antigen could interfere with the assembly process of the proteins into VLPs or modify the antigen conformation and consequently limit their use as vaccine. By contrast, the possibility of expressing full-length HCV envelope proteins instead of peptides, like the HBV–HCV system, appear to be a major advantage for the generation of anti-HCV NAbs, especially due to the right conformation of the expressed envelope proteins.

Recombinant retrovirus murine leukemia virus Gag-based VLPs

Retroviruses also offer an opportunity to display complete glycoproteins in their native conformation on their surface. The authors demonstrated that many heterologous viral glycoproteins can be incorporated into retrovirus particles and mediate infectivity, this latter property indicating a conserved conformation allowing proper binding to the receptor and postbinding entry events. This process, known as pseudotyping, allows the engineering and production of numerous chimeric VLPs derived from lentivirus (HIV, simian immunodeficiency virus) or oncoretrovirus (murine leukemia virus; MLV). Infectious pseudoparticles have been designed by pseudotyping HCV glycoproteins onto MLV-Gag retroviral core particles (HCV pseudoparticles; HCVpp). HCVpp have a preferential tropism for hepatic cells and are specifically neutralized by anti-E2 monoclonal Abs as well as sera of HCV-infected patients, revealing that E1E2 complexes assembled on HCVpp mimic those displayed by parenteral HCV [29]. As a

result, HCVpp have been proposed as a powerful and highly versatile tool to investigate the early events of HCV infection in detail, to identify HCV receptors and to detect and measure the NAbs from HCV patient sera. Alternatively, HCV-pseudotyped retrovirus-derived VLPs containing no viral genome (HCV-retroVLPs) have been generated for vaccine application [30]. The molecular details of these pseudoparticles were more recently studied and cryo-transmission electron microscopy revealed HCVpp and HCV-retroVLPs as regular 100-nm spherical structures containing the dense retroviral nucleocapsid surrounded by a lipid bilayer [31,32] in which E1E2 protein complexes were detected (**Figure 3.1C**).

When their immunogenicity was tested, it was observed that HCV-retroVLPs induced poor Ab responses in mice, particularly against E1 even after several injections [32]. As a result, structural modifications of envelope glycoproteins have been proposed to increase their integration at the surface of the particles and to improve their immunogenicity. Therefore, the C-terminal hydrophobic regions of both E1 and E2 proteins containing signals that are responsible for retaining these proteins in the endoplasmic reticulum [33,34] have been replaced with foreign transmembrane and cytoplasmic domains of glycoprotein from vesicular stomatitis virus (VSV). The authors observed that chimeric HCV-E1G glycoproteins improves E1 antigen incorporation into retrovirus-based VLPs [32] and allow the production of retroVLPs pseudotyped with the E1 protein alone (**Figure 3.1C**). Combining both retroVLPs pseudotyped with HCV-E1E2 or -E1G, the authors demonstrated that recombinant retroVLPs induced high-titer Abs, as well as NAb, in both mouse and macaque, in prime-boost strategies using HCV-recombinant viral vectors [32] or DNA vaccine [35] for priming. Importantly, E1-specific Abs were only detected when retroVLPs pseudotyped with HCV-E1G were injected, demonstrating that separate E1 and E2 immunizations are required to elicit an anti-E1 Ab response. Moreover, only VLPs were able to elicit significant levels of anti-E1 Abs in contrast with recombinant adenovirus and measles vectors, demonstrating the potential of the retrovirus-based platform. Most importantly, the NAb generated after HCV-retroVLP immunization were shown to cross-neutralize all of the HCV genotypes tested [32], demonstrating the interest of the retroVLPs to induce broad NAb.

VLP-based genetic vaccines

While VLPs are attractive candidates to generate neutralizing responses, their production may represent a limitation. In contrast with nonenveloped VLPs, which are formed by expression and self-assembly *in vitro* of structural proteins, production of enveloped VLPs, including HCV- or recombinant-derived VLPs, is often complex, requiring the expression of

multiple proteins in cell culture systems. Strategies based on *in vivo* VLP formation have been proposed. Genetic vaccines encoding structural proteins that self-assemble and form HCV-derived or -recombinant VLPs have been developed (**Table 3.2**).

Plasmid DNA encoding HCV recombinant retrovirus based VLPs

To circumvent the *in vitro* production of retroVLPs, DNA plasmids generating recombinant retroVLPs (plasmo-retroVLPs) has been proposed as vaccine candidates. The recombinant retroVLPs are thus formed *in situ* from transfected cell expressing Mo-MLV Gag and antigen encoding plasmid. The authors previously demonstrated that plasmo-retroVLPs induce significantly better antigen-specific responses and antiviral immune protection than 'classic' DNA vaccines [36,37]. This established that plasmo-retroVLPs represent a notable improvement over DNA vaccines, related to the retroVLP formation, and this strategy was used in HCV vaccine development. The authors demonstrated that HCV plasmo-retroVLPs are better immunogens for boosting cellular and humoral immune responses in primed animals than expression control plasmid expressing nonparticulate HCV-E1E2 antigens [30]. The immunogenicity and efficacy of HCV plasmo-retroVLPs as a vaccine candidate in the absence of recombinant virus vector priming was also demonstrated more recently and optimized protocols to induce HCV-specific broad cellular and humoral immune responses were defined [35]. Notably, a combination of plasmo-retroVLPs with retroVLPs in a heterologous prime-boost strategy induced HCV-specific immunity. Remarkably, both anti-E1 and -E2 Abs were induced in this prime-boost vaccination scheme, confirming also the authors previous observations that significant levels of anti-E1 Abs can only be obtained when E1 is dissociated from E2. Moreover, exploiting the advantage of DNA vaccination, the authors established a multigenotype vaccine approach combining plasmo-retroVLPs that express E1E2-antigens from different HCV strains and that display HCV-NS3 (**Figure 3.1C**), a key protective antigen since NS3-specific T responses have been linked to viral clearance. Altogether, these results exemplify the flexibility of the authors retrovirus-based platform and its unique ability to generate HCV-specific broad and complete immune responses after prime-boost immunization. The potential of this strategy using plasmo-retroVLPs was recently confirmed in macaques [BELLIER B, KLATZMANN D, UNPUBLISHED DATA] and should have important implications for the development of anti-HCV vaccines.

Recombinant VSV vector expressing HCV-VLPs

Rhabdoviruses, including VSV, have utility as viral vector for gene expression. VSV has a simple genome organization with only five genes (N,

P, M, G and L) and is unable to undergo reassortment or integration. Several groups have focused on using VSV as a candidate for HCV vaccination. Recombinant VSV (rVSV) has been engineered to express HCV core, E1 and E2 proteins (rVSV-C/E1/E2) that can assemble to form HCV-VLPs possessing properties similar to the ultrastructural properties of HCV virions [38]. Intravenous or intraperitoneal immunization of BALB/c mice with rVSV-C/E1/E2 resulted in significant HCV-core and E2-specific Ab responses [39]. However, this study did not reveal *in vivo* HCV-VLP formation after rVSV injection. In contrast with the retrovirus Gag, which induces the budding and the release of VLPs, HCV structural proteins assembled in HCV-VLPs are not spontaneously released from expressing cells [12]. As a result, spontaneous HCV-VLP release is limited in vaccine strategy using rVSV and thus limits the interest of this strategy. Similarly, DNA immunization encoding core, E1 and E2 antigens has the advantage of combining multiple antigens but does not induce VLP formation.

Conclusion

The prospects for HCV vaccines are better today than they have ever been. This is partly due to overall advances in vaccine technology and methods used to analyze and characterize immune responses, but it also stems from advances in our specific understanding of natural HCV immunity, HCV replication and kinetics and the development of new tools to test Ab immune responses. However, there is still a great deal of missing information. We still do not know which types of immune responses correlate with protection or clearance of HCV during natural infection. New vaccine studies also need to include higher level immunological analyses examining multifunctional activities of T cells and neutralizing epitope mapping. It is still too early to assess the cross-protection activity of the new vaccine candidates, mainly due to the difficulties of challenging large numbers of chimpanzees with multiple heterologous genotypes. The development of a small-animal model that is immunocompetent would advance the field enormously, although care should always be taken in extrapolating data to humans as many immune response studies in mice have not translated well to humans and primates.

To date, few vaccine candidates have progressed to Phase I/II trials, but the published data on both the efficacy and safety of these vaccines are limited. With several different vaccine approaches in various stages of development it appears likely that additional clinical trials will be implemented in the next 5 years. Among the most promising of these vaccines, VLPs, which have so many favorable immunological characteristics, should soon be tested in a clinical setting. The development of VLP-based

vaccines is supported by their successful development for the prevention of persistent viral infections, such as HBV and human papillomavirus. Other studies of VLP-based vaccination strategies have also shown promising results and led to Phase I testing against a number of disease indications, including seasonal and pandemic influenza, hepatitis B, malaria and HIV (reviewed in [40]). The large-scale vaccine manufacturing process could represent a bottleneck for HCV-VLP-based vaccine development. By contrast, the ease of large-scale production of clinical-grade plasmid DNAs makes genetic vaccines that express HCV-recombinant VLPs attractive vaccine candidates. All these encouraging data suggest that it should be possible to develop a prophylactic HCV vaccine in the near future.

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Summary.

- Hepatitis C virus (HCV) infects more than 130 million people globally and is now the leading cause of liver transplantation worldwide.
- Broad and strong T-cell responses may be induced to neutralize the HCV infection.
- The size, particulate nature and dense, repetitive structure of virus-like particles are the basis for their innate immunogenicity and their efficacy in inducing neutralizing antibody and T-cell responses.
- HCV-derived and -recombinant virus-like particles are considered as promising HCV vaccine candidates.

References

- 1 Shepard CW, Finelli L, Alter MJ. Global epidemiology of hepatitis C virus infection. *Lancet Infect. Dis.* 5(9), 558–567 (2005).
- 2 Stoll-Keller F, Barth H, Fafí-Kremer S, Zeisel MB, Baumert TF. Development of hepatitis C virus vaccines: challenges and progress. *Expert Rev. Vaccines* 8(3), 333–345 (2009).
- 3 Torresi J, Johnson D, Wedemeyer H. Progress in the development of preventive and therapeutic vaccines for hepatitis C virus. *J. Hepatol.* 54(6), 1273–1285 (2011).
- 4 Roohvand F, Kossari N. Advances in hepatitis C virus vaccines, Part one: advances in basic knowledge for hepatitis C virus vaccine design. *Expert Opin. Ther. Pat.* 21(12), 1811–1830 (2011).
- 5 Roohvand F, Kossari N. Advances in hepatitis C virus vaccines, part two: advances in hepatitis C virus vaccine formulations and modalities. *Expert Opin. Ther. Pat.* 22(4), 391–415 (2012).
- 6 Yu CI, Chiang BL. A new insight into hepatitis C vaccine development.

- J. Biomed. Biotechnol.* 2010, 548280 (2010).
- 7 Feinstone SM, Hu DJ, Major ME. Prospects for prophylactic and therapeutic vaccines against hepatitis C virus. *Clin. Infect. Dis.* 55(Suppl. 1), S25–S32 (2012).
- 8 Spohn G, Bachmann MF. Exploiting viral properties for the rational design of modern vaccines. *Expert Rev. Vaccines* 7(1), 43–54 (2008).
- 9 Chackerian AA, Oldham ER, Murphy EE, Schmitz J, Pflanz S, Kastelein RA. IL-1 receptor accessory protein and ST2 comprise the IL-33 receptor complex. *J. Immunol.* 179(4), 2551–2555 (2007).
- 10 Baumert TF, Vergalla J, Satoi J et al. Hepatitis C virus-like particles synthesized in insect cells as a potential vaccine candidate. *Gastroenterology* 117(6), 1397–1407 (1999).
- 11 Triyatni M, Vergalla J, Davis AR, Hadlock KG, Foung SK, Liang TJ. Structural features of envelope proteins on hepatitis C virus-like particles as determined by anti-envelope monoclonal antibodies and CD81 binding. *Virology* 298(1), 124–132 (2002).
- 12 Baumert TF, Ito S, Wong DT, Liang TJ. Hepatitis C virus structural proteins assemble into viruslike particles in insect cells. *J. Virol.* 72(5), 3827–3836 (1998).
- 13 Lechmann M, Murata K, Satoi J, Vergalla J, Baumert TF, Liang TJ. Hepatitis C virus-like particles induce virus-specific humoral and cellular immune responses in mice. *Hepatology* 34(2), 417–423 (2001).
- 14 Murata K, Lechmann M, Qiao M, Gunji T, Alter HJ, Liang TJ. Immunization with hepatitis C virus-like particles protects mice from recombinant hepatitis C virus–vaccinia infection. *Proc. Natl Acad. Sci. USA* 100(11), 6753–6758 (2003).
- 15 Elmowald GA, Qiao M, Jeong SH et al. Immunization with hepatitis C virus-like particles results in control of hepatitis C virus infection in chimpanzees. *Proc. Natl Acad. Sci. USA* 104(20), 8427–8432 (2007).
- 16 Ulrich R, Nassal M, Meisel H, Kruger DH. Core particles of hepatitis B virus as carrier for foreign epitopes. *Adv. Virus Res.* 50, 141–182 (1998).
- 17 Mihailova M, Boos M, Petrovskis I et al. Recombinant virus-like particles as a carrier of B- and T-cell epitopes of hepatitis C virus (HCV). *Vaccine* 24(20), 4369–4377 (2006).
- 18 Sominskaya I, Skrastina D, Dislers A et al. Construction and immunological evaluation of multivalent hepatitis B virus (HBV) core virus-like particles carrying HBV and HCV epitopes. *Clin. Vaccine Immunol.* 17(6), 1027–1033 (2010).
- 19 Schodel F, Moriarty AM, Peterson DL et al. The position of heterologous epitopes inserted in hepatitis B virus core particles determines their immunogenicity. *J. Virol.* 66(1), 106–114 (1992).
- 20 Bruss V, Gerhardt E, Vieluf K, Wunderlich G. Functions of the large hepatitis B virus surface protein in viral particle morphogenesis. *Intervirology* 39(1–2), 23–31 (1996).
- 21 Netter HJ, Macnaughton TB, Woo WP, Tindle R, Gowans EJ. Antigenicity and immunogenicity of novel chimeric hepatitis B surface antigen particles with exposed hepatitis C virus epitopes. *J. Virol.* 75(5), 2130–2141 (2001).
- 22 Vietheer PT, Boo I, Drummer HE, Netter HJ. Immunizations with chimeric hepatitis B virus-like particles to induce potential anti-hepatitis C virus neutralizing antibodies. *Antivir. Ther.* 12(4), 477–487 (2007).
- 23 Patient R, Houroux C, Vaudin P, Pages JC, Roingeard P. Chimeric hepatitis B and C viruses envelope proteins can form subviral particles: implications for the design of new vaccine strategies. *N. Biotechnol.* 25(4), 226–234 (2009).
- 24 Beaumont E, Patient R, Houroux C, Dimier-Poisson I, Roingeard P. Chimeric HBV-HCV envelope proteins elicit broadly neutralizing antibodies and constitute a potential bivalent prophylactic vaccine. *Hepatology* 57(4), 1303–1313 (2012).
- 25 Saini M, Vrati S. A Japanese encephalitis virus peptide present on Johnson grass mosaic virus-like particles induces virus-neutralizing antibodies and protects mice against lethal challenge. *J. Virol.* 77(6), 3487–3494 (2003).
- 26 Tremblay MH, Majean N, Gagne ME et al. Effect of mutations K97A and E128A on RNA binding and self assembly of papaya mosaic potexvirus

- coat protein. *FEBS J.* 273(1), 14–25 (2006).
- 27 Denis J, Majeanu N, Acosta-Ramirez E *et al.* Immunogenicity of papaya mosaic virus-like particles fused to a hepatitis C virus epitope: evidence for the critical function of multimerization. *Virology* 363(1), 59–68 (2007).
- 28 Leclerc D, Beauseigle D, Denis J *et al.* Proteasome-independent major histocompatibility complex class I cross-presentation mediated by papaya mosaic virus-like particles leads to expansion of specific human T cells. *J. Virol.* 81(3), 1319–1326 (2007).
- 29 Bartosch B, Dubuisson J, Cosset FL. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. *J. Exp. Med.* 197(5), 633–642 (2003).
- 30 Desjardins D, Huret C, Dalba C *et al.* Recombinant retrovirus-like particle forming DNA vaccines in prime-boost immunization and their use for hepatitis C virus vaccine development. *J. Gene Med.* 11(4), 313–325 (2009).
- 31 Bonnafous P, Perrault M, Le Bihan O *et al.* Characterization of hepatitis C virus pseudoparticles by cryo-transmission electron microscopy using functionalized magnetic nanobeads. *J. Gen. Virol.* 91(Pt 8), 1919–1930 (2010).
- 32 Garrone P, Fluckiger AC, Mangeot PE *et al.* A prime-boost strategy using virus-like particles pseudotyped for HCV proteins triggers broadly neutralizing antibodies in macaques. *Sci. Transl. Med.* 3(94), 94ra71 (2011).
- 33 Cocquerel L, Duvet S, Meunier JC *et al.* The transmembrane domain of hepatitis C virus glycoprotein E1 is a signal for static retention in the endoplasmic reticulum. *J. Virol.* 73(4), 2641–2649 (1999).
- 34 Flint M, McKeating JA. The C-terminal region of the hepatitis C virus E1 glycoprotein confers localization within the endoplasmic reticulum. *J. Gen. Virol.* 80(Pt 8), 1943–1947 (1999).
- 35 Huret C, Desjardins D, Miyalou M *et al.* Recombinant retrovirus-derived virus-like particle-based vaccines induce hepatitis C virus-specific cellular and neutralizing immune responses in mice. *Vaccine* 31(11), 1540–1547 (2013).
- 36 Bellier B, Dalba C, Clerc B *et al.* DNA vaccines encoding retrovirus-based virus-like particles induce efficient immune responses without adjuvant. *Vaccine* 24(14), 2643–2655 (2006).
- 37 Bellier B, Huret C, Miyalou M *et al.* DNA vaccines expressing retrovirus-like particles are efficient immunogens to induce neutralizing antibodies. *Vaccine* 27(42), 5772–5780 (2009).
- 38 Ezelle HJ, Markovic D, Barber GN. Generation of hepatitis C virus-like particles by use of a recombinant vesicular stomatitis virus vector. *J. Virol.* 76(23), 12325–12334 (2002).
- 39 Majid AM, Ezelle H, Shah S, Barber GN. Evaluating replication-defective vesicular stomatitis virus as a vaccine vehicle. *J. Virol.* 80(14), 6993–7008 (2006).
- 40 Buonaguro FM, Tornesello ML, Buonaguro L. New adjuvants in evolving vaccine strategies. *Expert Opin. Biol. Ther.* 11(7), 827–832 (2011).
- 41 Qiao M, Murata K, Davis AR, Jeong SH, Liang TJ. Hepatitis C virus-like particles combined with novel adjuvant systems enhance virus-specific immune responses. *Hepatology* 37(1), 52–59 (2003).
- 42 Jeong SH, Qiao M, Nascimbeni M *et al.* Immunization with hepatitis C virus-like particles induces humoral and cellular immune responses in nonhuman primates. *J. Virol.* 78(13), 6995–7003 (2004).
- 43 Netter HJ, Woo WP, Tindle R, Macfarlan RI, Gowans EJ. Immunogenicity of recombinant HBsAg/HCV particles in mice pre-immunised with hepatitis B virus-specific vaccine. *Vaccine* 21(21–22), 2692–2697 (2003).
- 44 Duenas-Carrera S, Vina A, Martinez R *et al.* Immunization with a DNA vaccine encoding the hepatitis-C-virus structural antigens elicits a specific immune response against the capsid and envelope proteins in rabbits and *Macaca irus* (crab-eating macaque monkeys). *Biotechnol. Appl. Biochem.* 39(Pt 2), 249–255 (2004).

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Chapter

4

Evaluation of human rotavirus VLP vaccines in neonatal gnotobiotic pigs

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Marli P Azevedo, Anastasia N Vlasova & Linda J Saif

We discuss here rotavirus virus-like particle (VLP) vaccines as an alternative approach to oral live-attenuated rotavirus vaccines and their efficacy in a gnotobiotic pig model. Rotavirus VLPs were evaluated in different doses, routes of administration, combined with live-attenuated virus and adjuvants. A VLP vaccine composed of rotavirus VP2 and VP6 was immunogenic in gnotobiotic pigs when inoculated intranasally; however, this vaccine failed to confer protection. A combination of oral-attenuated human rotavirus and intranasal 2/6-VLP vaccines conferred immunogenicity, partial protection against a human rotavirus challenge and induced IFN- γ -producing T cells in the ileum of pigs in similar frequencies to human rotavirus infections. Vaccination through a combination of mucosal inductive sites and live-attenuated vaccine combined with VLP vaccines was the most effective regimen, compared with the use of a single route or a single vaccine alone. Moreover, if formulated with neutralizing antigens, VLP vaccines may constitute a better approach in populations with high maternal antibodies.

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Rotaviruses (RV) are nonenveloped icosahedral viruses, with a double-stranded segmented RNA genome. The viral capsid is formed by three concentric protein layers. The viral proteins VP1, VP2 and VP3 form the core, which is surrounded by an inner layer composed of VP6. RV are classified in groups A to G based on VP6 characteristics [1]. VP4 (protease-sensitive protein) and VP7 (glycoprotein) compose the outermost layer and represent the viral neutralizing (VN) antigens. A dual nomenclature system was created based on the diversity of G (VP7) and P (VP4) proteins. In humans, 12 G and 15 P types have been reported [2].

RV is the most common cause of infantile diarrhea worldwide accounting for 453,000 deaths in infants and young children yearly. RV-associated deaths are more frequent in developing countries [3]. Two RV vaccines are currently available: Rotarix® (GlaxoSmithKline Biologic) a monovalent vaccine derived from a clinical human isolate of serotype G1P1A[8]; and Rotateq® (Merck) a pentavalent vaccine containing human RV (HRV) VP7 proteins of serotypes G1, G2, G3 and G4 and genotype P1A[8] on a G6P5 bovine RV background (WC3 strain) [4]. Clinical trials of the current RV vaccines reported at least 85% protection rates against severe diarrhea [4–6]. In developing countries, trials of these second-generation vaccines induced overall efficacies of 51% against RV diarrhea [7,8].

Consequently, there is a need to develop vaccines that are more efficacious in developing countries, and among other obstacles, will circumvent maternal antibody interference [4,9]. Immunity to RV occurs after successive natural HRV infections in children, with protection rates against reinfection and diarrhea increasing after each subsequent infection. In this scenario, secondary infections are most often caused by different G serotypes [10].

Protection against RV infection is complex and not totally understood. Both intestinal IgA antibody-secreting cell (ASC) numbers (in pigs) and serum IgA antibody titers (in pigs, mice and humans) have been correlated with protection against reinfection [11–13]. In humans, both serum neutralizing and IgA antibodies have been correlated with protection against RV diarrhea [12,14]. Although serum IgA antibodies have been used as a marker of vaccine seroconversion in children [15], analysis of other data by Angel *et al.* [16] suggest that a correlation does not exist between vaccine-induced serum IgA and protection against RV diarrhea.

As an alternative to live vaccines, virus-like particles (VLPs) have been evaluated as recombinant nonreplicating candidate vaccines. They lack viral nucleic acids and therefore are noninfectious. But in contrast to live vaccines, nonreplicating vaccines administered orally require effective mucosal adjuvants to magnify their immunogenicity and to overcome oral

tolerance, and effective delivery systems to prevent antigen degradation in the stomach. RV VLPs have been produced by coexpression of the VP2, VP6, VP4 and/or VP7 using baculovirus expression systems; however, the presence of VP2 has been shown to be essential for particle formation and stability [17,18].

We discuss here the use of RV 2/6-VLP vaccines to induce protective immunity against HRV infection in the **gnotobiotic pig** model of HRV infection and disease.

Gnotobiotic pig model of HRV infection & disease

Gnotobiotic pigs are a unique animal model to assess HRV disease pathogenesis and to evaluate RV vaccines in the presence or absence of a defined gut microflora or maternal antibodies. Because of the sow's placental type (epitheliochorial) that prohibits the *in utero* passage of immunoglobulins, pigs are born devoid of circulating maternal antibodies, which are acquired post-partum via the colostrum after suckling the sow [19]. Colostrum-deprived gnotobiotic pigs can be reconstituted with circulating only or circulating and intestinal passive maternal antibodies to mimic bottle- versus breast-fed infants, respectively. Neonatal pigs resemble infants in their physiology, anatomy and in the development of mucosal immunity [20,21]. They are immunocompetent at birth, but immunologically immature and, as outbred animals, they are more representative of human population heterogeneity [22]. HRV-infected gnotobiotic pigs exhibit diarrhea, transient viremia and intestinal lesions (villous atrophy) mimicking that in children [23,24]. The gnotobiotic pig model is susceptible to HRV diarrhea for at least 8 weeks of age, which is the time necessary to assess protective immunity against disease upon challenge [13].

In summary, similarities between the gastrointestinal tract physiology of gnotobiotic pigs and humans, the development of mucosal immunity and their extended susceptibility to infection with HRV strains indicate that they are a highly relevant animal model for the study of HRV-induced disease and immunity.

RV 2/6-VLP vaccines evaluated in gnotobiotic pigs

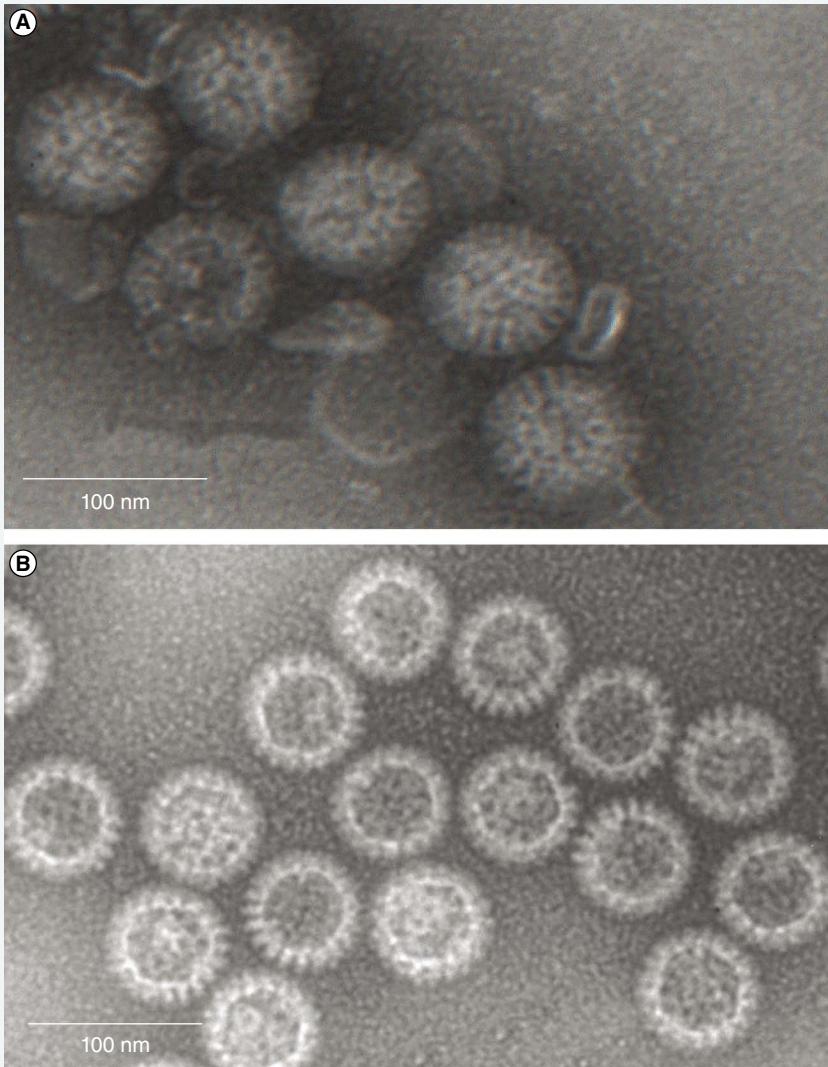
The first VLP vaccine to be evaluated in the gnotobiotic pig model for its immunogenicity and protective efficacy was a double-layered 2/6-VLP vaccine [25]. In that study, RV 2/6-VLPs were formed by the coexpression of the VP2 protein of the bovine RF strain and the VP6 protein of the simian



Gnotobiotic pigs: pigs that have been rendered free of contaminants, into which known microorganisms can be introduced for research purposes.

RV SA11 or human Wa strains in a baculovirus expression system. Coexpression of VP2 and VP6 resulted in spontaneous assembly into double-layered 2/6-VLPs (**Figure 4.1**).

Figure 4.1. Electron micrographs of intact human rotaviruses and 2/6-virus-like particles.



(A) Human rotaviruses and **(B)** 2/6-virus-like particles.

In our studies, neonatal pigs were inoculated with 250 µg of group A RV 2/6-VLP given with or without 5 µg of mutant LT-R192G (mLT) as adjuvant in two or three



2/6-VLP+mLT or ISCOM adjuvant were immunogenic but not protective against human rotavirus (HRV) diarrhea in gnotobiotic pigs.

intranasal dose regimens (**Table 4.1**). The 2/6-VLP-mLT vaccine induced higher levels of intestinal IgA, higher numbers of IgA ASC and memory B-cell responses compared with 2/6-VLP vaccine without mLT. As expected for 2/6-VLP vaccines lacking the RV-neutralizing antigens, no pigs developed VN antibodies and the 2/6-VLP vaccine failed to induce protection against diarrhea after oral virulent Wa HRV (VirHRV) challenge [25]. The failure of the 2/6-VLP vaccine to induce protective immunity in this animal model is likely to be due to the lack of induction of VN antibodies generated against the external capsid proteins, VP4 and VP7.

In further attempts to increase the immunogenicity of the 2/6-VLP vaccine, 250-µg doses were administered in association with 1.25 g of immunostimulating complex matrix (ISCOM) as adjuvant. ISCOM was previously reported to improve the efficacy of subunit vaccines [26,27]. The 2/6-VLP-ISCOM vaccine failed to induce detectable levels of RV-neutralizing antibody as expected and also failed to induce serum IgA antibody titers at challenge with VirHRV. A delayed serum IgG antibody response was also observed when compared with three doses of live AttHRV (mimic live attenuated RV vaccine). Three oral doses of 2/6-VLP-ISCOM also failed to induce protection in gnotobiotic pigs [28,29]. Thus, 2/6-VLP administered intranasally with mLT or ISCOM adjuvants were immunogenic, but did not confer protection against RV infection or diarrhea in gnotobiotic pigs.

Boosting live AttHRV vaccine protection with 2/6-VLP

Because the 2/6-VLP vaccine failed to induce protection against HRV diarrhea and virus shedding, a 2/6-VLP composed of RF BRV VP2 and Wa HRV VP6 (major antigenic protein Wa VP6 homologous to the challenge VirHRV Wa strain) was evaluated in a combined regimen with live-attenuated oral RV (AttHRV) vaccine (**Table 4.1**). In all our studies, we used the tissue culture-attenuated HRV Wa strain, which has a similar genetic G/P type to the licensed RV vaccine Rotarix (G1P1A[8]). These studies were designed to reduce the number of doses of live-attenuated vaccines needed to induce protection in a gnotobiotic pig model and thus, minimizing the potential adverse effects of live vaccines.

When 250 µg/5 µg of 2/6-VLP+mLT was used as intranasal booster after oral priming with live AttHRV vaccine, protection rates of 44% against diarrhea and 58% against virus shedding were observed [30]. Although not statistically different, these rates were slightly lower than rates observed

Table 4.1. Human rotavirus 2/6-virus like particle vaccines evaluated in neonatal gnotobiotic pigs.

Priming dose	Amount	Route	Booster doses	Amount	Route	Adjuvant	Note	Protection diarrhea (%)	Protection virus shed (%)	Ref.
2/6-VLP [#]	250 µg	IN	2/6-VLP 2x [†]	250 µg	IN	mLT		0	0	[25]
AttHRV [§]	5 × 10 ⁷ FFU	O	2/6-VLP 2x [†]	250 µg	IN	mLT		44	58	[30,32]
2/6-VLP [†]	250 µg	IN	2/6-VLP [†] / AttHRV [§]	250 µ/5 × 10 ⁷ FFU	O	mLT		25	17	[30,32]
2/6-VLP [†]	250 µg	O	2/6-VLP 2x	250 µg	O	ISCOM		0	0	[28,29]
AttHRV [§]	5 × 10 ⁷ FFU	O	2/6-VLP 2x	250 µg	O	ISCOM		50	75	[28,29]
2/6-VLP [†]	250 µg	IN	2/6-VLP 2x	250 µg	IN	ISCOM		0	0	[31,34,39]
AttHRV [§]	5 × 10 ⁷ FFU	O	2/6-VLP 2x	50 or 100 µg	IN	ISCOM	0 or 33	33		[31,34,39]
AttHRV [§]	5 × 10 ⁷ FFU	IN	2/6-VLP 2x	250 µg	IN	ISCOM		17	50	[31,34,39]
AttHRV [§]	5 × 10 ⁷ FFU	O	2/6-VLP 2x	250 µg	IN	ISCOM		71	71	[31,34,39]
AttHRV [§]	5 × 10 ⁷ FFU	O	2/6-VLP 2x	250 µg	IN	ISCOM	High or low maternal antibody	50	50 or 67	[42,43]
2/6-VLP [†]	250 µg	IN	2/6-VLP 2x	250 µg	IN	ISCOM	High or low maternal antibody	29 or 0	0	[42,43]

Protection rates evaluated after challenge with Wa Viral HRV at 10⁶ FFU dose.[†]RF VP2/Wa VP6.[‡]RF VP2/SA11 VP6.[§]Wa strain.

FFU: Focus-forming unit; IN: Intranasal; ISCOM: Immunostimulating complex matrix; mLT: Mutant LT-R192G; O: Oral; VLP: Virus-like particle.

after three oral doses of live AttHRV vaccine, 44 and 67%, respectively [31]. However, when 2/6-VLP was used as prime-boost intranasally, followed by a final boost with oral AttHRV vaccine

(2/6-VLP-mLT+AttHRV), only a 25% protection rate against diarrhea was observed [30]. Prior to challenge all pigs primed with oral AttHRV and boosted with two-times VLPs intranasally seroconverted with serum IgA antibodies to HRV and had significantly higher intestinal IgA antibody titers, measured against the intact RV particles. In this group and in the group receiving three doses of live AttHRV, VN antibodies correlated with RV-specific IgA antibody titers in the serum. Higher HRV-specific antibody titers in serum were significantly correlated with protection. Serum IgA antibody titers, but not IgM or IgG, were highly correlated with the corresponding antibody isotype in the intestine among vaccinated groups [32].

In a subsequent study, two doses of 2/6-VLP (250 µg) adjuvanted with ISCOM (1.25 g) were administered as oral booster vaccine after one oral prime dose of live AttHRV vaccine. The combined vaccine regimen, using a single oral route of immunization induced 44 and 50% protection rates against diarrhea and virus shedding respectively, after the highest dose of VLP [28,29]. Intriguingly, the 2/6-VLP-ISCOM vaccine boosted VN antibody titers and protection after oral priming with AttHRV. One possible explanation is that 2/6-VLP boosted responses against all RV proteins through cross-reactive T helper cells, providing cognate help to external viral protein-specific B cells [33]. This vaccine regimen induced intestinal and systemic IgA antibodies and intestinal IgA ASC numbers comparable to three oral doses of live AttHRV.

In conclusion, two oral doses of 2/6-VLP-ISCOM or two intranasal doses of 2/6-VLP-mLT vaccines used as booster after oral priming with live-attenuated RV-induced intestinal and systemic ASC responses and protection rates similar to three doses of live oral AttHRV vaccine.

Use of combination routes & vaccine combinations in prime-boost regimens to induce protection, IgA antibody & ASC responses in gnotobiotic pigs

The impact of mucosal prime/boost vaccine regimens and vaccine type (live-attenuated vs nonreplicating) on protection, serum and intestinal antibody and on ASC responses was determined. The 2/6-VLP intranasal dose needed to boost immune response to live AttHRV vaccine was also examined. Different booster doses of 2/6-VLP (25, 100 and 250 µg)



A combination of priming with AttHRV+2/6-VLPmLT or ISCOM boosters induced partial protection against HRV diarrhea, similar to the protection elicited by 3xAttHRV vaccines.



Combining replicating AttHRV and nonreplicating virus-like particle (VLP) vaccines and different routes of vaccination improved protection against HRV diarrhea.

induced a dose-response effect on the protection rates against diarrhea in pigs primed orally with live AttHRV (**Table 4.1**). Protection against diarrhea was observed only after doses of at least 100 µg and the

duration of virus shedding was decreased significantly with a 2/6-VLP 250-µg dose. The dose-response effects were also reflected in the ASC responses. IgA ASC responses in the intestine increased proportionally with increasing 2/6-VLP booster doses. Induction of IgA ASC in the spleen also required the highest dose (250 µg) [31]. Intestinal IgA antibody titers to HRV were significantly higher in the prime-boost oral/intranasal AttHRV-2/6-VLP-ISCOM group than in all other groups and VN antibody titers were similar to those after three oral doses of live AttHRV [34].

The effect of oral versus intranasal priming with AttHRV using the optimal dose of 2/6-VLP (250 µg) and ISCOM adjuvant was then assessed. The highest protection rates (71%) against virus shedding and diarrhea were induced by oral priming with live virus followed by two intranasal 2/6-VLP-ISCOM booster doses. Other routes were explored in this study and evaluated for protection rates against virus shedding and diarrhea, respectively: intranasal priming/boosting with AttHRV-2/6-VLP-ISCOM induced only 17 and 50% protection; three intranasal doses of live AttHRV induced 50 and 67% protection rates; and three oral doses of live AttHRV induced 44 and 67% protection rates. Differences between the use of 2/6-VLP intranasal as vaccine booster and the different routes of immunization to prime used in that study were reflected by the intestinal ASC responses, which were up to 11-fold higher for IgA ASC and up to 12-fold higher for IgG ASC responses in the group receiving the oral/intranasal AttHRV-2/6-VLP-ISCOM vaccine compared to the other vaccine regimens and routes tested [31].

Oral versus intranasal priming effectiveness can be attributed to the largest mass of lymphoid tissue located in the gut, compared to more restricted lymphoid tissue in the respiratory tract [35]. However, the gut may be more limiting for VLP vaccines in the absence of an effective delivery system. On the other hand, it is possible that local antibodies induced by the oral priming dose may partially block the oral booster doses. Thus, both the type of booster and the route of vaccination may play a role to overcome the blockage. In summary, vaccination through a combination of mucosal inductive sites and live attenuated vaccine combined with VLP vaccines were the most effective regimens compared to single route of vaccination or repeated vaccination with live AttHRV alone or 2/6-VLP vaccine alone.

T-cell responses to VLP vaccines in gnotobiotic pigs

RV-specific cell-mediated immune responses have been also associated with protection in humans, pigs and mice [36–38].

Few studies have been conducted to determine cell-mediated immunity after VLP administration in mice or in piglets. RV-specific IFN- γ producing CD4 $^{+}$, CD8 $^{+}$ and CD4 $^{+}$ CD8 $^{+}$ T-cell responses were examined in gnotobiotic pigs infected with VirHRV, vaccinated with live AttHRV or oral priming and intranasal boosting with AttHRV-2/6-VLP-ISCOM regimen (**Table 4.1**). Flow cytometer analysis of RV *in vitro* restimulated cells revealed that the combination of oral live AttHRV and intranasal 2/6-VLP vaccine induced RV-specific IFN- γ -producing CD4 $^{+}$ T cells in ileum of gnotobiotic pigs with frequencies similar to a low dose of VirHRV, whereas oral AttHRV vaccine alone was less effective [39]. In our previous studies, 10^6 FFU of VirHRV induced a 100% protection rate against diarrhea upon subsequent rechallenge and 10^5 FFU (used in this study) induced an 87% protection rate.

The AttHRV-VLP-ISCOM induced spleen RV-specific IFN- γ CD4 $^{+}$, IFN- γ CD8 $^{+}$ and IFN- γ CD4 $^{+}$ CD8 $^{+}$ T cells similar to three oral doses of AttHRV vaccine. Most importantly, 2/6-VLP as booster after AttHRV priming induced higher intestinal RV-specific IFN- γ -producing T cells than oral AttHRV or 2/6-VLP vaccines alone. The AttHRV-2/6-VLP-ISCOM vaccine induced significantly higher IFN- γ CD4 $^{+}$ and CD8 $^{+}$ T-cell responses than VirHRV challenge alone (mimicking natural infection), which can be attributed to the vaccine delivery through multiple mucosal inductive sites in combination with the use of 2/6-VLP-ISCOM as booster. In both groups, VirHRV and AttHRV-2/6-VLP-ISCOM, protection rates against RV diarrhea upon VirHRV challenge significantly correlated with frequencies of intestinal IFN- γ -producing T cells, suggesting their role in protective immunity. Thus, multiple vaccine types and inoculation routes, when optimally combined, induced not only robust intestinal B-cell responses but also higher T-cell responses than single formulation/route vaccines (oral AttHRV) in a gnotobiotic pig model.

Obstacles for RV vaccines & RV VLP vaccines

Effect of maternal antibodies

The presence of circulating maternal antibodies and high levels of maternal antibodies in breast milk may play a role in the efficacy of oral live RV vaccines in developing countries [40]. Studies of maternal antibody effects on protection and immune responses to RV VLP vaccines were



An optimal combination of vaccine types and inoculation routes induced not only robust intestinal B-cell responses, but also higher T-cell responses than single formulation/route vaccines.

conducted in gnotobiotic pigs. Normal sow sera with low RV antibodies were used to mimic maternal RV antibody titers in women in industrialized countries versus the use of high titer hyperimmune sow serum to mimic maternal RV antibody titers in women in developing countries [41].

In the first study, low-RV-antibody-titer sow serum was injected into the peritoneal cavity of newborn gnotobiotic pigs to provide circulating passive antibodies. Piglets were then vaccinated intranasally with three doses of 250 µg of 2/6-VLP-ISCOM or vaccinated orally with AttHRV and boosted with two intranasal doses of 250 µg of 2/6-VLP-ISCOM (**Table 4.1**). Low-titer passive antibody did not suppress induction of IgA effector and memory B cells, but impaired their homing to effector sites. Low-titer passive maternal antibody suppressed IgG ASC responses in the 2/6-VLP group, but not in the AttHRV group. However, low passive maternal antibody titers suppressed systemic memory IgA and IgG ASC in both groups. In the intestine, low or no inhibition was observed with the AttHRV-VLP vaccine regimen, and enhancement of intestinal IgM and IgA ASC responses was observed for both vaccines. Nguyen *et al.* postulated that at low levels, passive antibodies may enhance uptake, processing and presentation of viral proteins when complexed with low amounts of antibody [42]. The AttHRV-VLP vaccine regimen partially overcame the suppressive effect of the low-titer maternal antibody and conferred partial protection against VirHRV challenge. Although not statistically significant, protection rates in the magnitude of 67 and 50% against virus shedding and diarrhea were observed, respectively, in the AttHRV-VLP group in the presence of passive maternal antibody, compared with 71% protection rates against virus shedding and diarrhea observed for the same vaccine regimen in the absence of passive maternal antibody serum [42].

In a subsequent study, the effects of high RV maternal antibody titers (HRV hyperimmune sow serum) were evaluated [43]. Pigs were injected with sow serum and vaccinated as described earlier. High levels of passive circulating RV antibodies induced significant suppression of intestinal IgA and IgG effector, memory B cells and antibody titers in pigs receiving the AttHRV-VLP-ISCOM vaccine. Although not statistically significant, protection rates in the AttHRV-VLP vaccine group were also affected. Protection against diarrhea and virus shedding were observed in 50% of the pigs receiving AttHRV-VLP-ISCOM, compared to 71% of pigs that did not receive maternal antibody. As expected, protection was not induced by the 2/6-VLP vaccines alone; however, their low intestinal IgA and IgM ASC responses were not affected [43]. These results suggest that a nonreplicating vaccine may be more effective in the presence of

high levels of maternal antibodies. Studies using 2/4/6/7VLPs are needed to extend and clarify these findings.



Nonreplicating vaccines may be more effective in the presence of high levels of maternal antibodies than live-attenuated vaccines.

Effects of passive immunity using VLP

vaccines, derived from the bovine RF strain, were also studied in other animal models. Vaccination of cows (**Table 4.2**) with SA11 2/4/6/7VLP increased VN and IgG₁ antibody titers in colostrum by 70- and 64-fold, respectively, compared with control cows, conferring complete protection to calves against diarrhea after challenge with virulent bovine RV (**Table 4.3**) [44]. Only partial protection was seen in calves receiving colostrum from cows vaccinated with SA11 2/6-VLP [45]. Vaccination of cows with 250 µg of Indiana strain (P[5]G6) 2/4/6/7VLP, 250 µg 2292B (P[11]G10) 2/4/6/7VLP or combined (125 µg each) IND/2292B 2/4/6/7VLP with incomplete Freund's adjuvant induced in colostrum and milk IgG₁ and IgA antibodies, with homologous and heterologous VN activities. The combined IND/2292B 2/4/6/7VLP vaccine induced VN antibody responses to bovine RV in serum, colostrum and milk comparable to the individual VLP. Thus, VLP of two or more serotypes can be combined to confer multiserotypic immunity [46].

Our studies have shown that the presence of maternal antibodies can enhance or suppress neonatal immune responses to RV vaccines and these effects have also been observed in other animal models. However, as suggested by Nguyen *et al.* [42], the effect may depend on the ratio

Table 4.2. Lactogenic antibody responses in cows vaccinated with rotavirus virus-like particles.

Priming dose	Amount	Route	Booster	Amount	Route	Adjuvant	Antibody responses	Ref.
2/6/4/7-SA11 VLP	250 µg or 100 µg	IM	2/6-VLP	250 µg	IMm	IFA	VLPs enhanced serum, colostrum and milk antibodies	[44]
2/6-SA11 VLP	250 µg	IM	2/6-VLP	250 µg	IMm	IFA		
2/6/4/7-IND VLP	250 µg	IM	2/6/4/7-VLP IND	250 µg	IMm	IFA	High viral neutralizing and IgG ₁ in serum and milk and high IgA in milk	[46]
2/6/4/7-2292B VLP	250 µg	IM	2/6/4/7-VLP 2292B	250 µg	IMm	IFA		
INDVLP+2292B VLP	125 µg + 125 µg	IM		125 µg + 125 µg	IMm	IFA		

IFA: Incomplete Freund's adjuvant; IM: Intramuscular; IMm: Intramammary; VLP: Virus-like particle.

Table 4.3. Passive protection induced in calves by lactogenic antibodies from cows immunized with rotavirus virus-like particles.

Feed	Challenge			Mean days shedding	Mean days with diarrhea
	Virus strain	Dose	Route		
Colostrum from 2/6/4/7-SA11 VLP vaccinated cows	Virulent IND BRV	5×10^7 FFU	Oral	1.6	0
Colostrum from 2/6-SA11 VLP vaccinated cows	Virulent IND BRV	5×10^7 FFU	Oral	3.4	0.3
Normal colostrum	Virulent IND BRV	5×10^7 FFU	Oral	5.8	7.6
No colostrum	Virulent IND BRV	5×10^7 FFU	Oral	6.0	3.6

FFU: Focus-forming units; VLP: Virus-like particle.

Data taken from [45].

between maternal antibody levels and the amount of VLP administered. Thus further studies should explore different doses of VLPs, as well as different VLP constructs, such as triple-layered VLPs and/or multivalent VLPs. Because of the window of HRV susceptibility of gnotobiotic pigs, although longer than other animal models, the long-term effects of the presence of maternal antibodies at the time of HRV vaccination remain to be defined.

Generation of new RV reassortants in vaccinated population

Another challenge regarding the use of live attenuated RV vaccines is the fact that they could contribute to the generation of new reassortants RV, which were suggested to contribute to the modest increased incidence of diarrhea with one of the vaccines [47]. Reassortment between RV vaccines and circulating strains has been reported, with strains carrying at least one gene (NSP2) identical to the one the Rotated vaccine [48] and a human–bovine double reassortant (G1P[8]) [47]. Patton [49] suggested that generation of reassortant RV among these vaccines and the circulating strains of HRV should be higher in developing countries, where the rates of coinfection can reach 20% compared with 5% reported in developing countries [50,51]. These recombination events may not interfere with vaccine efficacy in the short term, but may contribute to an increased pool of divergent strains, with unknown longer term consequences.

Conclusion

Triple-layered VLPs (VP2, VP6, VP4 and VP7) are antigenically similar to intact RV, whereas double-layered VLPs (VP2 and



Replacing or reducing the number of live attenuated oral vaccine doses by nonreplicating vaccines can prevent reassortment between vaccine and circulating strains.

VP6) lack the VN antigens. Because they are noninfectious, they may reduce the risk of side effects associated with live vaccines, such as intussusception or diarrhea [47]. They are potent nonreplicating immunogens. The rationale to use 2/6-VLP as a possible RV vaccine was based on the facts that: VP2 and VP6 are the most abundant proteins, representing 15% and more than 60% of the total virion mass, respectively; VP6 is highly antigenic and contains antigenic determinants shared by all group A RV [52]; 2/6-VLP are highly stable [53]; and double-layered inactivated RV particles administered intranasally to mice induced at least partial protection against RV infection [54]. However, in a RV naive neonatal animal diarrhea model, a priming dose of AttHRV vaccine was needed before boosting with 2/6-VLP to achieve at least partial protection using this strategy. The use of multiple doses of live-attenuated vaccine was reduced to a single dose. Such vaccine regimens, although requiring priming with live attenuated vaccine may at least reduce the risk associated with live virus boosters or, by using the intranasal route, avoid the side effects of such vaccines or circumvent other local intestinal factors (e.g., intestinal parasites, maternal antibodies) common in infants in developing countries. Importantly, the use of 2/6-VLP boosters given by the intranasal route may overcome the suppressive effects of maternal antibodies on live oral vaccines [43]. Future VLP vaccine studies in gnotobiotic pigs should explore if 2/4/6/7VLP for priming or given alone in multiple doses may replace live-attenuated RV vaccines.

Disclaimer

The views contained herein are those of the authors and do not necessarily reflect those of the US FDA.

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Summary

- A 2/6-virus-like particle (VLP) vaccine even with adjuvants (mutant LT-R192G [mLT], immuno-stimulating complex [ISCOM]) failed to induce protection in a neonatal gnotobiotic piglet model.
- Use of ISCOM as adjuvant for VLP vaccines increased protection, compared with mLT when used as boosters with live vaccine priming.
- The combination of live-attenuated and 2/6-VLP vaccines increased protection rates comparable to three doses of live-attenuated vaccine.
- The combination of live-attenuated and VLP vaccines induced IFN- γ -producing T cells in the ileum of gnotobiotic pigs with frequencies similar to infection with VirHRV.
- Route of immunization influences the efficacy of VLP vaccines.
- Lack of available and licensed mucosal adjuvants and delivery systems impedes the application of mucosal VLP vaccines.
- Maternal antibody levels in developed versus developing countries are among the obstacles for infant mucosal vaccines.
- VLP vaccines were efficacious as maternal rotavirus vaccines to provide passive immunity in calves.

References

- 1 Estes M. *Rotavirus and Their Replication*. Knipe D, Holey PM (Eds). Lippincott Williams & Wilkins, PA, USA (2001).
- 2 Matthijnssens J, Van Ranst M. Genotype constellation and evolution of group A rotaviruses infecting humans. *Curr. Opin. Virol.* 2(4), 426–433 (2012).
- 3 Tate JE, Burton AH, Boschi-Pinto C, Steele AD, Duque J, Parashar UD. 2008 estimate of worldwide rotavirus-associated mortality in children younger than 5 years before the introduction of universal rotavirus vaccination programmes: a systematic review and meta-analysis. *Lancet Infect. Dis.* 12(2), 136–141 (2012).
- 4 Vesikari T, Matson DO, Dennehy P et al. Safety and efficacy of a pentavalent human-bovine (WC3) reassortant rotavirus vaccine. *N. Engl. J. Med.* 354(1), 23–33 (2006).
- 5 Yen C, Tate JE, Patel MM et al. Rotavirus vaccines: update on global impact and future priorities. *Hum. Vaccin.* 7(12), 1282–1290 (2011).
- 6 Ruiz-Palacios GM, Perez-Schael I, Velazquez FR et al. Safety and efficacy of an attenuated vaccine against severe rotavirus gastroenteritis. *N. Engl. J. Med.* 354(1), 11–22 (2006).
- 7 Jiang V, Jiang B, Tate J, Parashar UD, Patel MM. Performance of rotavirus vaccines in developed and developing countries. *Hum. Vaccin.* 6(7), 532–542 (2010).
- 8 Jiang B, Patel M, Parashar U. Rotavirus vaccines for global use: what are the remaining issues and challenges? *Hum. Vaccin.* 6(5), 425–427 (2010).
- 9 Greenberg HB, Estes MK. Rotaviruses: from pathogenesis to vaccination. *Gastroenterology* 136(6), 1939–1951 (2009).
- 10 Velazquez FR, Matson DO, Calva JJ et al. Rotavirus infections in infants as protection against subsequent infections. *N. Engl. J. Med.* 335(14), 1022–1028 (1996).
- 11 To TL, Ward LA, Yuan L, Saif LJ. Serum and intestinal isotype antibody responses and correlates of protective immunity to human rotavirus in a gnotobiotic pig model of disease. *J. Gen. Virol.* 79(Pt 11), 2661–2672 (1998).
- 12 Velazquez FR, Matson DO, Guerrero ML et al. Serum antibody as a marker of protection against natural rotavirus infection and disease. *J. Infect. Dis.* 182(6), 1602–1609 (2000).
- 13 Yuan L, Ward LA, Rosen BI, To TL, Saif LJ. Systematic and intestinal antibody-secreting cell responses and correlates of protective immunity to human rotavirus in a gnotobiotic pig model of

Human rotavirus VLP vaccines in neonatal gnotobiotic pigs

- disease. *J. Virol.* 70(5), 3075–3083 (1996).
- 14 Chiba S, Yokoyama T, Nakata S *et al.* Protective effect of naturally acquired homotypic and heterotypic rotavirus antibodies. *Lancet* 2(8504), 417–421 (1986).
- 15 Franco MA, Angel J, Greenberg HB. Immunity and correlates of protection for rotavirus vaccines. *Vaccine* 24(15), 2718–2731 (2006).
- 16 Angel J, Franco MA, Greenberg HB. Rotavirus immune responses and correlates of protection. *Curr. Opin. Virol.* 2(4), 419–425 (2012).
- 17 Madore HP, Estes MK, Zarley CD *et al.* Biochemical and immunologic comparison of virus-like particles for a rotavirus subunit vaccine. *Vaccine* 17(19), 2461–2471 (1999).
- 18 Crawford SE, Labbe M, Cohen J, Burroughs MH, Zhou YJ, Estes MK. Characterization of virus-like particles produced by the expression of rotavirus capsid proteins in insect cells. *J. Virol.* 68(9), 5945–5952 (1994).
- 19 Friess AE, Sinowitz F, Skolek-Winnisch R, Trautner W. The placenta of the pig. II. The ultrastructure of the areolae. *Anat. Embryol. (Berl.)* 163(1), 43–53 (1981).
- 20 Butler JE, Lemke CD, Weber P, Sinkora M, Lager KM. Antibody repertoire development in fetal and neonatal piglets: XIX. Undiversified B cells with hydrophobic HCDR3s preferentially proliferate in the porcine reproductive and respiratory syndrome. *J. Immunol.* 178(10), 6320–6331 (2007).
- 21 Yuan L, Saif LJ. Induction of mucosal immune responses and protection against enteric viruses: rotavirus infection of gnotobiotic pigs as a model. *Vet. Immunol. Immunopathol.* 87(3–4), 147–160 (2002).
- 22 Hammerberg C, Schurig GG, Ochs DL. Immunodeficiency in young pigs. *Am. J. Vet. Res.* 50(6), 868–874 (1989).
- 23 Azevedo MS, Yuan L, Jeong KI *et al.* Viremia and nasal and rectal shedding of rotavirus in gnotobiotic pigs inoculated with Wa human rotavirus. *J. Virol.* 79(9), 5428–5436 (2005).
- 24 Ward LA, Rosen BI, Yuan L, Saif LJ. Pathogenesis of an attenuated and a virulent strain of group A human rotavirus in neonatal gnotobiotic pigs. *J. Gen. Virol.* 77(Pt 7), 1431–1441 (1996).
- 25 Yuan L, Geyer A, Hodgins DC *et al.* Intranasal administration of 2/6-rotavirus-like particles with mutant *Escherichia coli* heat-labile toxin (LT-R192G) induces antibody-secreting cell responses but not protective immunity in gnotobiotic pigs. *J. Virol.* 74(19), 8843–8853 (2000).
- 26 Hu KF, Elvander M, Merza M, Akerblom L, Brandenburg A, Morein B. The immunostimulating complex (ISCOM) is an efficient mucosal delivery system for respiratory syncytial virus (RSV) envelope antigens inducing high local and systemic antibody responses. *Clin. Exp. Immunol.* 113(2), 235–243 (1998).
- 27 Mowat AM, Donachie AM. ISCOMS – a novel strategy for mucosal immunization? *Immunol. Today* 12(11), 383–385 (1991).
- 28 Iosef C, Van Nguyen T, Jeong K *et al.* Systemic and intestinal antibody secreting cell responses and protection in gnotobiotic pigs immunized orally with attenuated Wa human rotavirus and Wa 2/6-rotavirus-like-particles associated with immunostimulating complexes. *Vaccine* 20(13–14), 1741–1753 (2002).
- 29 Nguyen TV, Iosef C, Jeong K *et al.* Protection and antibody responses to oral priming by attenuated human rotavirus followed by oral boosting with 2/6-rotavirus-like particles with immunostimulating complexes in gnotobiotic pigs. *Vaccine* 21(25–26), 4059–4070 (2003).
- 30 Yuan L, Iosef C, Azevedo MS *et al.* Protective immunity and antibody-secreting cell responses elicited by combined oral attenuated Wa human rotavirus and intranasal Wa 2/6-VLPs with mutant *Escherichia coli* heat-labile toxin in gnotobiotic pigs. *J. Virol.* 75(19), 9229–9238 (2001).
- 31 Azevedo MS, Gonzalez AM, Yuan L *et al.* An oral versus intranasal prime/boost regimen using attenuated human rotavirus or VP2 and VP6 virus-like particles with immunostimulating complexes influences protection and antibody-secreting cell responses to rotavirus in a neonatal gnotobiotic pig model. *Clin. Vaccine Immunol.* 17(3), 420–428 (2010).

- 32 Azevedo MS, Yuan L, Josef C et al. Magnitude of serum and intestinal antibody responses induced by sequential replicating and nonreplicating rotavirus vaccines in gnotobiotic pigs and correlation with protection. *Clin. Diagn. Lab. Immunol.* 11(1), 12–20 (2004).
- 33 Scherle PA, Gerhard W. Differential ability of B cells specific for external vs. internal influenza virus proteins to respond to help from influenza virus-specific T-cell clones *in vivo*. *Proc. Natl Acad. Sci. USA* 85(12), 4446–4450 (1988).
- 34 Gonzalez AM, Nguyen TV, Azevedo MS et al. Antibody responses to human rotavirus (HRV) in gnotobiotic pigs following a new prime/boost vaccine strategy using oral attenuated HRV priming and intranasal VP2/6 rotavirus-like particle (VLP) boosting with ISCOM. *Clin. Exp. Immunol.* 135(3), 361–372 (2004).
- 35 Brandtzaeg P, Halstensen TS, Kett K et al. Immunobiology and immunopathology of human gut mucosa: humoral immunity and intraepithelial lymphocytes. *Gastroenterology* 97(6), 1562–1584 (1989).
- 36 Franco MA, Greenberg HB. Immunity to rotavirus infection in mice. *J. Infect. Dis.* 179(Suppl. 3), S466–S469 (1999).
- 37 Jaimes MC, Rojas OL, Gonzalez AM et al. Frequencies of virus-specific CD4⁽⁺⁾ and CD8⁽⁺⁾ T lymphocytes secreting gamma interferon after acute natural rotavirus infection in children and adults. *J. Virol.* 76(10), 4741–4749 (2002).
- 38 Ward LA, Yuan L, Rosen BI, To TL, Saif LJ. Development of mucosal and systemic lymphoproliferative responses and protective immunity to human group A rotaviruses in a gnotobiotic pig model. *Clin. Diagn. Lab. Immunol.* 3(3), 342–350 (1996).
- 39 Yuan L, Wen K, Azevedo MS, Gonzalez AM, Zhang W, Saif LJ. Virus-specific intestinal IFN-gamma producing T cell responses induced by human rotavirus infection and vaccines are correlated with protection against rotavirus diarrhea in gnotobiotic pigs. *Vaccine* 26(26), 3322–3331 (2008).
- 40 Chan J, Nirwati H, Triasih R et al. Maternal antibodies to rotavirus: could they interfere with live rotavirus vaccines in developing countries? *Vaccine* 29(6), 1242–1247 (2011).
- 41 Moon SS, Wang Y, Shane AL et al. Inhibitory effect of breast milk on infectivity of live oral rotavirus vaccines. *Pediatr. Infect. Dis. J.* 29(10), 919–923 (2010).
- 42 Nguyen TV, Yuan L, Azevedo MS et al. Low titer maternal antibodies can both enhance and suppress B cell responses to a combined live attenuated human rotavirus and VLP-ISCOM vaccine. *Vaccine* 24(13), 2302–2316 (2006).
- 43 Nguyen TV, Yuan L, Azevedo MS et al. High titers of circulating maternal antibodies suppress effector and memory B-cell responses induced by an attenuated rotavirus priming and rotavirus-like particle-immunostimulating complex boosting vaccine regimen. *Clin. Vaccine Immunol.* 13(4), 475–485 (2006).
- 44 Fernandez FM, Conner ME, Parwani AV et al. Isotype-specific antibody responses to rotavirus and virus proteins in cows inoculated with subunit vaccines composed of recombinant SA11 rotavirus core-like particles (CLP) or virus-like particles (VLP). *Vaccine* 14(14), 1303–1312 (1996).
- 45 Fernandez FM, Conner ME, Hodgins DC et al. Passive immunity to bovine rotavirus in newborn calves fed colostrum supplements from cows immunized with recombinant SA11 rotavirus core-like particle (CLP) or virus-like particle (VLP) vaccines. *Vaccine* 16(5), 507–516 (1998).
- 46 Kim Y, Nielsen PR, Hodgins D, Chang KO, Saif LJ. Lactogenic antibody responses in cows vaccinated with recombinant bovine rotavirus-like particles (VLPs) of two serotypes or inactivated bovine rotavirus vaccines. *Vaccine* 20(7–8), 1248–1258 (2002).
- 47 Hemming M, Vesikari T. Vaccine-derived human-bovine double reassortant rotavirus in infants with acute gastroenteritis. *Pediatr. Infect. Dis. J.* 31(9), 992–994 (2012).
- 48 Bucardo F, Rippinger CM, Svensson L, Patton JT. Vaccine-derived NSP2 segment in rotaviruses from

Human rotavirus VLP vaccines in neonatal gnotobiotic pigs

- vaccinated children with gastroenteritis in Nicaragua. *Infect. Genet. Evol.* 12(6), 1282–1294 (2012).
- 49 Patton JT. Rotavirus diversity and evolution in the post-vaccine world. *Discov. Med.* 13(68), 85–97 (2012).
- 50 Rotavirus vaccination coverage among infants aged 5 months – immunization information system sentinel sites, United States, June 2006–June 2009. *MMWR Morb. Mortal. Wkly Rep.* 59(17), 521–524 (2010).
- 51 Iturriza-Gomara M, Dallman T, Banyai K *et al.* Rotavirus genotypes co-circulating in Europe between 2006 and 2009 as determined by EuroRotaNet, a pan-European collaborative strain surveillance network. *Epidemiol. Infect.* 139(6), 895–909 (2011).
- 52 Estes MK, Kapikian AZ. Rotaviruses. In: *Fields Virology*. Knipe DM, Howley PE (Eds). Wolters Kluwer-Lippincott Williams and Wilkins, PA, USA, 1917–1974 (2007).
- 53 Charpilienne A, Lepault J, Rey F, Cohen J. Identification of rotavirus VP6 residues located at the interface with VP2 that are essential for capsid assembly and transcriptase activity. *J. Virol.* 76(15), 7822–7831 (2002).
- 54 McNeal MM, Rae MN, Bean JA, Ward RL. Antibody-dependent and -independent protection following intranasal immunization of mice with rotavirus particles. *J. Virol.* 73(9), 7565–7573 (1999).

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Chapter

5

Presenting heterologous epitopes with hepatitis B core-based virus-like particles

Sarah De Baets, Kenny Roose, Bert Schepens & Xavier Saelens

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Since the first effort to recombinantly express the capsid protein of hepatitis B virus (HBV), namely **hepatitis B core protein (HBc)** in bacteria, the remarkable virion-like structure that is formed by this protein has fueled interest in unraveling its structural and antigenic properties. Initial studies proved HBc **virus-like particles (VLPs)** possess strong immunogenic properties, which can be conveyed to linked antigens. More than 35 years later, numerous studies have been performed using HBc as a carrier protein for antigens derived from over a dozen different pathogens and diseases. This chapter focuses on the development and use of HBc as a VLP platform to present heterologous antigens to the mammalian immune system. The authors summarize the possible molecular strategies that permit fusion of antigens to HBc, as well as the available recombinant expression platforms. Some of the key innate and adaptive immunostimulatory mechanisms that help explain the remarkable immunogenicity of some of the recombinant HBc-VLPs are also reviewed. Finally, the

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Aa

Hepatitis B core protein (HBc): the capsid protein of hepatitis B virus (HBV), which forms dimers that make up the icosahedral nucleocapsid of HBV.

Virus-like particle: noninfectious and genome-free assemblies of viral structural proteins (such as HBc) that form a capsid resembling the native virus.

preparation and outcome of clinical trials performed with two novel HBc VLP-based vaccine candidates are discussed.

Hepatitis B virus

HBV is responsible for a vast global disease burden: more than 2 billion people have

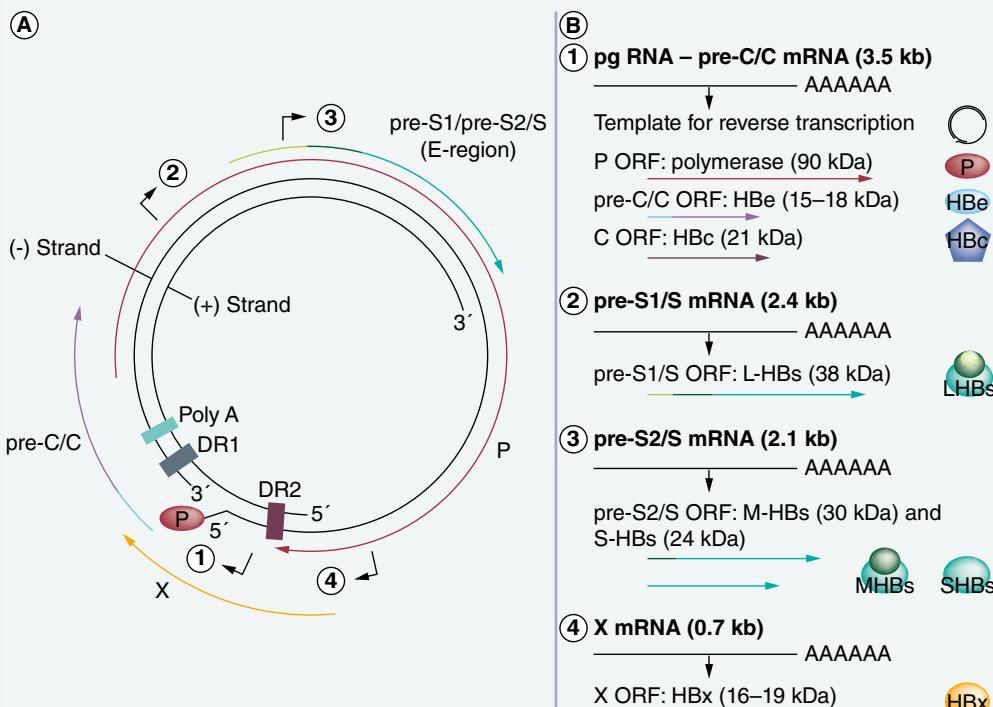
been, or are currently, infected with HBV. Approximately 400 million of these people are unable to clear the virus and become chronically infected. Eventually, such a chronic infection will lead to liver cirrhosis or hepatocellular carcinoma, which, in the absence of treatment, is usually fatal. It is estimated that each year 600,000 people die as a result of liver failure caused by HBV infection [1]. The genomic replication strategy of HBV and related viruses, classified as *Hepadnaviridae*, is remarkable. The majority of infectious HBV virions (42–47 nm in diameter) contain a circular DNA genome with an approximate size of 3200 nucleotides. One strand of the genomic DNA is complete, whereas the complementary strand is incomplete (**Figure 5.1A**). Despite their DNA genome, members of the *Hepadnaviridae* family replicate through a reverse transcription step. In the infected hepatocyte, a pregenomic RNA (pgRNA) is synthesized by the host cell RNA polymerase. This pgRNA is then used as a template for the viral reverse transcriptase to generate progeny genomic DNA.

HBV-infected hepatocytes produce spherical, enveloped virions. Three different proteins are embedded in the lipid envelope that presumably surrounds the virion: large (L), middle (M) and small (S) surface antigens. These glycoproteins are present on the virion surface as homo- or heterodimers that are linked by disulfide bridges [2]. All three proteins are translated from a single open reading frame in the viral genome, named the E-region (**Figure 5.1**). This open reading frame is translated from the first start codon (L), the second (M; 108 or 119 codons downstream) or third (S; 55 codons downstream from the M start codon), implying that the three hepatitis B surface antigen (HBsAg) molecules share a 226 amino acid residue long C-terminal sequence (i.e., the region that is identical to S). The N-terminal 55 amino acid residues region of M is named pre-S2 and the 108–119 N-terminal region that is unique to L is named pre-S1. S, M and L share an N-glycosylation site at amino acid residue position 309 (numbering for L) and the three proteins are membrane anchored by a C-terminal hydrophobic domain.

The noninfectious, subviral filamentous and 22-nm spherical particles that are present in the serum of infected patients are comprised of HBsAg S and M with only trace amounts of L, and have been used as prophylactic

Presenting heterologous epitopes with hepatitis B core-based VLPs

Figure 5.1. Hepatitis B virus genome organization and coding strategy.



(A) Schematic representation of the hepatitis B virus genome (black lines: genomic DNA). The genome is composed of a complete but nicked negative sense DNA strand (~3.2 kb) and an incomplete positive sense DNA strand. Complementary regions in their 5' ends ensure circularization. DR1 and DR2 play an important role during genome replication. Four main distinctive mRNA transcripts are encoded by the negative sense strand. Transcription of these mRNAs is controlled by their respective promoters, indicated by the numbered arrows. For all, the same PolyA site is used. These transcripts contain the open reading frames (colored arrows) for the hepatitis B virus proteins. The pgRNA also serves as template for the generation of progeny genomes, a process that is catalyzed by the viral P (a reverse transcriptase). **(B)** The four main mRNA transcripts derived from the hepatitis B virus genome and the proteins for which they code. The largest species, the pgRNA, is used as template for the production of progeny DNA genomes.
 DR: Direct repeat; HBC: Hepatitis B core protein; HBe: Hepatitis B precore protein; L-HB: Large hepatitis B surface antigen; M-HB: Middle hepatitis B surface antigen; ORF: open reading frame; P: Polymerase; PolyA: Polyadenylation; pgRNA: Pregenomic RNA.

vaccines against HBV [2]. Most currently licensed HBV vaccines are now recombinantly produced and composed of *Saccharomyces cerevisiae*-produced S or mammalian cell-produced S and M antigens. There is evidence, however, that the N-terminal part of the L protein harbors receptor-binding activity [2], and contains B- and T-cell epitopes that are well recognized, unique to L and absent in M and S [2].



The hepatitis B surface antigen is a heterogenous protein consisting of homo- and hetero-dimers of the small, middle and large forms of the protein. hepatitis B surface antigen is currently used as a recombinant HBV vaccine.

HBc: the building block for icosahedral capsid formation

The HBV envelope surrounds the nucleocapsid: an icosahedral structure with a diameter of approximately 30 nm,

comprised of the HBc protein. Two HBc proteins dimerize to form the building blocks for the icosahedral nucleocapsid, comprising 180 or 240 copies of HBc proteins [2]. Those with 180 copies have an icosahedral $T = 3$ symmetry and a diameter of approximately 30 nm, while nucleocapsids with 240 HBc copies have a diameter of 34 nm and a $T = 4$ symmetry. Both types of viral particles are detectable in the liver of infected patients.

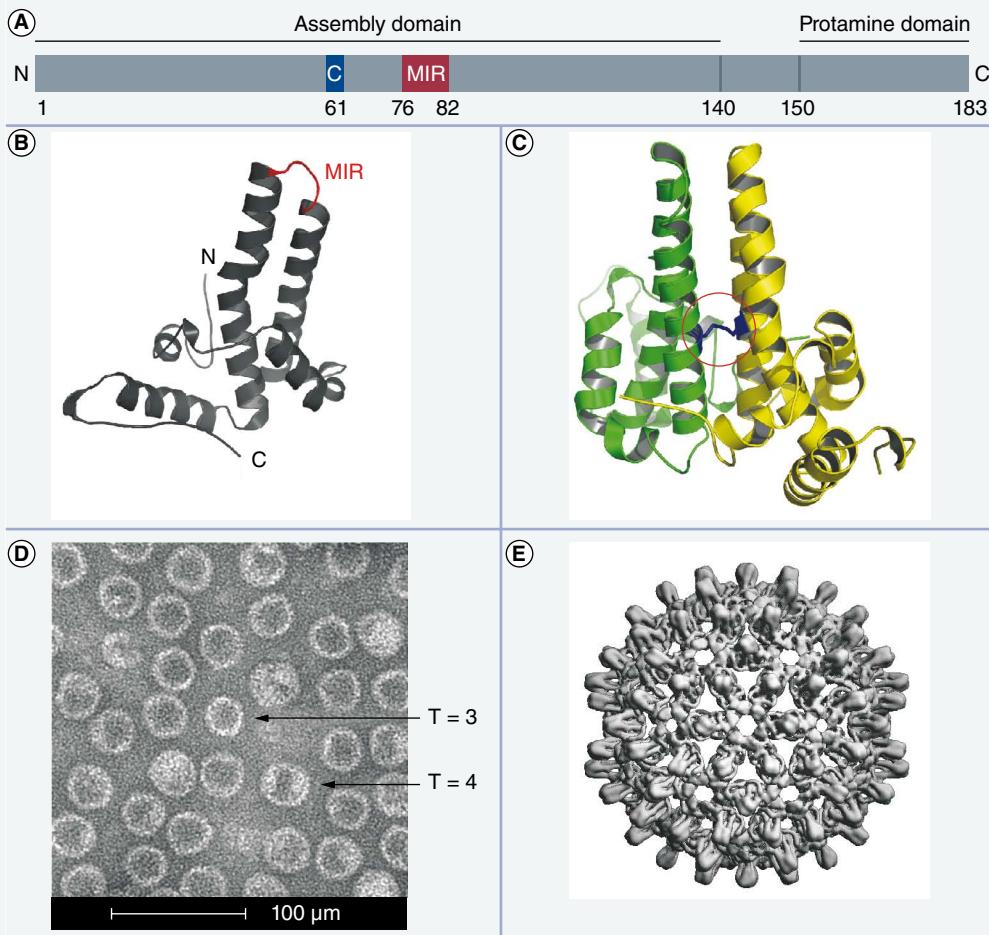
HBc is translated from a 3.5-kb mRNA species, representing the pgRNA that encodes the polymerase and HBc (Figure 5.2). HBc is a 183–185 amino acid residue-long structural protein (Figure 5.2A). The N-terminal part of HBc (amino acid residues 1–140) represents the assembly domain, as this region is both necessary and sufficient to form dimers that can self-assemble into capsids. The C-terminal part (residues 150–183) of HBc is a so-called ‘protamine’ domain, which is arginine rich and has a high affinity for RNA [3]. The 3D structure of HBc is made up of α -helices that are connected by loops (Figure 5.2B) [2]. HBc dimers are characterized by a central bundle of four helices that are connected by a disulfide bridge between the cysteine residues at position 61 in the two monomers (Figure 5.2C). The loops that connect the antiparallel helices within an HBc monomer are oriented distally from the inner capsid core, and form spikes consisting of a bundle of four helices in each HBc dimer. The tips of these spikes are known as the major immunodominant region (MIR) of HBc. These MIRs are extremely immunogenic, and insertion of heterologous epitopes in the loops that make up the MIR or replacement of parts of the loop by heterologous epitopes has been extensively exploited to create highly immunogenic recombinant VLPs.

Recombinant HBc particles as epitope carriers

Expression of HBc outside its natural context results in the formation of spherical capsids (Figures 5.2D & 5.2E). Interestingly, high-level expression and self-assembly of HBc into VLPs has been accomplished in many different expression systems, including prokaryotic (e.g., *Escherichia coli*, *Salmonella* and *Bacillus subtilis*), lower eukaryotic (e.g., *Saccharomyces cerevisiae*, *Pichia pastoris* and *Aspergillus niger*), mammalian (e.g., *Xenopus* oocytes, insect cells and HeLa cells) and plant expression systems. Regardless of the expression



Aa Major immunodominant region: the region at the tip of the spike formed by an HBc dimer. This region is highly immunogenic and can be used for the insertion of heterologous sequences.

Figure 5.2. Structure of hepatitis B core protein.

(A) Primary structure of hepatitis B core protein (HBc). Amino acids 76–82 form the MIR. This region, together with the N-terminal region, is commonly used for the insertion of foreign epitopes. The C at position 61 forms a disulfide bridge with a corresponding C 61 in a second monomer, for the formation of HBc dimers.

(B & C) Structure of an HBc monomer (residues 1–142; the MIR is shown in red). **(C)** Structure of an HBc dimer. The two monomers are coloured yellow and green. HBc dimers are characterized by a central bundle of four helices, connected by a disulfide bridge between the C residues at position 61 in the two monomers (blue and circled in red). **(D)** Electron micrographs of purified *Escherichia coli*-produced HBc particles, truncated at amino acid position 150. Smaller particles consist of 180 monomers and have a T = 3 symmetry; larger particles are made up of 240 monomers and have a T = 4 symmetry [SCHOTSAERT M, DERYCKE R, UNPUBLISHED DATA]. **(E)** Cryo-electron microscopy picture of HBc particles (residues 1–149) with T = 4 icosahedral symmetry.

C: Cysteine; MIR: Major immunodominant region.

(B & C) Data taken from [43]. **(E)** Was kindly provided by RA Crowther (Medical Research Council Laboratory of Molecular Biology, Cambridge, UK).



Recombinant HBC particles have been developed as a vaccine against different pathogens or pathologies by fusion with foreign epitopes. This can be done genetically, by fusing the epitope to the N- or C-terminus or inserting it into the major immunodominant region (MIR) of HBC; chemically, using HBC particles bearing a Cys residue in the MIR; or physically, by inserting proteins in the MIR interacting with sequences fused to the foreign epitope. All three sites accommodate fairly large insertion without many problems, as long as the virus-like particle formation is not impaired.

system used, the production of HBC only-based VLPs will result in naked, non-enveloped VLPs, since envelopment of HBC VLPs strictly depends on the coexpression of HBsAg proteins.

The first evidence of the merit of HBC as an antigen carrier was reported by Clarke *et al.* in 1987 [4]. They demonstrated the remarkably heightened immunogenicity of the 141–160 peptide derived from the VP1 protein of the foot-and-mouth disease

virus (FMDV) when this peptide was fused to the N-terminus of HBC. Extending HBC with a heterologous sequence such as the FMDV VP1 peptide did not compromise VLP formation, and immunization of mice with the FMDV peptide epitope-displaying, HBC-based VLPs induced a protective immune response.

The HBC protein lends itself to accommodate foreign epitopes in various ways. Through genetic fusion antigens can be linked in three different ways: to the N-terminus or C-terminus of HBC, or inserted in the MIR of HBC. Heterologous antigens are often inserted between two residues of the MIR (residues 76–82), or inserted in a way that replaces the MIR completely. For C-terminal fusions the nucleic acid-binding, protamine-like domain (residues 150–183) is generally replaced by the antigen of interest. Since the protamine region is dispensable for VLP formation, this does not usually compromise particle formation. A direct effect of this deletion is the inability of HBC particles to retain nucleic acids and loss of the C-terminal Cys residue at position 183. This loss may affect particle stability and, therefore, in some studies a C-terminal Cys-residue is added to truncated HBC [5].

A direct comparison of the three potential insertion sites showed the MIR to be superior in inducing antigen-specific antibodies, with the added effect that the dominant anti-HBC response is strongly impaired [6]. The same study showed that increasing the distance between the antigen fused to the N-terminus and the HBC carrier increases the accessibility and immunogenicity of the antigen, a finding that is corroborated by other examples [5,6]. This and subsequent studies proved all three potential insertion sites to be very resilient in containing large sequences of over 100 amino acids.

Is there an upper limit on the length of the polypeptide that can be inserted in the MIR? The main restriction is the specific structure of the

loop: the two α -helices of one monomer form a four-helix bundle in the HBc dimers (**Figures 5.2B & 5.2C**). As a result, the N- and C-terminal ends of the MIR are in close proximity, and genetically grafting exogenous sequences at the MIR should not lead to displacement of these MIR termini in space. The group of Michael Nassal *et al.* succeeded in inserting entire proteins, with their own specific folds, into the MIR, constructing so-called ‘whole-chain proteins’ [7]. Dependent on the inserted protein, different avenues of engineering can be followed. For some proteins, the N- and C-terminal ends are naturally in close proximity of each other, as is the case for the 258 residue green fluorescent protein and the 189 residue outer surface lipoprotein C of *Borrelia burgdorferi* [8]. For such proteins, direct insertion in the MIR allows recombinant particle formation. For proteins with opposed N- and C-termini, long flexible linkers have to be included to allow insertion in the loop of HBc, as has been shown for the 255 amino acid outer surface lipoprotein A of *B. burgdorferi* [9]. An alternative for the forced insertion through linkers is the introduction of a sequence-specific proteolytic cleavage site at one side of the inserted protein. Coexpression of the corresponding protease (e.g., tobacco etch virus protease) can then relieve the induced tension at the site of insertion and allow proper folding and assembly of the recombinant protein and the HBc particle [10].

Although a number of attractive and ingenious designs for creating recombinant HBc particles have been developed, none of these are a guarantee for ending up with a soluble, correctly assembled and particulate protein. Furthermore, when humoral responses are aimed for, a native folding of the inserted epitope is a prerequisite for effective vaccine design. Fusion to or insertion in HBc could potentially influence this in a negative way. An alternative method to produce antigen-bearing HBc particles relies on chemical conjugation of the antigen and the carrier. Jegerlehner *et al.*, for example, designed an HBc protein in which the MIR contains an engineered Lys residue, which allows chemical crosslinking of a Cys-bearing antigen to the engineered HBc particles using a heterobifunctional crosslinker [11]. This method can bypass cumbersome genetic engineering steps, or optimization of expression, stability and purification of antigen-HBc fusions. More recently, a similarly elegant way was developed, in which the 111 amino acid substrate-binding domain of bacterial molecular chaperone DNaK was first inserted in the HBc MIR [12]. These chimeric HBc fusions readily formed VLPs. Subsequently the researchers demonstrated that these particles could be uploaded with an antigen of interest when the latter was conjugated to a NRLLLTG peptide motif, to which DNaK specifically binds.

A large number of antigens and epitopes have been fused to HBc as vaccine development strategies (**Table 5.1**). In the section titled ‘Examples of antigen–HBc fusions as vaccine formulations’ the authors provide a more in depth discussion on two examples, emphasizing the rationale of vaccine design, and preclinical and clinical developments.

Adjuvant properties of HBc as carrier platform

As natural or recombinant antigens, HBc VLPs induce a strong adaptive immune response, characterized by significant T-cell responses and high titers of antibodies directed to both the carrier and coupled antigen [6,13]. At the base of the inherent adjuvant properties of HBc lies its particulate

Table 5.1. Overview of hepatitis B core protein virus-like particle based vaccination studies.

Pathogen	Antigen/epitopes	HBc fusion	Ref.
FMDV	VP1 VP1, VP4	N-terminal MIR	[4] [32]
Hantavirus	Nucleocapsid protein	MIR	[34]
HIV	gag env env V3 region	N-terminal C-terminal MIR	[37]
<i>Mycobacterium tuberculosis</i>	ESAT-6, CFP-10	MIR	[38]
Dengue virus	EDIII	MIR	[39]
HBV	Pre-S1	C-terminal MIR	[40] [41]
<i>Borrelia burgdorferi</i>	OspA, OspC	MIR	[7]
HPV	E7	C-terminal	[42]
HCV	HCc T-cell epitope HCc N-terminus	MIR C-terminal	[41]
<i>Plasmodium falciparum</i>	CSP B-cell epitope, T1 and T* T-cell epitopes	N-terminal (T*), MIR (B, T1)	[23,24]
IAV	M2e	N-terminal	[30,31]
HCC	HBx, PADRE T-cell epitope MAGE-1, MAGE-3, AFP1, AFP2	C-terminal, MIR C-terminal	[18]
HBc-expressing melanoma	HBc-CpG VLP-loaded DC		[19]

DC: Dendritic cell; FMDV: Foot-and-mouth disease virus; HBc: Hepatitis B core protein; HBV: Hepatitis B virus; HCc: Hepatitis C coreprotein; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HPV: Human papillomavirus; IAV: Influenza A virus; MIR: Major immunodominant region; Osp: Outer surface lipoprotein; VLP: Virus-like particle.

nature. Specifically, HBc particles can act as both T-helper (Th) cell-dependent and -independent antigens [14]. For the former, the presence in HBc of Th epitopes, which are recognized by a wide range of murine and human haplotypes, drives immune responses towards HBc, as well as to linked antigens [15]. Moreover, HBc-derived T-cell help can act in an intermolecular or intrastructural fashion. For example, priming of HBc epitope-specific Th cells by HBc-only vaccination can induce the production of antibodies against HBsAg after a subsequent boost with HBV particles, containing both HBc and HBsAg [16]. The Th-priming effects of HBc can, thus, easily convey adaptive responses to linked or associated epitopes, a feature making HBc a powerful carrier protein and adjuvant.

A productive adaptive immune response in a vertebrate host starts with the uptake of antigens by antigen-presenting cells (APCs), followed by T-cell activation. Dendritic cells (DCs) are professional APCs, which are usually associated with sparking up an immune answer to foreign antigens. In the case of HBc, however, the primary trigger comes from B cells acting as APCs. In unprimed animals, HBc VLPs are taken up actively by a defined splenic population of B cells, B1a and B1b cells, through interaction with the membrane Ig B-cell receptor. These B cells subsequently activate Th cells, initiating humoral and cellular responses directed to HBc [17]. The B1 cells are part of the innate immune system and carry germline-derived variable heavy-chain and variable light-chain chains, which might specifically recognize the structural repeat of the MIR in HBc VLPs rather than a defined stretch of amino acids [17]. Later in the immune response, antibodies binding HBc or coupled antigens will be produced and will allow the formation of immune complexes with HBc VLPs. These complexes are easily recognized by DCs and allow crosspriming [17]. Furthermore, DCs uploaded *in vitro* with HBc VLPs can be used as an adoptive vaccination strategy in experimental settings [18,19].

The quintessential property of HBc VLPs, their particulate nature, is at the basis of their inherent Th-independent adjuvant properties. This ensures a regular shape and spacing of epitopes, such as the HBc MIR with a fixed spacing of approximately 10 nm [2], and allows crosslinking of the B-cell receptor and activation of the B cell in a completely T-cell independent fashion. HBc was shown to induce a humoral response in athymic mice [14]. Even more interesting, this feature can be transferred to a linked or recombinantly inserted antigen, as long as the regularity of the HBc VLP remains undisturbed.

When using full-length HBc (183 residues), the protamine-like C-terminal domain will ensure encapsidation of nucleic acid species, typically RNA

derived from the production host. This encapsidated RNA is protected against hydrolysis by exogenous RNase, and augments the immune response to the particulate antigen in a TLR7-dependent fashion [17,20].

Examples of antigen-HBc fusions as vaccine formulations

Plasmodium falciparum

Malaria is caused by the transmission of *Plasmodium* parasites, mainly *Plasmodium falciparum* and, to a lesser extent, *Plasmodium vivax*, by certain *Anopheles* mosquito species. With an annual burden of over 500 million infections, largely in developing countries, malaria has been a focus for vaccine development for decades. Schödel *et al.* employed HBc as a scaffold for immunogenic presentation in the MIR of *Plasmodium* epitopes derived from the major surface protein of the sporozoite, CSP [21]. High immunogenicity of rodent malaria parasite *Plasmodium berghei* CSP-derived epitopes was observed in a number of inbred mouse strains tested, and proved to be protective in Balb/c mice against homologous challenge. However, the immunogenicity of epitopes from the human *P. falciparum* parasite was rather low, encouraging further optimization of constructs. The addition of a CSP-derived T-cell epitope in the MIR and to the C-terminus of the HBc protein, stabilized by retaining the C-terminal Cys residue, dramatically improved immunogenicity of the *P. falciparum* B-cell epitope in Balb/c mice and other inbred strains [22]. The inclusion of parasite-derived T-cell epitopes would allow a boosting effect and the induction of memory T cells upon infection of a vaccinated individual with *Plasmodium*. The resulting prototypical vaccine, termed ICC-1132, showed promising results in preclinical testing (performed by Apovia Inc., CA, USA) regarding the induction of antibody titers in mice and primates, murine and human-specific T-cell responses, and boosting of immune responses induced by *Plasmodium* infection [23]. These findings encouraged the Phase I/II clinical testing of ICC-1132, renamed Malaria Vax, combined with a number of different adjuvant systems. However, the immunogenicity and protective capacity of this malaria vaccine candidate remained low in the different settings tested, warranting further optimization of the vaccine and its delivery [24].

A quite similar route of constructing a *P. falciparum* vaccine was undertaken by GlaxoSmithKline (Middlesex, UK). They combined known B- and T-cell epitopes derived from CSP and fused them to the HBsAg protein, constructing the so-called 'RTS, S' vaccine. Combined with GlaxoSmithKline's frontline adjuvant systems (AS), based on monophosphoryl lipid A and the saponin QS21, the malaria vaccine

candidate RTS,S/AS was designed. After initial clinical studies showing promising results (reviewed in [25]), RTS,S/AS has now advanced into Phase III clinical testing in multiple sub-Saharan African countries. This clearly illustrates how a rational design, complemented with the use of powerful adjuvants for clinical use, can push a vaccine design into further development.



M2 protein is a transmembrane protein encoded by segment seven of the influenza A genome. M2 functions as a pH-sensitive, proton-selective channel, allowing uncoating of the viral genome during entry and safeguarding the structure of the acid-sensitive hemagglutinin structure during intracellular transport. M2 is also involved in virus assembly, pinching off the viral membrane during budding and evasion of host immune responses.

A broad-spectrum influenza A vaccine is capable of protecting the vaccinee against a wide variety of influenza strains.

Influenza A virus

As a basis for a broad influenza A vaccine, the ectodomain (M2e) of the M2 protein of this virus has been extensively examined by many groups. The stringent functional constraints of the protein along with the coding strategy of the virus, in which the M2 reading frame overlaps partially with that of M1, most likely do not allow for a wide variability in sequence. Indeed, sequence analysis of influenza A viruses of different subtypes or derived from different hosts shows a strong conservation of the 23 amino acid sequence of M2e [26,27].

In their initial study, Neirynck *et al.* genetically fused a single copy of the M2e sequence close to the N-terminus of full-length HBC [28]. The recombinant, *E. coli*-produced protein formed stable particles with the M2e moiety exposed on their surface. These M2e VLPs proved to be a favorable format to induce M2e-specific antibodies, which provided clear and long-lasting protection (at least 6 months) against influenza A virus challenge in laboratory mice. In follow-up studies, different M2e–HBC formats were designed and assayed [5,29,30]. The systematic preclinical assessments led to the selection of a construct, containing three tandem M2e copies fused N-terminally to the intermediate length (163 amino acid) HBC, retaining its nucleic acid-binding properties, to move forward into clinical testing. In a Phase I clinical trial performed by Acambis Inc. (now Sanofi-Pasteur, Lyon, France), healthy volunteers were immunized with this broad-spectrum influenza A vaccine candidate, termed Acam-Flu-A. It was found that the vaccine was well tolerated and induced significant seroconversion in vaccinees, stimulating further evaluation of the vaccine [31].

Zhang *et al.* reasoned mucosal delivery of an M2e-based vaccine would be a more advantageous approach to vaccinate [32]. They constructed an M2e–HBC particle, containing one N-terminal M2e copy and one in the MIR, with or without the addition of the B subunit of the heat-labile

enterotoxin of *E. coli* (LTB) fused N terminally to the protein. Both intranasal and oral delivery of the vaccines showed enhanced humoral responses for the LTB fusions, both for IgG and IgA antibody subtypes. Likewise, Ameiss *et al.* used an attenuated *Salmonella typhimurium* strain as delivery and adjuvant system for vaccination with Woodchuck hepatitis core protein M2e particles [33]. Compared with nonmucosal delivery of purified particles, the oral or intranasal immunization routes gave higher IgG_{2a} anti-M2e titers, indicative of a Th1-skewed response, and slightly better protection against influenza A challenge.

Could pre-existing anti-HBc immunity interfere with HBc-antigen vaccination?

One issue that needs to be addressed when using HBc as a basis for a VLP-based vaccine delivery platform is the fact that HBc is a natural antigen. Indeed, HBV infection leads to the induction of an anti-HBc response, and pre-existing HBc-specific antibodies may blunt the response to an HBc-based VLP displaying heterologous epitopes of interest. In their initial study on malaria vaccine development, Schödel *et al.* found that initial HBc carrier-only immunization did not interfere significantly with a secondary antigen-specific response [21]. Likewise, anti-HBc immunity does not inhibit immune responses to hantavirus using a viral nucleocapsid antigen, nor does it inhibit anti-M2e immunity and protection against influenza A infection in mice that were previously vaccinated with recombinant unsubstituted HBc particles [30,34]. It is noteworthy that in the two former studies the epitope of interest was inserted in the MIR of HBc, while in the latter the three M2e copies N terminal of HBc appeared more immunogenic than HBc itself in the hybrid particles, potentially by masking the MIR through the long N-terminal extensions [30]. This suggests that lowering the HBc-specific immunogenicity in the recall response by disrupting or masking the MIR, could circumvent potential silencing by pre-existing anti-HBc antibodies [35].

Another more practical issue that can be raised, is that HBV infection-induced antibody responses are used for serological identification of infected individuals. This suggests that the use of HBc-based vaccines may lead to confusion in the interpretation of this screening strategy. Conversely, chronic HBV-infected patients are characterized by a low immunological responsiveness to HBc, with low-to-undetectable humoral and cellular responses to the antigen [36]. This limits the use of HBc-based vaccines in such persons, since the format relies on the T-cell inducing properties of the core protein [36]. Some research groups have alluded to

this problem by proposing the use of the core protein of nonhuman *Hepadnaviridae* family members. The most evident ones are those derived from rodent viruses, such as woodchuck or ground squirrel hepatitis virus core proteins.

Conclusion

With the initial study of Clarke *et al.* (1987) showing improved immunogenicity of an FMDV epitope fused to HBc compared with other known carrier systems, a new chapter in vaccination strategy was introduced [4]. Since then, a number of proteinaceous particulate carriers that form VLPs have been described and widely tested. The specific characteristics of HBc, however, have maintained it as one of the most attractive platforms for vaccine design. The flexibility of the protein in adding antigens N terminally, C terminally or in the immunodominant loop region, ranging in length from a couple of amino acids to over 100 residues, is a key feature. This and the detailed knowledge of its expression in a range of hosts and its purification strategies render it a technically appealing format. The specific immunostimulatory characteristics, both on the level of T- and B-cell immunity, underline the adjuvant action of HBc exerted on linked antigens. Issues surrounding potential silencing due to anti-HBc immunity in HBV-primed people can probably be avoided or could be solved by the use of core proteins from related hepadnaviruses. With many diseases being targeted by HBc–antigen fusions in preclinical settings, more and more evidence and knowledge is gathered. This has led to the progression of two HBc fusions into clinical testing, and although initial results are not uniformly satisfying, the merit of HBc as a basis for prophylactic and therapeutic vaccines is generally accepted.

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Summary

- The structural characteristics of hepatitis B core protein (HBc) have enticed numerous research groups to investigate the determinants involved in particle formation, frontlining research in virus-like particle (VLP) structure.
- As a carrier platform, the flexibility of HBc is unknown regarding the length of antigens, which can be linked, the use of three potential insertion sites in the protein, the possible replacement of the major immunodominant region or C-terminal sequence by antigens, and the use of both prokaryotic and eukaryotic expression systems.
- Immunologically, HBc VLPs represent a unique system, being active as both a T-cell-dependent and -independent antigen and using primarily B cells as antigen-presenting cells for initiating an immune response.
- Of the antigen–HBc fusions tested, two have passed Phase I clinical trials (Malariaavax [Apovia Inc., CA, USA] and Acam-Flu-A™ [Sanofi-Pasteur, Lyon, France]); with further optimization of formulation these and other HBc VLP vaccines can move further in clinical testing.
- In acute or chronically hepatitis B virus-infected patients, the use of HBc-based vaccines might pose a problem, although initial murine studies contradict this. If so, alternative hepadnaviral core proteins, possessing similar antigenic and immunologic properties, can be used.

References

- 1 Shepard CW, Simard EP, Finelli L, Fiore AE, Bell BP. Hepatitis B virus infection: epidemiology and vaccination. *Epidemiol. Rev.* 28, 112–125 (2006).
- 2 Schadler S, Hildt E. HBV life cycle: entry and morphogenesis. *Viruses* 1(2), 185–209 (2009).
- 3 Porterfield JZ, Dhason MS, Loeb DD, Nassal M, Stray SJ, Zlotnick A. Full-length hepatitis B virus core protein packages viral and heterologous RNA with similarly high levels of cooperativity. *J. Virol.* 84(14), 7174–7184 (2010).
- 4 Clarke BE, Newton SE, Carroll AR *et al.* Improved immunogenicity of a peptide epitope after fusion to hepatitis B core protein. *Nature* 330(6146), 381–384 (1987).
- 5 De Filette M, Min Jou W, Birkett A *et al.* Universal influenza A vaccine: optimization of M2-based constructs. *Virology* 337(1), 149–161 (2005).
- 6 Schödel F, Moriarty AM, Peterson DL *et al.* The position of heterologous epitopes inserted in hepatitis B virus core particles determines their immunogenicity. *J. Virol.* 66(1), 106–114 (1992).
- 7 Nassal M, Skamel C, Vogel M *et al.* Development of hepatitis B virus capsids into a whole-chain protein antigen display platform: new particulate lyme disease vaccines. *Int. J. Med. Microbiol.* 298(1–2), 135–142 (2008).
- 8 Skamel C, Ploss M, Böttcher B *et al.* Hepatitis B virus capsid-like particles can display the complete, dimeric outer surface protein C and stimulate production of protective antibody responses against *Borrelia burgdorferi* infection. *J. Biol. Chem.* 281(25), 17474–17481 (2006).
- 9 Nassal M, Skamel C, Kratz PA, Wallich R, Stehle T, Simon MM. A fusion product of the complete *Borrelia burgdorferi* outer surface protein A (OspA) and the hepatitis B virus capsid protein is highly immunogenic and induces protective immunity similar to that seen with an effective lipidated OspA vaccine formula. *Eur. J. Immunol.* 35(2), 655–665 (2005).
- 10 Walker A, Skamel C, Vorreiter J, Nassal M. Internal core protein cleavage leaves the hepatitis B virus capsid intact and enhances its capacity for surface display of heterologous whole chain proteins. *J. Biol. Chem.* 283(48), 33508–33515 (2008).
- 11 Jegerlehner A, Tissot A, Lechner F *et al.* A molecular

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- assembly system that renders antigens of choice highly repetitive for induction of protective B cell responses. *Vaccine* 20(25), 3104–3112 (2002).
- 12 Wang XJ, Gu K, Xiong QY *et al.* A novel virus-like particle based on hepatitis B core antigen and substrate-binding domain of bacterial molecular chaperone DnaK. *Vaccine* 27(52), 7377–7384 (2009).
- 13 Schwarz K, Meijerink E, Speiser DE *et al.* Efficient homologous prime-boost strategies for T cell vaccination based on virus-like particles. *Eur. J. Immunol.* 35(3), 816–821 (2005).
- 14 Milich DR, McLachlan A. The nucleocapsid of hepatitis B virus is both a T-cell-independent and a T-cell-dependent antigen. *Science* 234(4782), 1398–1401 (1986).
- 15 Milich DR, Peterson DL, Schödel F, Jones JE, Hughes JL. Preferential recognition of hepatitis B nucleocapsid antigens by Th1 or Th2 cells is epitope and major histocompatibility complex dependent. *J. Virol.* 69(5), 2776–2785 (1995).
- 16 Milich DR, McLachlan A, Thornton GB, Hughes JL. Antibody production to the nucleocapsid and envelope of the hepatitis B virus primed by a single synthetic T cell site. *Nature* 329(6139), 547–549 (1987).
- 17 Lee BO, Tucker A, Frelin L *et al.* Interaction of the hepatitis B core antigen and the innate immune system. *J. Immunol.* 182(11), 6670–6681 (2009).
- 18 Ding FX, Wang F, Lu YM *et al.* Multiepitope peptide-loaded virus-like particles as a vaccine against hepatitis B virus-related hepatocellular carcinoma. *Hepatology* 49(5), 1492–1502 (2009).
- 19 Song S, Zhang K, You H *et al.* Significant anti-tumour activity of adoptively transferred T cells elicited by intratumoral dendritic cell vaccine injection through enhancing the ratio of CD8⁺ T cell/regulatory T cells in tumour. *Clin. Exp. Immunol.* 162(1), 75–83 (2010).
- 20 Ibanez LI, Roose K, De Filette M *et al.* M2e-displaying virus-like particles with associated RNA promote T helper 1 type adaptive immunity against influenza A. *PLoS ONE* 8(3), e59081 (2013).
- 21 Schödel F, Wirtz R, Peterson D *et al.* Immunity to malaria elicited by hybrid hepatitis B virus core particles carrying circumsporozoite protein epitopes. *J. Exp. Med.* 180(3), 1037 (1994).
- 22 Sällberg M, Hughes J, Jones J, Phillips TR, Milich DR. A malaria vaccine candidate based on a hepatitis B virus core platform. *Intervirology* 45(4–6), 350–361 (2002).
- 23 Birkett A, Lyons K, Schmidt A *et al.* A modified hepatitis B virus core particle containing multiple epitopes of the *Plasmodium falciparum* circumsporozoite protein provides a highly immunogenic malaria vaccine in preclinical analyses in rodent and primate hosts. *Infect. Immun.* 70(12), 6860–6870 (2002).
- 24 Gregson AL, Oliveira G, Othoro C *et al.* Phase I trial of an alhydrogel adjuvanted hepatitis B core virus-like particle containing epitopes of *Plasmodium falciparum* circumsporozoite protein. *PLoS ONE* 3(2), e1556 (2008).
- 25 Cohen J, Nussenzweig V, Nussenzweig R, Vekemans J, Leach A. From the circumsporozoite protein to the RTS, S/AS candidate vaccine. *Hum. Vaccin.* 6(1), 90–96 (2010).
- 26 Roose K, Fiers W, Saelens X. Pandemic preparedness: toward a universal influenza vaccine. *Drug News Perspect.* 22(2), 80–92 (2009).
- 27 Roose K, Schotsaert M, Bakkouri KE, Schepens B, Fiers W, Saelens X. Cutting edge approaches toward novel and cross-protective influenza vaccines. *Dev. Novel Vaccine* 205–232 (2012).
- 28 Neirynck S, Deroo T, Saelens X, Vanlandschoot P, Jou WM, Fiers W. A universal influenza A vaccine based on the extracellular domain of the M2 protein. *Nat. Med.* 5(10), 1157–1163 (1999).
- 29 De Filette M, Ramne A, Birkett A *et al.* The universal influenza vaccine M2e–HBc administered intranasally in combination with the adjuvant CTA1-DD provides complete protection. *Vaccine* 24(5), 544–551 (2006).
- 30 De Filette M, Martens W, Smet A *et al.* Universal influenza A M2e–HBc vaccine protects against disease even in the presence of pre-existing anti-HBc antibodies. *Vaccine* 26(51), 6503–6507 (2008).
- 31 Fiers W, De Filette M, El Bakkouri K *et al.* M2e-based universal influenza A vaccine. *Vaccine* 27(45), 6280 (2009).
- 32 Zhang GG, Li DX, Zhang HH, Zeng YM, Chen L. Enhancement of mucosal

- immune response against the M2eHBc⁺ antigen in mice with the fusion expression products of LTB and M2eHBc⁺ through mucosal immunization route. *Vet. Res. Commun.* 33(7), 735 (2009).
- 33 Ameiss K, Ashraf S, Kong W *et al.* Delivery of woodchuck hepatitis virus-like particle presented influenza M2e by recombinant attenuated *Salmonella* displaying a delayed lysis phenotype. *Vaccine* 28(41), 6704–6713 (2010).
- 34 Geldmacher A, Skrastina D, Borisova G *et al.* A hantavirus nucleocapsid protein segment exposed on hepatitis B virus core particles is highly immunogenic in mice when applied without adjuvants or in the presence of pre-existing anti-core antibodies. *Vaccine* 23(30), 3973–3983 (2005).
- 35 De Filette M, Fiers W, Martens W *et al.* Improved design and intranasal delivery of an M2e-based human influenza A vaccine. *Vaccine* 24(44), 6597–6601 (2006).
- 36 Baumert TF, Thimme R, Von Weizsäcker F. Pathogenesis of hepatitis B virus infection. *World J. Gastroenterol.* 13(1), 82 (2007).
- 37 Takeda S, Shiosaki K, Kaneda Y *et al.* Hemagglutinating virus of Japan protein is efficient for induction of CD4⁺ T-cell response by a hepatitis B core particle-based HIV vaccine. *Clin. Immunol.* 112(1), 92–105 (2004).
- 38 Dhanasooraj D, Kumar RA, Mundayoos S. Development of a vaccine delivery system using hepatitis B core antigen based VLPs to deliver mycobacterial antigens. *BMC Infect. Dis.* 12(Suppl. 1), 9 (2012).
- 39 Arora U, Tyagi P, Swaminathan S, Khanna N. Chimeric hepatitis B core antigen virus-like particles displaying the envelope domain III of dengue virus type 2. *J. Nanobiotechnol.* 10(1), 30 (2012).
- 40 Yue Q, Hu X, Yin W *et al.* Immune responses to recombinant *Mycobacterium smegmatis* expressing fused core protein and preS1 peptide of hepatitis B virus in mice. *J. Virol. Methods* 141(1), 41–48 (2007).
- 41 Sominskaya I, Skrastina D, Dislers A *et al.* Construction and immunological evaluation of multivalent hepatitis B virus (HBV) core virus-like particles carrying HBV and HCV epitopes. *Clin. Vaccine Immunol.* 17(6), 1027–1033 (2010).
- 42 Pumpens P, Razanskas R, Pushko P *et al.* Evaluation of HBs, HBC, and frCP virus-like particles for expression of human papillomavirus 16 E7 oncoprotein epitopes. *Intervirology* 45(1), 24–32 (2002).
- 43 Zlotnick A, Cheng N, Conway JF *et al.* Dimorphism of hepatitis B virus capsids is strongly influenced by the C-terminus of the capsid protein. *Biochemistry* 35(23), 7412–7421 (1996).

About the Authors



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Masaaki Kawano has been engaged in the study of the *in vitro* assembly of simian virus 40 (SV40) virus-like particles (VLPs) composed of VP1 capsid for 10 years, from the beginning of his career. He has currently focused on the development of a novel vaccine carrier consisting of SV40 VP1 for cytotoxic T lymphocyte (CTL)-based vaccines against latent infection and cancer.



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Masanori Matsui has been working for more than 25 years on the molecular mechanism of CTL activation and recognition, starting with the extensive mutational analyses of HLA-A2 to clarify critical interactions between MHC class I, CTL and antigenic peptides in antigen presentation. In the last 10 years, he has worked to develop novel CTL-based vaccines against pathogenic viruses and tumors using HLA class I transgenic mice.



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Hiroshi Handa has worked on molecular biology for more than 40 years. In the last 30 years, he has focused on the study of human RNA polymerase II transcription, leading to the discovery of DRB sensitivity inducing factor (DSIF) and negative elongation factor (NELF), which affect transcription elongation by RNA polymerase II. He has also developed affinity nanobeads called Handa beads that enable the identification of targeting proteins for specific DNA sequences, bait-proteins, or even drugs with an extremely low background. In the last 15 years, he has been working in the construction of various bionanocapsules for biomedical applications using the SV40 VP1 capsid.

Chapter

6

SV40 virus-like particles as an effective delivery system and a vaccine platform

Masaaki Kawano, Masanori Matsui & Hiroshi Handa

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The authors have purified a major capsid protein, VP1 of simian virus 40 (SV40), using recombinant baculovirus, and have established the method of *in vitro* reassembly of SV40 virus-like particles (SV40-VLPs) from VP1-pentamers. In this reassembly, SV40-VLPs can encapsulate approximately 5-kb exogenous DNA shielded by histone or foreign proteins fused to minor capsid proteins VP2/3 and effectively deliver them into mammalian cells. Insertion of a particular foreign peptide into the surface loops of VP1 provides SV40-VLPs with the ability of cell targeting. Furthermore, SV40-VLPs appear to stimulate innate immunity as a natural adjuvant. Given these characteristics, SV40-VLPs may be a promising vaccine carrier to deliver heterologous antigens for the induction of cytotoxic T lymphocytes without artificial adjuvants. This chapter describes how SV40-VLPs have been developed and engineered, and discuss the potential benefits and challenges as a cytotoxic T-lymphocyte-based vaccine platform.

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Aa **Cytotoxic T lymphocytes (CTLs):** CD8⁺ T cells that specialize in eliminating virus-infected cells and cancerous cells. CTLs specifically recognize antigenic short peptides in association with MHC class I molecules on the surface of target cells, and bind to and kill them.

Virus-like particles: mimic the organization and conformation of authentic viruses, but are noninfectious because they do not contain the viral genome.

In the last 200 years, vaccines have controlled a variety of infectious diseases. However, we have not yet developed effective vaccines against many critical human pathogens, such as HIV, hepatitis C virus and *Mycobacterium tuberculosis*. Most, if not all, of these pathogens appear to cause persistent and latent infections. To develop successful vaccines against such diseases, it is essential for the vaccine not

only to induce neutralizing antibodies, but also to generate highly effective T cells; in particular, **cytotoxic T lymphocytes (CTLs)**. In the case of cancer vaccines, cellular immunity involving CTLs is thought to be more important than humoral immunity because the goal is to eradicate tumor cells.

Live-attenuated virus vaccines are extremely effective to stimulate protective immunity because they can abundantly induce both neutralizing antibodies and CTLs. In addition to the use of viruses as direct immunogens, many scientists have attempted to use them as a vaccine carrier for the delivery of foreign epitopes [1]. Viral vectors offer dramatic effectiveness to induce foreign antigen-specific immune responses. However, they have a variety of potential problems involving toxicity, unwanted immune and inflammatory responses, and genetic damage in the host cell caused by integration of virus genome. Furthermore, attenuated viruses can be reversed to virulence or cause severe complications in immunocompromised patients. Besides virus-mediated systems, there are several nonviral carriers for vaccine. These approaches, however, are almost always limited in their application due to their low efficiency of antigen delivery. Under these circumstances, **virus-like particles** (VLPs) have received much attention as a novel vaccine platform because of their safety and effectiveness [2]. VLPs are comprised of multiple copies of viral surface structural proteins, such as the capsid or the envelope without including viral genomes, and therefore mimic the conformation of a native virus but are incapable of causing infection themselves. Since VLPs retain the efficient entry activity of the virus into the host cell without replication, they are a much safer alternative than attenuated viruses as an efficient vaccine carrier in the antigen delivery system [2]. Particulate VLPs are readily taken up into antigen-presenting cells (APCs), thereby priming long-lasting cellular immune responses in addition to antibody responses.

Aa **Simian virus 40 (SV40):** a small, nonenveloped tumor DNA virus that belongs to the *Polyomaviridae* family.

Simian virus 40 (SV40) is a small, nonenveloped tumor DNA virus that belongs to the *Polyomaviridae* family. The

capsid of SV40 is 40–45 nm in diameter and is formed by 72 pentamer subunits of the VP1 major capsid protein (360 molecules in total) and 72 molecules of the VP2/3 minor capsid proteins [3]. VP1-pentamers are self-assembled into VLPs of 45 nm in diameter without the aid of any cellular factors. When VP1 is genetically modified to insert foreign epitopes following self-assembly [4], SV40-VLPs hold antigenic epitopes to prime immune responses. Furthermore, SV40-VLPs can encapsulate various materials such as DNA [5,6] and proteins [7] as antigens. Therefore, SV40-VLPs may be a promising platform for the antigen delivery system. The capsid structure of papillomavirus is quite similar to that of SV40, and VLPs of the virus have been generated for the development of vaccine to inhibit virus infection and the antigen delivery system. SV40 was first discovered as a contaminated material in the culture of rhesus monkey kidney cells for the preparation of polio vaccine. Although there is no evidence that SV40 is harmful to humans who received polio vaccine, there has been a debate whether SV40 is linked to various cancers in humans, including mesothelioma [8]. However, in the case of SV40-VLPs composed of VP1 alone, it is considered to be safe for humans to use them as a vaccine material because there is no evidence that SV40 VP1 protein has acute or chronic cytotoxicity against human cells.

This chapter describes how SV40-VLPs have been developed and engineered, and attempts to address the potential of SV40-VLPs as a platform for the CTL-based vaccine.

Preparation of VP1-pentamers from purified SV40-VLPs

The authors first developed the method to prepare SV40-VLPs consisting of a single component, VP1, from insect Sf-9 cells using the baculovirus expression system. This insect cell-based system produces large amounts of correctly folded recombinant proteins without the risk of culturing opportunistic pathogens. Baculovirus does not bring any particular threats to vaccinated individuals due to the narrow host range. When expressed in insect cells using recombinant baculovirus, VP1 was self-assembled into VLPs of 45 nm in diameter. SV40-VLPs were purified from sonicated cell lysate containing 1% sodium deoxycholic acid by two rounds of the cesium chloride density gradient.

The authors next prepared VP1-pentamers from the purified SV40-VLP fraction [9]. This fraction in the cesium chloride-containing buffer was firstly dialyzed with the physiological buffer containing 20 mM Tris-HCl (pH 7.9), 100 mM NaCl and 0.1% NP-40.



The baculovirus expression system is frequently used for the preparation of a recombinant protein that needs the appropriate post-translational processing and folding, which are difficult to accomplish in the bacterial expression system.

A calcium-chelating agent, ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid and a disulfide bond reducing agent, dithiothreitol, were then added to the fraction, leading to the dissociation of SV40-VLPs into VP1-pentamers (**Figure 6.1A**). After the disassembly of SV40-VLPs, VP1-pentamers were purified by gel filtration using HPLC. The purified molecules were present in a highly homogenous and homo-VP1-pentermeric form.

In vitro reassembly of SV40-VLPs from VP1-pentamers

In order to determine the buffer condition to promote the *in vitro* reassembly of SV40-VLPs, solution containing highly purified VP1-pentamers was dialyzed with various kinds of buffers (**Figure 6.1B**) [9]. In the buffer containing 2 M $(\text{NH}_4)_2\text{SO}_4$, and 2 mM CaCl_2 , pH 7.2 at 4°C, VP1-pentamers reassembled into three different-sized spherical particles of 45, 25–35 and 20 nm in diameter (**Figure 6.1B**). In the buffer containing 1 M NaCl, 2 mM CaCl_2 , pH 7.2, at room temperature, VP1-pentamers reassembled into tubular structures as well as the three different-sized spherical particles (**Figure 6.1B**). By contrast, VP1-pentamers preferentially reassembled into small spherical particles of 20 nm in diameter in the calcium-ion-free buffer containing 1 M NaCl, pH 7.2, at 4°C. These results suggest that the calcium ion is essential for the formation of spherical 45-nm SV40-VLPs. On the other hand, VP1-pentamers did not form any particular structures but just aggregates in the physiological salt solution (150 mM NaCl, 2 mM CaCl_2) at pH 7.0. Moreover, they turned into very long tubular structures [9] and aberrant spherical structures [10] in the same physiological salt solution at pH 5.0 and pH 9.0, respectively.

The main problem in the reassembly was that morphologically normal 45-nm spherical particles were not formed under the physiological condition. The formation of 45-nm spherical particles was observed only under high-salt conditions as described above, whereas other diverse structures such as aggregates and tubular structures were formed under the physiological condition. Therefore, it was supposed that additional factors were required for the formation of 45-nm SV40-VLPs in the physiological buffer. Since viral genome is packaged into SV40 particles through multiple interactions between the DNA genome, the N-terminal region of VP1, and the C-terminal region of minor capsid proteins, VP2/3, the authors presumed that dsDNA of viral genome and/or VP2/3 might contribute to the assembly of 45-nm SV40-VLPs from VP1-pentamers under the physiological condition. As expected, it was found that VP1-pentamers formed 45-nm spherical particles in the presence of dsDNA or dsDNA arranged with cellular histones plus VP2/3 in the physiological salt buffer at neutral pH at room temperature (**Figure 6.1C**) [5,10]. In this case, the

resultant particles encapsulated dsDNA and VP2/3. The functional domain of VP2/3, which is important for the promotion of capsid formation, was explored with a series of VP2/3 deletion mutants. It was found that a region common to VP2 and VP3 (amino acids 119–272) was required to promote VP1 pentamer assembly [10]. In addition, 750 bp or longer dsDNA was necessary for the reassembly of 45-nm spherical particles from VP1-pentamers [5]. In the presence of dsDNA of less than 750 bp, VP1-pentamers assembled into spherical particles of 20–45 nm in diameter [5].

Delivery of foreign large DNA & proteins into cells by SV40-VLPs

Since wild-type SV40 particles, which are similar in size and shape to 45-nm spherical particles, accommodate 5243 bp minichromosomal DNA of SV40, the authors next examined whether and to what extent naked DNA was packaged within 45-nm spherical particles. For this purpose, assembly reactions were performed with DNA of various lengths, followed by the incubation with DNase I to digest unpackaged DNA. It was shown that DNA of less than 2 kb remained intact from DNase I treatment in the assembly solution, whereas larger DNA was digested into approximately 2-kb fragments by DNase I, suggesting that 45-nm spherical particles can accommodate up to 2-kb DNA (**Figure 6.1C,i**) [5,6]. By contrast, the cellular histone-coordinated viral genome of SV40 that is 5324 bp in length can be encapsidated in the SV40 capsid, suggesting that histone-induced DNA compaction may enable large DNA of more than 2 kb to be encapsulated into SV40-VLPs. In fact, it has been demonstrated that approximately 5-kb nucleosomal dsDNA with histones can be successfully packaged into SV40-VLPs in the DNA-mediated *in vitro* reassembly (**Figure 6.1C,ii**) [6]. Our future goal is to accomplish the *in vitro* reconstitution of SV40 particles that have the efficient activity of gene transduction equivalent to that of SV40 virion. Therefore, the authors next examined whether histones had an effect on the gene transfer activity of VLPs. When incubated with mammalian cells, SV40-VLPs containing nucleosomal DNA carrying a reporter gene yielded a significantly higher level of gene expression than VLPs containing the corresponding naked DNA [6]. The elevated gene expression resulted mainly from the enhanced association of particles with the cell surface and from the facilitation of subsequent uptake into cells. It was also revealed that the efficiency of gene transfer was significantly improved by the encapsidation of VP2/3 and hyperacetylated histones (**Figure 6.1C**) [6]. In our system, the DNA transduction efficiency of SV40-VLPs containing both hyperacetylated histone-coordinated nucleosomal DNA and VP2/3 was 2% of the efficiency generated by SV40 virions [6].

Figure 6.1. Overview of simian virus 40 VP1 engineering.

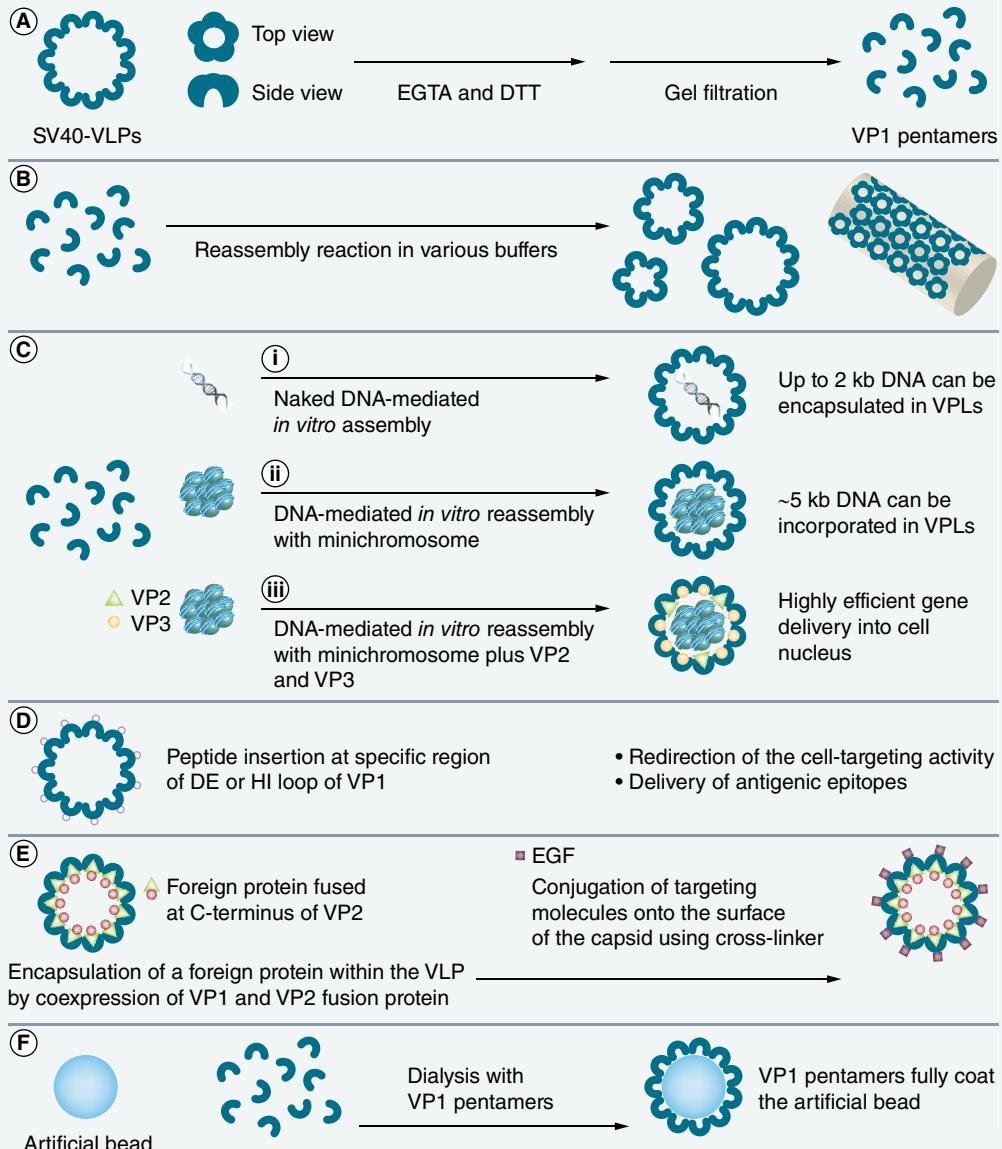


Figure 6.1 (cont.); see previous page. **(A)** Preparation of VP1-pentamers from SV40-VLPs in the presence of EGTA and DTT. **(B)** *In vitro* reassembly of various particle structures from VP1-pentamers under high-salt conditions. **(C)** DNA-mediated reassembly of SV40-VLPs with **(i)** naked DNA, **(ii)** minichromosomal DNA, and **(iii)** minichromosomal DNA and VP2/3. **(D)** Insertion of a particular foreign peptide into ED and HI loops provides SV40-VLP with the ability of active cell-targeting or antigenic epitope delivery. **(E)** Encapsulation of a foreign protein fused to VP2 in SV40-VLPs, followed by addition of EGF-specific targeting of tumor cells. **(F)** The coating of an artificial bead with VP1-pentamers ensures stable dispersion even in the body fluid.
DTT: Dithiothreitol; EGTA: Ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid; SV40: Simian virus 40; VLP: Virus-like particle.

In addition to large DNA, foreign proteins can also be packaged in SV40-VLPs. As described above, minor capsid proteins VP2/3 contribute to the assembly of SV40-VLPs from VP1-pentamers under the physiological condition. In this reassembly, VP2/3 proteins line the inside of SV40-VLPs. When a heterologous protein was fused to the C-terminus of VP2/3, this foreign protein was efficiently incorporated into SV40-VLPs during particle assembly [7]. Since the C-terminus of VP2/3 is directed toward the interior area of the particle, the C-terminally fused foreign protein is located in the inside of the particle, and therefore avoids interfering with the assembly of SV40-VLPs from VP1-pentamers. We used green fluorescent protein (GFP) as a model protein. In brief, recombinant VP1 and GFP-fused VP2/3 (VP2/3-GFP) were synthesized in SF-9 cells in the baculovirus expression system, and VP2/3-GFP was stably encapsulated in SV40-VLPs (**Figure 6.1E**). When cells were incubated with SV40-VLPs containing VP2/3-GFP, strong green fluorescence was observed both on the cell membrane and inside the cells [7]. These results indicate that SV40-VLPs encapsulating GFP retained their ability to bind to and enter cells, and also show that GFP was delivered successfully into mammalian cells by SV40-VLPs. Using this system, a biologically active material such as yeast cytosine deaminase could also be delivered into cells by SV40-VLPs [7].

Addition of the cell-targeting ability to SV40 VLPs

One of the most desirable requirements for the drug-delivery system is the ability of *in vivo* targeting of the intended tissues or cells. To develop the cell-type specific delivery system, the authors attempted to insert a foreign peptide onto the surface of SV40-VLPs by genetically manipulating VP1 (**Figure 6.1D**). The authors first identified two sites, called DE and HI loops, within the surface loops of VP1 that can accommodate the display of a foreign short peptide on the surface of VLPs without disrupting the assembly of VLPs [4]. BC, DE and HI loops of VP1 are involved in the cell attachment and entry of SV40 into host cells. These three loops can grab sialic acid, especially in ganglioside GM1, on the cell surface [11]. Recently,

it has been shown that sialylated glycans facilitate infectious entry of *Polyomaviridae* [12] and mutations in the GM1 binding site altered ganglioside usage and cell tropism [13], indicating that the interaction between the three loops and ganglioside is important for the proper entry of SV40 virus. We also found that insertion of three consecutive RGD integrin-binding motifs (3×RGD) into DE and HI loops strongly inhibited the GM1-dependent cell attachment of SV40-VLPs [4]. Instead, SV40-VLPs carrying 3×RGD motifs bound to the cell surface in an integrin $\alpha_v\beta_3$ -dependent manner [4]. Therefore, insertion of a particular foreign peptide into DE and HI loops provides SV40-VLPs with the ability of active cell targeting (**Figure 6.1D**). In addition, loss of the binding of SV40-VLPs to ganglioside GM1 should reduce the nonspecific binding to cells.

Because the EGF receptor (EGFR) is overexpressed on the surface of various tumor cells such as lung cancer, breast cancer and kidney cancer, it is possible to target certain tumor cells by selective binding to EGFR. The authors constructed SV40-VLPs conjugated with EGF using the heterobifunctional crosslinker, SM(PEG)₂ (Thermo Scientific, MA, USA) (**Figure 6.1E**) [14]. In this study, the authors used mutant SV40-VLPs (SV40-VLPs^{N138C}) composed of mutant VP1 (VP1^{N138C}) with an amino-acid substitution from asparagine (N) to cysteine (C) at position 138. The amino-acid residue at position 138 of VP1 is located in the DE loop on the outside surface of VLPs [4]. Since SM(PEG)₂ crosslinks between primary amines of EGF and sulphydryl groups of cysteine residues on the surface of VLPs [14], EGF preferably resides on the outside surface of VLPs^{N138C}. Therefore, it was expected that EGF-conjugated VLPs^{N138C} could target EGFR-expressing tumor cells more efficiently than EGF-conjugated wild-type VLPs with much less EGF on their surface. In addition, the mutation at position 138 elicits the conformational change of the DE loop, and thereby disrupts the binding of VLPs to ganglioside GM1 on various cells. Thus, EGF-conjugated VLPs^{N138C} are supposed to be engaged in EGFR-expressing tumor-specific targeting.

Since SV40-VLPs composed of VP1-pentamers are highly dispersible *in vivo* even in the high concentration, it was assumed that VP1-pentamers might be useful for coating the surface of artificial beads and micelles to improve *in vivo* dispersibility for medical use. Citrate-coated magnetic nanoparticles (CMNPs) were developed as an MRI contrast agent [15]. It is preferable for MRI contrast agents to be dispersible in the body fluid. However, CMNPs were dispersible in water but not in phosphate-buffered saline or serum. The authors found that the coating of CMNPs with VP1-pentamers (VP1-CMNPs) ensured stable dispersion of CMNPs even in the body fluid (**Figure 6.1F**) [16]. To equip VP1-CMNPs with the tumor-targeting ability,

EGF was conjugated to CMNPs coated with VP1^{N138C} (VP1^{N138C}-CMNPs) using a crosslinker, SM(PEG). It was found that the association of EGFR-expressing A431 cells with EGF-conjugated VP1^{N138C}-CMNPs was significantly inhibited by the addition of free EGF in a dose-dependent manner [16]. These data indicate that EGF-conjugated VP1^{N138C}-CMNPs can specifically target EGFR-expressing tumor cells, which would be useful as a highly dispersible MRI contrast agent.

Application of SV40-VLPs to a vaccine carrier

In addition to the delivery of diagnostic and/or therapeutic materials as described above, SV40-VLPs are also very attractive to deliver immunogenic epitopes as a vaccine carrier. In particular, VLPs are able to display great ability as a CTL-based vaccine platform because they effectively deliver heterologous antigens into the antigen-processing pathways needed to stimulate MHC class I-restricted CTL responses.

It is well known that CTLs play an important part of the immune responses against viral infections and tumors. CTLs generally recognize naturally processed, antigenic short peptides composed of approximately nine amino acids in association with MHC class I molecules on the surface of APCs, and lyse target cells, including virus-infected cells or tumor cells. Most of these peptides result from the degradation of endogenously synthesized proteins such as viral proteins or tumor antigens in the cytosol and are supplied to class I molecules in the endoplasmic reticulum via the transporters associated with antigen processing. Therefore, for the development of a CTL-based vaccine, it is crucial to find a vehicle that can safely and effectively deliver antigenic proteins into the cytosol. Vaccine vehicles that have been tried to date include attenuated viruses, intracellular bacteria [17] and naked DNA [18], although any one was not sufficient to satisfy both efficiency and safety. By contrast, VLPs safely mimic the distinguished ability of live viruses with great efficiency in the generation of strong and long-lasting CTL responses [19]. In the case of SV40-VLPs, another advantage is that it is possible to add the cell-targeting ability to them. For example, **dendritic cells** (DCs) are the most powerful APCs to initiate and maintain T-cell immune responses, and therefore, should be one of the major targets for the antigen delivery system of SV40-VLPs.

In general, subunit vaccines are poorly immunogenic, and therefore usually contain **adjuvants** for the enhancement of immune responses. A variety of adjuvants have been widely used for experiments.



Dendritic cells: the most powerful antigen-presenting cells that process antigens, present them to the immune system, and initiate immune responses. They also act as messengers between the innate and adaptive immunity.

Aa

Adjuvant: a pharmacological or immunological agent that is often included in vaccines to enhance the recipient's immune response to the target antigen.

Pattern recognition: a process to recognize the characteristic features of pathogens through pattern recognition receptors in innate immunity.

However, few adjuvants have been licensed for clinical use around the world due to the risk of side effects. Furthermore, the most commonly used adjuvants for humans, alum [20] and the oil-based formulation MF59 [21] are useful for the augmentation of antibody responses, but not for cellular immune responses. In our experiments, it

should be noted that an adjuvant is not necessary for the epitope-specific CTL induction caused by the immunization with SV40-VP1s carrying an antigenic epitope, suggesting that SV40-VP1s have a natural adjuvant effect on CTL induction.

The mammalian immune system is comprised of innate and acquired immunity. Acquired immunity such as B- and T-cell immune responses is involved in the specific recognition and elimination of pathogens in the late phase of infection as well as the immunological memory. By contrast, innate immunity is the first line of host defense through the **pattern recognition** of various microorganisms via a limited number of germline-encoded pattern-recognition receptors such as Toll-like receptors and retinoic acid-inducible gene I-like receptors. Innate immunity plays an important role in the modulation of quality and quantity of acquired immune responses. Recent advances in immunology have revealed that most adjuvants enhance immune responses by triggering innate immune responses, rather than by direct effects on the lymphocytes [22]. The authors found that SV40-VP1s carrying an epitope strongly stimulated lymphocytes to upregulate the expression of maturation markers, CD80, CD86 and CD40. Although the mechanism of this action is still unclear, their data suggest that SV40-VP1s themselves stimulate innate immunity as a natural adjuvant.

What needs to be done for the successful development of SV40-VLP vaccine?

Since DCs play a central role for the induction of adaptive immune responses, a vaccine targeting DCs would be useful for the effective immunization to augment T-cell immunity. Several C-type lectin receptors (CLRs) are more or less specifically expressed by DCs, and are potential candidates for targeting molecules on the surface of DCs [23]. There are mainly two approaches to targeting



A subunit vaccine consists of an antigenic protein fragment derived from a virus without viral whole particles or genomes, and provides a protective immune response in the recipient. Examples include the hepatitis B vaccine that is composed of only the hepatitis B surface antigen, the virus-like particle vaccine against human papillomavirus, and the hemagglutinin subunit vaccine against influenza virus.

CLRs. One is a strategy based on the binding of natural receptor ligands [24,25] and the second strategy is to use CLR-specific antibodies [26–28]. To target SV40-VLPs to DCs, it is possible to insert particular CRL-recognition sequences derived from certain CLR ligands in the DE loop or HI loop of SV40-VLPs. A recent study reported a DC-targeted lentivector enveloped with a Sindbis virus-derived glycoprotein that are specific to the DC-specific surface protein, DC-SIGN [24,25].

In general, multiple immunizations are required for effective vaccines. Similar to viral vector-derived vaccines, however, SV40-VLP-mediated vaccines may potentially limit the effectiveness of multiple administrations due to the induction of anti-VLP immunity involving neutralizing antibodies. To lower the intrinsic immunogenicity of SV40-VLPs, we could use SV40 VP1 mutants that escape neutralization by monoclonal antibodies [29] for the construction of SV40-VLPs. These particles elicit low VLP-specific immune responses compared with wild-type SV40-VLPs, and might be used for repetitive immunization. Alternatively, the heterologous prime–boost immunization could circumvent the problem associated with the VLP-specific immunity. This strategy involves priming with naked DNA, followed by boosting with a viral vector, and generates stronger immune responses compared with regimens using a homologous boost. SV40-VLPs should be very good at boosting DNA-primed immune responses. Furthermore, it was shown that mucosal immunization of vaccinia-immune mice with a recombinant vaccinia virus expressing HIV gp160 induced strong HIV-specific serum antibodies and CTL responses. Therefore, mucosal vaccination may overcome the barrier caused by pre-existing immunity and can also be applicable for SV40-VLP immunization. Recently, it was demonstrated that immunization with adenoviral vector-infected cells elicited strong cellular immune responses specific for a target antigen but induced much weaker anti-adenovirus antibodies in adenovirus-seronegative macaques than in macaques injected with the adenovirus [30]. Thus, immunization with VLP-pulsed cells may enable repeated application of VLP-based vaccines for consistently eliciting T-cell responses toward target antigens.

The epitope approach to vaccine development offers several potential advantages. One of them is that epitopes are relatively safer than antigenic proteins of origin, as exemplified by the case of E6 and E7 proteins of human papillomavirus that are associated with cervical carcinoma. However, the large degree of HLA polymorphism is a major obstacle to be overcome in the development of epitope-based vaccines. Moreover, broad T-cell immune responses that are directed against multiple epitopes are desirable because the virus is less likely to

accumulate escape mutations under broad immune responses. As described before, SV40-VLPs can encapsulate large foreign DNA or heterologous proteins fused to the C-terminus of VP2/3, and deliver them into mammalian cells. Therefore, it might be possible for SV40-VLPs to carry a large antigen in the form of a protein or long DNA that contains multiple epitopes associated with a variety of HLA class I molecules.

SV40-VLPs are also applicable to a platform for the CTL-based cancer vaccine. However, in cancer there are at least three significant barriers to be overcome [31]. First, since most cancer antigens are closely related or identical to self-antigens, it is often difficult to induce therapeutic immune responses. Second, DCs have to receive a suitable maturation signal, allowing them to differentiate properly to promote protective immunity. Without such a signal, DCs would induce tolerance leading to T-cell deletion, anergy or the generation of regulatory T cells. Finally, effector T cells have to overcome immunosuppression in the tumor bed.

Conclusion

Here, we have shown that SV40-VLPs are promising as a platform candidate for the CTL-based vaccine. However, several important issues remain to be solved for further development of the SV40-VLP-based vaccine as described above. At the same time, we should abide by all stringent rules and regulations set forth in the preparation of biological drug products demanded by Good Manufacturing Practice for Imported Drugs and Medical Devices, and overcome the difficulty of the establishment of the methodology of the large amount preparation of the equal quality vaccine platforms. In this regard, the minimum unit for sufficient CTL induction should be identified from the SV40 VP1-based material because the organic synthesis of the smallest part would reduce the time and cost of preparation, and minimize biological contamination during the purification steps.

Financial & competing interests disclosure

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Summary.

- Simian virus 40 (SV40) virus-like particles (VLPs) are purified from the lysate of SF-9 insect using the baculovirus expression system, and VP1-pentamers were prepared from purified SV40-VLPs.
- Minor capsid proteins, VP2/3 or dsDNA contribute to the *in vitro* reassembly of 45-nm SV40-VLPs from VP1-pentamers under the physiological condition.
- SV40-VLPs can encapsulate approximately 5-kb dsDNA shielded by histone or foreign proteins fused to VP2/3, and effectively deliver them into mammalian cells.
- Insertion of a particular foreign peptide into the DE and HI loops provides SV40-VLPs with the ability of active cell targeting.
- SV40-VLPs are very attractive to deliver immunogenic epitopes as a vaccine delivery system with characteristics of natural adjuvants.
- Successful SV40-VLP-based vaccine will need to target DCs, escape from neutralizing antibodies, and overcome HLA polymorphisms.

References

- | | | | |
|---|--|---|--|
| 1 | Liu MA. Immunologic basis of vaccine vectors. <i>Immunity</i> 33(4), 504–515 (2010). | Berk AJ, Handa H. <i>In vitro</i> reconstitution of SV40 particles that are composed of VP1/2/3 capsid proteins and nucleosomal DNA and direct efficient gene transfer. <i>Virology</i> 420(1), 1–9 (2011). | capsid protein VP1 into particles. <i>J. Biol. Chem.</i> 281(15), 10164–10173 (2006). |
| 2 | Roy P, Noad R. Virus-like particles as a vaccine delivery system: myths and facts. <i>Hum. Vaccin. A</i> (1), 5–12 (2008). | Inoue T, Kawano MA, Takahashi RU <i>et al.</i> Engineering of SV40-based nano-capsules for delivery of heterologous proteins as fusions with the minor capsid proteins VP2/3. <i>J. Biotechnol.</i> 134(1–2), 181–192 (2008). | 11 Ewers H, Romer W, Smith AE <i>et al.</i> GM1 structure determines SV40-induced membrane invagination and infection. <i>Nat. Cell Biol.</i> 12(1), 11–18 (2010). |
| 3 | Daniels R, Rusan NM, Wadsworth P, Hebert DN. SV40 VP2 and VP3 insertion into ER membranes is controlled by the capsid protein VP1: implications for DNA translocation out of the ER. <i>Mol. Cell</i> 24(6), 955–966 (2006). | 7 Qi F, Carbone M, Yang H, Gaudino G. Simian virus 40 transformation, malignant mesothelioma and brain tumors. <i>Expert Rev. Respir. Med.</i> 5(5), 683–697 (2011). | 12 Schowalter RM, Pastrana DV, Buck CB. Glycosaminoglycans and sialylated glycans sequentially facilitate merkel cell polyomavirus infectious entry. <i>PLoS Pathog.</i> 7(7), e1002161 (2011). |
| 4 | Takahashi RU, Kanesashi SN, Inoue T <i>et al.</i> Presentation of functional foreign peptides on the surface of SV40 virus-like particles. <i>J. Biotechnol.</i> 135(4), 385–392 (2008). | 9 Kanesashi SN, Ishizu K, Kawano MA <i>et al.</i> Simian virus 40 VP1 capsid protein forms polymorphic assemblies <i>in vitro</i> . <i>J. Gen. Virol.</i> 84, 1899–1905 (2003). | 13 Magaldi T, Buch MH, Murata H <i>et al.</i> Mutations in the GM1 binding site of simian virus 40 VP1 alter receptor usage and cell tropism. <i>J. Virol.</i> 86(13), 7028–7042 (2012). |
| 5 | Tsukamoto H, Kawano MA, Inoue T <i>et al.</i> Evidence that SV40 VP1-DNA interactions contribute to the assembly of 40-nm spherical viral particles. <i>Genes Cells</i> 12(11), 1267–1279 (2007). | 10 Kawano MA, Inoue T, Tsukamoto H <i>et al.</i> The VP2/VP3 minor capsid protein of simian virus 40 promotes the <i>in vitro</i> assembly of the major | 14 Kitai Y, Fukuda H, Enomoto T <i>et al.</i> Cell selective targeting of a simian virus 40 virus-like particle conjugated to epidermal growth factor. <i>J. Biotechnol.</i> 155(2), 251–256 (2011). |
| 6 | Enomoto T, Kukimoto I, Kawano MA, Yamaguchi Y, | | 15 Hatakeyama M, Kishi H, Kita Y <i>et al.</i> A two-step ligand exchange reaction generates |

- highly water-dispersed magnetic nanoparticles for biomedical applications. *J. Mater. Chem.* 21(16), 5959–5966 (2011).
- 16 Enomoto T, Kawano M, Fukuda H *et al.* Viral protein-coating of magnetic nanoparticles using simian virus 40 VP1. *J. Biotechnol.* 167(1), 8–15 (2013).
- 17 Singh R, Wallencha A. Cancer immunotherapy using recombinant listeria monocytogenes: transition from bench to clinic. *Hum. Vaccin.* 7(5), 497–505 (2011).
- 18 Saade F, Petrovsky N. Technologies for enhanced efficacy of DNA vaccines. *Expert Rev. Vaccines* 11(2), 189–209 (2012).
- 19 Buonaguro L, Tagliamonte M, Tornesello ML, Buonaguro FM. Developments in virus-like particle-based vaccines for infectious diseases and cancer. *Expert Rev. Vaccines* 10(11), 1569–1583 (2011).
- 20 Marrack P, McKee AS, Munk M. Towards an understanding of the adjuvant action of aluminium. *Nat. Rev. Immunol.* 9(4), 287–293 (2009).
- 21 O'Hagan DT, Ott GS, De Gregorio E, Seubert A. The mechanism of action of MF59 – an innately attractive adjuvant formulation. *Vaccine* 30(29), 4341–4348 (2012).
- 22 McKee AS, Munk M, Marrack P. How do adjuvants work? Important considerations for new generation adjuvants. *Immunity* 27(5), 687–690 (2007).
- 23 Tacken PJ, de Vries IJM, Torensma R, Figdor CG. Dendritic-cell immunotherapy: from *ex vivo* loading to *in vivo* targeting. *Nat. Rev. Immunol.* 7(10), 790–802 (2007).
- 24 Yang L, Yang H, Rideout K *et al.* Engineered lentivector targeting of dendritic cells for *in vivo* immunization. *Nat. Biotechnol.* 26(3), 326–334 (2008).
- 25 Dai B, Yang L, Yang H *et al.* HIV-1 Gag-specific immunity induced by a lentivector-based vaccine directed to dendritic cells. *Proc. Natl Acad. Sci. USA* 106(48), 20382–20387 (2009).
- 26 Bonifaz LC, Bonnyay DP, Charalambous A *et al.* *In vivo* targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. *J. Exp. Med.* 199(6), 815–824 (2004).
- 27 Trumpfheller C, Finke JS, López CB *et al.* Intensified and protective CD4⁺ T cell immunity in mice with anti-dendritic cell HIV gag fusion antibody vaccine. *J. Exp. Med.* 203(3), 607–617 (2006).
- 28 Dakappagari N, Maruyama T, Renshaw M *et al.* Internalizing antibodies to the C-type lectins, L-SIGN and DC-SIGN, inhibit viral glycoprotein binding and deliver antigen to human dendritic cells for the induction of T cell responses. *J. Immunol.* 176(1), 426–440 (2006).
- 29 Murata H, Teferedegne B, Sheng L, Lewis AM, Peden K. Identification of a neutralization epitope in the VP1 capsid protein of SV40. *Virology* 381(1), 116–122 (2008).
- 30 Sun C, Feng L, Zhang Y *et al.* Circumventing antivector immunity by using adenovirus-infected blood cells for repeated application of adenovirus-vectored vaccines: proof of concept in rhesus macaques. *J. Virol.* 86(20), 11031–11042 (2012).
- 31 Mellman I, Coukos G, Dranoff G. Cancer immunotherapy comes of age. *Nature* 480(7378), 480–489 (2011).

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Plant-produced virus-like particle vaccines

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Nunzia Scotti &
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Virus-like particles (VLPs) are possibly the best candidates for safe, immunogenic, efficacious and inexpensive vaccines, for both animals and humans. Well-characterized human and animal viruses such as hepatitis B and C, HIV and papillomaviruses, rotaviruses, norovirus, foot-and-mouth disease viruses and even influenza A virus proteins have all been successfully investigated for VLP formation. Proteins have been produced in transgenic plants and via transient expression techniques; simple structures and structures depending on more than one protein, naked and enveloped particles, and peptides displayed on other viruses have all been made. There have been multiple proofs of concept, and more than a few proofs of efficacy. This chapter covers the history of VLP production in plants, and explores a few examples in detail to illustrate the potential of such a mode of production for human and animal medicine.

Aa

Virus-like particle (VLP): composed of subunit proteins and possibly envelope only.

Hepatitis B surface antigen: an outer membrane protein encoded by the S gene of hepatitis B virus. The S gene is generally divided into three domains: these are S, preS1 and preS2, all of which contain epitopes able to induce antibody production, while preS2 also includes T-cell activation epitopes.

Transient expression: foreign protein expression elicited during the lifetime of a normal plant by means of a recombinant expression system introduced into the mature plant, such as a bacterium or a virus.

Virus-like particles (VLPs) are increasingly being used as vaccine candidates for both animals and humans; an advantage over whole-virus-derived products are that no infectious material is involved at any stage. VLPs are also highly immunogenic and are self-adjuvanting in terms of stimulating both Th1- and Th2-type immune responses. One disadvantage is the need for relatively high doses as they do not replicate, meaning that they have to be manufactured at high yield as well as inexpensively. VLP-based

recombinant hepatitis B virus (HBV) and human papillomavirus (HPV) VLP vaccines made in yeast and insect cells are currently available for human use; no animal products have yet been registered.

Plants as production vehicles for vaccines

The use of plants as reaction vessels to produce high-value biological pharmaceuticals has been actively explored since 1989, at first for monoclonal antibodies [1]. One of the first vaccine candidates made in plants was **hepatitis B surface antigen** (HBsAg) in 1992 [2]; this was followed by many others, reviewed in detail recently [3]. This chapter will therefore cover VLP-specific applications and new developments in expression technology.

The wide variety of types of plants and plant tissues and cells historically used for vaccine production, as well as expression via transgenesis or **transient expression**, has been covered in detail elsewhere [3,4]. Presently, the use of ‘deconstructed’ plant viral vectors and of somatic gene transfer to plant cells via whole-plant infiltration with *Agrobacterium tumefaciens* – a process now known as agroinfiltration – has become a viable alternative to the previous use of stably transformed plants.

Hepatitis B vaccines

HBV is the etiologic agent of liver cirrhosis or hepatocellular carcinoma and causes more than 600,000 deaths per year worldwide [5]. Although a highly effective HBV vaccine based on HBsAg VLPs has been available since 1996, several alternative and cheaper strategies based on plant-derived HBsAg VLPs have been developed (reviewed in [6]). Since 1992, HBV vaccine production – mostly based on HBsAg protein – has been widely explored in a number of **transgenic plants** or plant cell

Aa

Transgenic plant: plant containing foreign DNA sequences integrated into its chromosomes.

systems. In this chapter, we will summarize the more recent and successful examples.

In terms of production yield, the best results (up to 295 µg/g fresh weight [FW]) were obtained using the MagnICON®

(Nomad Bioscience, Munich, Germany) transient expression system [7]. The HBsAg correctly assembled into VLPs displaying the protective ‘a’ determinant, and the partially purified HBsAg elicited specific antibodies in mice, showing an immunogenicity equivalent to the licensed recombinant yeast-derived vaccine antigen.

Other examples of plant-based HBsAg vaccine production have been focused on their use for oral immunization. One of the major problems of oral immunization is the induction of tolerance that suppresses antigen-specific immune responses, partly due to regulatory T cells blocking the proliferation of CD4⁺ and CD8⁺ effector T cells [8]. Kostrzak *et al.* tested the effects of orally administered plant-based vaccines on regulatory T cells and specific immune response, using low doses of HBsAg (0.5–100 ng VLPs) in lyophilized tobacco leaves in a prime–boost immunization scheme [9]. Regulatory T cells increased linearly at high antigen doses and dry plant tissue mass, while the highest humoral IgA and IgG responses correlated with the lowest antigen dose. These results suggest that the oral tolerance is linked to both antigen dose and plant tissue.

Oral immunizations have also been tested using lettuce and maize expressing HBsAg [10–12]. The objective of Pniewski and colleagues was to develop a prototype of an oral vaccine based on the second generation of primary (T_0) transgenic lettuce plants (T_1) that stably expressed HBsAg at >10 µg/g FW, where the HBsAg assembled into VLPs displaying the protective ‘a’ determinant [12]. They administered mice orally with lyophilized lettuce tissues containing 100 ng of HBsAg VLP with a 60-day interval between prime and boost, and observed mucosal and systemic humoral specific responses at the protective level (10 mIU/ml). Furthermore, they verified that lyophilized tissues converted into tablets preserved the HBsAg content for at least 1 year at room temperature.

Hayden *et al.* chose maize as an oral vaccine delivery system for HBsAg, because it contains no allergenic or carcinogenic agents and provides a bioencapsulated environment, rich in protease inhibitors, that ensures antigen stability at room temperature [10,11]. They obtained the best expression with a construct containing the embryo-preferred promoter globulin-1, and further enhanced HBsAg yield up to



MagnICON® (Nomad Bioscience, Munich Germany): a ‘deconstructed’ tobacco mosaic virus vector system based on the *in planta* assembly of functional viral vectors by the recombination of separate provector modules.



A chimeric protein is a protein molecule containing amino acid sequences from another protein.

166 µg/g through a back-cross program, followed by germ enrichment and oil extraction. The germ-enriched maize (corresponding to 0.83 mg HBsAg) was used as boost in immunization experiments in mice primarily injected with commercial vaccine. Mice fed with the HBsAg germ showed a >50% increase in specific antibodies in both mucosal and systemic responses.

Pniewski *et al.* formulated a novel prototype of an oral tricomponent vaccine based on medium (M-HBsAg) and large (L-HBsAg) surface antigens containing S + preS2 and S + preS1 + preS1 domains, respectively [13]. This study demonstrated, in both lettuce and tobacco, that M-HBsAg was expressed at higher levels and appeared more stable after processing conditions than L-HBsAg. Alternatively, Hayden *et al.* obtained with an improved version of globulin-1 promoter a higher concentration of HBsAg in maize (up to 0.51% total soluable protein) and a protein tolerating 55°C for 1 month without degradation [10].

Papillomavirus vaccines

The production of papillomavirus vaccine candidates in plants has been well covered recently [14]; however, we will give a brief account of the history as well as of new developments, in order to illustrate important parameters affecting plant production.

High-risk variants of HPV (HR-HPVs) cause cancer of the cervix in women, and may in fact cause 5% of all cancers worldwide. The two HR-HPV types HPV-16 and -18 together cause approximately 70% of all cervical cancers [15]. The two highly successful prophylactic vaccines against HPV now available are Merck's Gardasil® (NJ, USA; HPV genital wart types 6 and 11, and HR types HPV-16 and -18), produced in yeast, and GlaxoSmithKline's Cervarix® (Middlesex, UK; HPV-16 and -18 only), made in insect cells via recombinant baculoviruses. Both vaccines protect against cervical intraepithelial neoplasia 2 or 3 associated with HPV-16 and -18, which may progress to cervical cancer [16].

Both vaccines are highly expensive, include only two oncogenic virus types, and do not cure established infections. In addition, they are delivered via intramuscular injection and require a cold chain [17]. Thus, there is room for improvement, both in terms of cost and in breadth of efficacy and delivery.

Papillomavirus VLPs may be made by expression of the major capsid protein L1 alone, or by coexpression of L1 and the minor protein L2 [18]. In 2003, Varsani and coworkers described expression of a full-length native HPV-16 L1 gene in transgenic *Nicotiana tabacum* [19]; another group expressed a plant codon-optimized HPV-11 L1 protein gene in transgenic potato tubers that assembled into recognizable VLPs [20]; a third expressed HPV VLPs from

a human codon-optimized HPV-16 *L1* gene in transgenic tobacco and potato [21]. While all of the products were immunogenic in experimental animals, yields were too low for meaningful production.

In 2006, there were two important proofs of efficacy of plant-produced papillomavirus vaccines. Cottontail rabbit papillomavirus (CRPV) L1 protein produced either in transgenic tobacco, or via recombinant tobacco mosaic virus (rTMV) in *Nicotiana benthamiana*, protected rabbits against development of tumors after virus challenge [22]. The display of CRPV or rabbit oral papillomavirus L2 protein-derived peptides on the surface of rTMV particles also completely (CRPV) or partially (rabbit oral papillomavirus) protected rabbits from cognate live virus challenge [23].

Further work on HPV-11 L1 showed that the native *L1* gene could be expressed at relatively high levels, and formed capsomers and VLPs, in transgenic *N. tabacum* and *Arabidopsis thaliana* and via rTMV expression in *N. benthamiana* [24].

Maclean *et al.* determined that human codon optimization of HPV-16 *L1* was better for gene expression than use of the native gene, or of *Nicotiana* spp. optimization, and that localization of protein to chloroplasts via the appropriate import signal resulted in significantly better accumulation (>0.5 g/kg whole plant) of protein than accumulation in cytoplasm or the endoplasmic reticulum [25]. Chloroplast-localized protein assembled efficiently into VLPs at high yield and the particles were highly effective immunogens in mice, resulting in high titers of neutralizing antibodies. A replicating Bean yellow dwarf virus-derived vector achieved an increase of over 50% in yields of HPV-16 L1 compared with using the conventional vector [26].

Transplastomic expression of L1 was also highly successful [27]: a HPV-16 *L1* gene in tobacco chloroplasts produced 3 g/kg wet weight of L1 protein, which assembled into VLPs, bound conformation-specific monoclonal antibodies and elicited neutralizing antibodies in injected mice. Recently, another group successfully expressed in chloroplasts a HPV-16 L1 protein modified to assemble only to pentameric capsomer level [28].

HPV-8 is a high-risk cutaneous papillomavirus associated with epidermodysplasia verruciformis and nonmelanoma skin cancer in immunocompromised individuals. While there is no vaccine presently, Matic *et al.* successfully expressed HPV-8 L1 VLPs at high yield via transient expression in *N. benthamiana*, to give the prospect of a niche vaccine [29].

HPV VLPs are also useful vehicles for surface display of other vaccine epitopes [30]. Recently, various chimeras of the HPV-16 L1 protein and the ectodomain of the influenza virus M2 ion channel protein (M2e) were

expressed via agroinfiltration in *N. benthamiana* [31]. Chimeras assembled correctly, and reacted with an anti-M2 monoclonal antibody.

The Rybicki laboratory has produced candidate second-generation HPV vaccines in plants based on HPV-16 L1 chimeras including portions of the minor L2 protein, which should allow elicitation of a wider range of cross-type neutralizing antibodies [30]. Preliminary success has been obtained with a L1:L2 (amino acid residues 108–120) construct as a helix 4 replacement, which expresses at very high yield and results in smaller-than-virion-sized VLPs [32] [PINEO C, HITZEROTH I, RYBICKI E, UNPUBLISHED DATA].

Bovine papillomaviruses (BPVs) are economically important in horses and cattle, as eight virus species cause mainly benign but also some carcinogenic tumors [33,34]. Commercial vaccines are generally crude preparations made from homogenized warts, as the viruses cannot be cultured, and yeast or insect cell-made subunit VLP vaccines are expensive. Transient expression of a *N. tabacum* codon-optimized BPV-1 L1 gene in *N. benthamiana* gave high (0.18 g/kg) yields of 30-nm VLPs, which were highly immunogenic in rabbits [35]. The authors conclude that theirs is the first report of a successful plant-produced candidate vaccine against any BPV.

Norwalk virus vaccines

Norwalk virus is the major cause of nonbacterial gastroenteritis in adult humans; moreover, it displays a high mutation rate in the sequence of the major virus capsid protein (VP1), which complicates efforts to make conventional vaccines. Plant-based VP1-VLP production has been pursued using different biotechnological methods and plant species. The first example of Norwalk virus capsid protein (NVCP) expression in transgenic tobacco and potato gave a very low protein yield in both species [36].

However, novel and rapid transient expression systems based on engineered plant viruses (MagnICON and geminiviral vectors) have been successfully used recently to give usable yields [37–39]. In particular, the MagnICON transient system in *N. benthamiana* plants produced approximately 0.8 mg/g leaf of properly assembled NVCP [39]. The plant-derived recombinant protein was partially purified and used at two different dosages (100 and 250 µg) for immunizations of CD1 mice with or without adjuvant (cholera toxin [CT]). All mice responded after the first and at each administration. The immune response was dose dependent and the highest titers were produced with 250 µg of plant-based NVCP in the presence of CT adjuvant.

This plant-based system was recently used by Lai and Chen to demonstrate its ability to produce scalable and current good manufacturing practice

(cGMP) compliant procedures for NVCP VLP extraction and purification [40]. They started with the establishment of greenhouse and bioprocessing facilities, designed to have a separated unidirectional flow of workers and biological materials. The master and working banks of *A. tumefaciens* harboring the NVCP expression vector and the seed bank of wild-type *N. benthamiana* were established and qualified by quality management system procedures. Furthermore, they developed efficient and scalable extraction and purification procedures for NVCP-VLPs, demonstrating the identity, purity and safety of plant-derived NVCP. The production cost of 1 g NVCP obtained with this method is US\$15, significantly lower than any other recombinant system.

Another transient expression system based on geminivirus-derived DNA replicon vectors allowed rapid production of a satisfactory NVCP-VLP yield of 0.34 and 0.2 g/kg leaves in *N. benthamiana* and lettuce, respectively [37,38].

HIV vaccines

HIV type 1 is the etiological agent of AIDS, probably the most important infectious disease threat in the world currently. Although several vaccine models based on structural (e.g., Env membrane glycoprotein and/or Gag capsid protein) or regulatory (e.g., Tat, Nef and Pol) proteins have been developed and tested in human clinical trials [41,42]. Most of them demonstrated no efficacy or a modest protection (31% efficacy) [43].

Recently, the feasibility of using plants to make regulatory and structural HIV proteins has been explored (reviewed in [44,45]); however, only a few studies have demonstrated production of HIV VLPs [46–48].

Meyers *et al.* expressed the Gag capsid polyprotein and its truncated versions (p17–p24 and p24) using *Agrobacterium*-based stable nuclear transformation and rTMV-based transient expression [47]. They obtained generally low expression levels, with a maximal level of 4.8 mg/kg fresh leaf weight of chloroplast-targeted p17–p24 produced by transient expression. The plant-derived p17–p24 was able to boost humoral and cellular responses in mice primed with a Gag DNA vaccine.

Scotti *et al.* investigated the factors potentially affecting Pr55^{gag} protein accumulation in plant cells using both transient and stable (nuclear and



Env glycoprotein: a glycosylated membrane polyprotein (glycoprotein [gp]160) of HIV that serves to form the viral envelope. It is composed of the surface protein (gp120) and the transmembrane protein (gp41). The gp160 precursor is cleaved by a cellular protease.

Gag capsid protein: a major structural polyprotein precursor (Pr55^{gag}) of HIV. It is composed of four distinct domains and two spacer peptides, the N-terminal matrix domain (p17), the central capsid domain (p24), the nucleocapsid domain (p7), the C-terminal p6 domain, and p1 and p2 spacer peptides located at the capsid domain/nucleocapsid domain and nucleocapsid domain/p6 junctions, respectively. Each domain is produced by cleavage of the Gag precursor by the *pol*-encoded protease during the maturation process.

plastid) expression systems [48]. Although the recombinant Pr55^{gag} accumulated in the chloroplasts with all expression systems, a significantly higher protein yield was achieved by plastid transformation. The highest accumulation level of Pr55^{gag} in transplastomic plants (up to 363 mg/kg FW) was obtained by modifying its N-terminus by fusion to the 5' untranslated region and the 42 N-terminal nucleotides of the plastid *rbcL* gene. Electron microscopy showed that the plastid-derived Pr55^{gag} protein assembled into VLPs.

More recently, a plant-optimized enveloped VLP based on Gag protein and a deconstructed form of glycoprotein 41 (dgp41), consisting of the membrane proximal external region, transmembrane domain and cytoplasmic tail, has been produced by the combination of stable nuclear transformation (for Gag) and MagnICON transient expression (for dgp41) [46]. A yield of approximately 22 mg/kg FW for Gag and 9 mg/kg FW for dgp41 was obtained. The coexpressed proteins had a sedimentation pattern similar to HIV type 1 VLPs expressed in insect cells or yeast, while dgp41 alone showed a different fractionation pattern, suggesting that the two proteins were colocalized in the same particles. Furthermore, they demonstrated by coexpressing both proteins that they assembled into VLPs, and budded through plant plasma membrane into the medium.

Although the highest Gag-VLP yield was obtained with plastid transformation [48], all of the results described here give serious credence to the real prospect of making important HIV vaccine components in plants.

Hepatitis C vaccines

Hepatitis C virus (HCV) causes contagious liver diseases, ranging from mild and transient to serious and chronic illnesses. HCV is characterized by a high mutation rate, especially in the N-terminal sequence of the putative envelope protein E2, known as hypervariable region 1; this feature complicates vaccine development [49].

Only a few studies of plant expression have been performed, mostly based on transient expression systems and focused on the evaluation of the R9 or E2 epitopes [50–54]. In particular, different plant viruses have been engineered to obtain chimeric particles by fusing the HCV epitopes to the B subunit of CT or to plant viral coat proteins. Although all chimeric particles elicited a specific immune response in mice, the highest specific immune response was obtained using chimeric potato virus X particles with R9 epitopes fused to the coat proteins via the 2A endoprotease coding sequence of foot-and-mouth disease virus (FMDV). A variable cross-reactivity between plant-derived chimeric particles and sera from patients

chronically infected with HCV was observed [52–54], probably due to different immunization schemes and the individual human sera.

To date, only one proof-of-concept study has focused on the expression of the HCV core antigen in transgenic tobacco plants [55]. The plant-based core antigen extracted from T₀ and T₁ progenies was able to react with polyclonal and monoclonal specific antibodies, and with a human serum from an infected patient, suggesting that it could be used either as a vaccine or as a reagent for the detection of infected patients.

FMDV vaccines

FMDV, genus *Aphthovirus*, family *Picornaviridae*, was one of the first viruses ever characterized, and has been studied intensively ever since [56]. There are seven serotypes of the virus, and many strains; there is no cross-protection between serotypes, and strains within a serotype may differ significantly as well. Thus, while there are successful killed whole-virus vaccines against all serotypes [57], and protective immunity is long lived, the continual emergence and re-emergence of different serotypes around the world means continuous vaccination programs are essential to control disease [58]. Given that much of the burden of disease is in developing countries, and in Africa the virus is also endemic in wild animal populations, achieving sufficient conventional vaccine coverage is a serious problem. A significant development in 2013 was the news that VLPs could be made at high yield in insect cells via recombinant baculoviruses, by coexpressing the P1 capsid polyprotein and the 3C proteinase [59].

Attempts to make FMDV vaccines in plants date back to the early days of biofarming, with varying degrees of success. For example, in 1993, the cowpea mosaic virus S protein gene was engineered to contain a FMDV VP1 peptide; an infectious vector produced cowpea mosaic virus virions displaying the peptide that reacted with FMDV-specific antiserum [60]. In 1998, protective immunity was elicited by immunization of mice with leaf extracts from transgenic *A. thaliana* expressing the VP1 subunit of FMDV [61]. This protein does not form any greater aggregate than pentamers; however, it also proved to be protective via feeding in transgenic alfalfa [62], and when made in *N. benthamiana* via recombinant TMV [63] and in transgenic potato [64].

Expression in transgenic alfalfa or tomato of the whole P1 polyprotein plus the 3C proteinase apparently resulted in VLP formation, in alfalfa at least [65,66], as well as protective efficacy in mice and guinea pigs, for the alfalfa and tomato products, respectively. Immunodominant peptides derived from VP1 have been fused to TMV [67] or Bamboo mosaic virus coat

proteins [68], with protection in guinea pigs and mice, and swine, respectively. An infectious clone of tobacco necrosis virus A displaying FMDV VP1-derived peptides on virion surfaces elicited humoral responses in mice after intramuscular injection, and systemic and mucosal responses after intranasal immunization [69].

Rotaviruses

Rotaviruses (genus *Rotavirus*; family *Reoviridae*) are pathogens with multicomponent dsRNA genomes, and cause approximately 110 million cases of human disease annually, with over 440,000 deaths in children under the age of 5 years [70]. Rotavirus infections in calves and piglets also cause significant economic losses. There are two live-attenuated human rotavirus vaccines; however, these are expensive in developing countries, and reassortment between vaccines and wild-type viruses may lead to novel pathogenic variants of rotavirus [71]. There are also concerns that the vaccines do not work as well in Africa as in Europe, due to differences in specific virus prevalences [72].

Reoviruses have four structural proteins in a multilayered capsid structure: VP2 forms an inner shell, overlaid by VP6; this is overlaid by VP7 for an outer shell, with VP4 forming spikes at fivefold rotational symmetry axes [73]. VP6 is an important group-specific antigen; VP7 and VP4 are the main type-specific and neutralization determinants. VP4 is cleaved during infection of cells into VP5 and VP8*, and anti-VP8* antibodies can also neutralize infectivity [74]. Virion-like VLPs were made by coexpression of all four structural proteins in insect cells [75], and scale-up was investigated for three-layer VP2/6/7 VLPs [76]; similar VLPs have also been produced in yeast [77]. An insect High-Five™ (Invitrogen, CA, USA) cell line, constitutively expressing VP2 protein of rotavirus RF strain and producing core-like VLP particles, has been recently established for vaccine development, gene therapy and drug delivery [78].

The first VLPs made in plants for rotaviruses were 'core' particles, or two-shell VP2/6 VLPs, expressed in fruits of transgenic tomato [79]. A later study used transgenic tobacco coexpressing VP2, 4 and 6 [80]: both VP2/6 and VP2/4/6 VLPs were made, and oral immunization of mice with total soluble protein extracts plus CT adjuvant elicited rotavirus-specific responses comparable with the attenuated rotavirus vaccine responses. The VP2/6/7 VLPs induced higher serum IgG and fecal IgA responses than VP2/6 VLPs.

Current studies in the Rybicki laboratory use capsid proteins of a local G9 P[6] isolate of human rotavirus that is not well matched to available commercial vaccines [81]. Expression of VP-2, 4 and 6 in *N. benthamiana*

via coagroinfiltration produced VP2/6 and VP2/6/4 VLPs in the cytosol, with yields as high as 1.1 g/kg of plant material, for batches of 100 g [82].

Bluetongue virus

Bluetongue is an insect-transmitted disease of ruminants, caused by multicomponent dsRNA viruses in the genus *Orbivirus*, family *Reoviridae*. There are 26 serotypes of the virus presently recognized worldwide [83]; virulence of different virus strains varies considerably, and serotypes do not cross-protect against each other after infection [84]. While it is endemic in Africa, the disease has relatively recently emerged as a serious and recurrent problem in Europe, with viruses in serotype 8 specifically having the greatest incidence [85].

There are long-established commercial vaccines against bluetongue viruses, including both attenuated and inactivated whole virus, and vaccination is one of the most effective ways of both controlling and eradicating the disease. However, serious side effects [86], and the worry that the vaccines might reassort with wild-type viruses, limit conventional vaccine use in Europe. Consequently, whole-killed vaccines are preferred, with large amounts of live virus having to be grown for each serotype in cell culture systems. There is much published work on VLP-based experimental vaccines for bluetongue viruses. Coexpression of viral structural proteins VP-2 and -5 (outer shell) and VP-3 and -7 (inner shell) [87] in a variety of expression systems results in formation of stable VLPs, which are highly immunogenic and protective [88].

Conventional cell-based production is still an expensive technology, however, which prompted the EU Seventh Framework Programme-funded PlaProVa programme to investigate plant production of VLPs [89]. Plant codon-optimized versions of the VP2, 3, 5 and 7 genes were tested in *N. benthamiana* using a variety of transient expression vectors via agroinfiltration, resulting in high concentrations of fully assembled particles. These were protective in a sheep challenge model, indicating significant potential for this vaccine approach [90].

Conclusion

Plant-made VLP vaccine candidates have been made for a wide variety of human and animal virus diseases, including what amount to 'biosimilars' for both HBV and HPVs. Moreover, plant-based production of novel HPV vaccine types and of novel chimeric HPV vaccines has been successful, as has production of human Norwalk virus and rotavirus and ruminant bluetongue virus VLPs. A variety of HIV vaccine candidates have been made at high yield, and in some cases demonstrated to have promising

immunogenicity in experimental animals. Proofs of efficacy have been shown for plant-produced animal papillomavirus, FMDV and bluetongue virus VLP vaccines. The fact that complex particles such as chimeric display vehicles composed of hepatitis B core antigen, HPV L1 or plant viruses, and multishelled rota- and orbi-virus capsids can be made, as well as particles such as HBsAg VLPs that are at least partly composed of lipid, is proof that plant-based expression systems can potentially do at least as well as conventional cell-based systems for these antigens. The options for accumulating protein in seeds and in chloroplasts, and for transgenic as well as transient expression, make plant-based production far more flexible than cell-based systems – and if costs really can be as low as predicted, then the future of biofarming for vaccine production is bright indeed.

Financial & competing interests disclosure

EP Rybicki has previously had ties with Era Biotech (Barcelona, Spain) as a Consultant and Collaborator, and presently works with Medicago Inc. (QC, Canada) as a Consultant. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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Summary

- Plant-based production systems are more flexible than cell-based systems.
- Virus-like particle vaccine candidates have been produced in plants at high yield for several human and animal diseases, and their immunogenicity demonstrated.
- Biotechnological hurdles related to the plant-based production of vaccine antigens under current good manufacturing practice have been overcome.
- Both biosimilar or generic vaccines can be produced in plants, as well as novel candidates.

References

- 1 Hiatt A, Cafferkey R, Bowdish K. Production of antibodies in transgenic plants. *Nature* 342, 76–78 (1989).
- 2 Mason HS, Lam DM, Arntzen CJ. Expression of hepatitis B surface antigen in transgenic plants. *Proc. Natl Acad. Sci. USA* 89, 11745–11749 (1992).
- 3 Rybicki EP. Plant-made vaccines for humans and animals. *Plant Biotechnol. J.* 8, 620–637 (2010).
- 4 Rybicki EP. Plant-produced vaccines: promise and reality. *Drug Discov. Today* 14, 16–24 (2009).
- 5 Buonaguro L, Tagliamonte M, Tornesello ML, Buonaguro FM. Developments in virus-like particle-based vaccines for infectious diseases and cancer. *Expert Rev. Vaccines* 10, 1569–1583 (2011).
- 6 Scotti N, Rybicki EP. Virus-like particles produced in plants as potential vaccines. *Expert Rev. Vaccines* 12, 211–224 (2013).
- 7 Huang Z, LePore K, Elkin G, Thanavala Y, Mason HS. High-yield rapid production of hepatitis B surface antigen in plant leaf by a viral expression

- system. *Plant Biotechnol. J.* 5, 202–209 (2008).
- 8 Faria AM, Weiner HL. Oral tolerance. *Immunol. Rev.* 206, 232–259 (2005).
- 9 Kostrzak A, Cervantes Gonzalez M, Guetard D *et al.* Oral administration of low doses of plant-based HBsAg induced antigen-specific IgAs and IgGs in mice, without increasing levels of regulatory T cells. *Vaccine* 27, 4798–4807 (2009).
- 10 Hayden CA, Egelkrot EM, Moscoso AM *et al.* Production of highly concentrated, heat-stable hepatitis B surface antigen in maize. *Plant Biotechnol. J.* 10, 979–984 (2012).
- 11 Hayden CA, Streatfield SJ, Lamphear BJ *et al.* Bioencapsulation of the hepatitis B surface antigen and its use as an effective oral immunogen. *Vaccine* 30, 2937–2942 (2012).
- 12 Pniewski T, Kapusta J, Bociag P *et al.* Low-dose oral immunization with lyophilized tissue of herbicide-resistant lettuce expressing hepatitis B surface antigen for prototype plant-derived vaccine tablet formulation. *J. Appl. Genet.* 52, 125–136 (2011).
- 13 Pniewski T, Kapusta J, Bociag P *et al.* Plant expression, lyophilisation and storage of HBV medium and large surface antigens for a prototype oral vaccine formulation. *Plant Cell. Rep.* 31, 585–595 (2012).
- 14 Giorgi C, Franconi R, Rybicki EP. Human papillomavirus vaccines in plants. *Expert Rev. Vaccines* 9, 913–924 (2010).
- 15 Bosch FX, Burchell AN, Schiffman M *et al.*
- Epidemiology and natural history of human papillomavirus infections and type-specific implications in cervical neoplasia. *Vaccine* 26(Suppl. 10), K1–K16 (2008).
- 16 Lu B, Kumar A, Castellsague X, Giuliano AR. Efficacy and safety of prophylactic vaccines against cervical HPV infection and diseases among women: a systematic review & meta-analysis. *BMC Infect. Dis.* 11, 13 (2011).
- 17 Haug CJ. Human papillomavirus vaccination – reasons for caution. *N. Engl. J. Med.* 359, 861–862 (2008).
- 18 Buonaguro FM, Tornesello ML, Buonaguro L. Virus-like particle vaccines and adjuvants: the HPV paradigm. *Expert Rev. Vaccines* 8, 1379–1398 (2009).
- 19 Varsani A, Williamson AL, Rose RC, Jaffer M, Rybicki EP. Expression of human papillomavirus type 16 major capsid protein in transgenic *Nicotiana tabacum* cv. Xanthi. *Arch. Virol.* 148, 1771–1786 (2003).
- 20 Warzecha H, Mason HS, Lane C *et al.* Oral immunogenicity of human papillomavirus-like particles expressed in potato. *J. Virol.* 77, 8702–8711 (2003).
- 21 Biemelt S, Sonnewald U, Galmbacher P, Willmitzer L, Muller M. Production of human papillomavirus type 16 virus-like particles in transgenic plants. *J. Virol.* 77, 9211–9220 (2003).
- 22 Kohl T, Hitzeroth II, Stewart D *et al.* Plant-produced cottontail rabbit papillomavirus L1 protein protects against tumor challenge: a proof-of-concept study. *Clin. Vaccine Immunol.* 13, 845–853 (2006).
- 23 Palmer KE, Benko A, Doucette SA *et al.* Protection of rabbits against cutaneous papillomavirus infection using recombinant tobacco mosaic virus containing L2 capsid epitopes. *Vaccine* 24, 5516–5525 (2006).
- 24 Kohl TO, Hitzeroth II, Christensen ND, Rybicki EP. Expression of HPV-11 L1 protein in transgenic *Arabidopsis thaliana* and *Nicotiana tabacum*. *BMC Biotechnol.* 7, 56 (2007).
- 25 Maclean J, Koekemoer M, Olivier AJ *et al.* Optimization of human papillomavirus type 16 (HPV-16) L1 expression in plants: comparison of the suitability of different HPV-16 L1 gene variants and different cell-compartment localization. *J. Gen. Virol.* 88, 1460–1469 (2007).
- 26 Regnard GL, Halley-Stott RP, Tanzer FL, Hitzeroth II, Rybicki EP. High level protein expression in plants through the use of a novel autonomously replicating geminivirus shuttle vector. *Plant Biotechnol. J.* 8, 38–46 (2010).
- 27 Fernandez-San Millan A, Ortigosa SM, Hervás-Stubbs S *et al.* Human papillomavirus L1 protein expressed in tobacco chloroplasts self-assembles into virus-like particles that are highly immunogenic. *Plant Biotechnol. J.* 6, 427–441 (2008).
- 28 Waheed MT, Thönes N, Müller M *et al.* Transplastomic expression of a modified human papillomavirus L1

- protein leading to the assembly of capsomeres in tobacco: a step towards cost-effective second-generation vaccines. *Transgenic Res.* 20, 271–282 (2011).
- 29 Matic S, Masenga V, Poli A *et al.* Comparative analysis of recombinant human papillomavirus 8 L1 production in plants by a variety of expression systems and purification methods. *Plant Biotechnol. J.* 10, 410–421 (2012).
- 30 Varsani A, Williamson AL, de Villiers D, Becker I, Christensen ND, Rybicki EP. Chimeric human papillomavirus type 16 (HPV-16) L1 particles presenting the common neutralizing epitope for the L2 minor capsid protein of HPV-6 and HPV-16. *J. Virol.* 77, 8386–8393 (2003).
- 31 Matic S, Rinaldi R, Masenga V, Noris E. Efficient production of chimeric human papillomavirus 16 L1 protein bearing the M2e influenza epitope in *Nicotiana benthamiana* plants. *BMC Biotechnol.* 11, 106 (2011).
- 32 Pineo C. Expression of chimaeric HPV-16 L1 protein in plants. [Master of Science thesis]. University of Cape Town, South Africa (2012).
- 33 Balcos LG, Borzacchiello G, Russo V, Popescu O, Roperto S, Roperto F. Association of bovine papillomavirus type-2 and urinary bladder tumours in cattle from Romania. *Res. Vet. Sci.* 85, 145–148 (2008).
- 34 Borzacchiello G, Roperto F. Bovine papillomaviruses, papillomas and cancer in cattle. *Vet. Res.* 39, 45 (2008).
- 35 Love AJ, Chapman SN, Matic S, Noris E, Lomonosoff GP, Taliantsky M. *In planta* production of a candidate vaccine against bovine papillomavirus type 1. *Planta* 236, 1305–1313 (2012).
- 36 Mason HS, Ball JM, Shi JJ, Jiang X, Estes MK, Arntzen CJ. Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. *Proc. Natl Acad. Sci. USA* 93, 5335–5340 (1996).
- 37 Huang Z, Chen Q, Hjelm B, Arntzen C, Mason H. A DNA replicon system for rapid high-level production of virus-like particles in plants. *Biotechnol. Bioeng.* 103, 706–714 (2009).
- 38 Lai H, He J, Engle M, Diamond MS, Chen Q. Robust production of virus-like particles and monoclonal antibodies with geminiviral replicon vectors in lettuce. *Plant Biotechnol. J.* 10, 95–104 (2012).
- 39 Santi L, Batchelor L, Huang Z *et al.* An efficient plant viral expression system generating orally immunogenic Norwalk virus-like particles. *Vaccine* 26, 1846–1854 (2008).
- 40 Lai H, Chen Q. Bioprocessing of plant-derived virus-like particles of Norwalk virus capsid protein under current good manufacture practice regulations. *Plant Cell Rep.* 31, 573–584 (2012).
- 41 Johnston MI, Fauci AS. An HIV vaccine – evolving concepts. *N. Engl. J. Med.* 356, 2073–2081 (2007).
- 42 Ross AL, Brave A, Scarlatti G, Manrique A, Buonaguro L. Progress towards development of an HIV vaccine: report of the AIDS Vaccine 2009 Conference. *Lancet Infect. Dis.* 10, 305–316 (2010).
- 43 Kim JH, Rerks-Ngarm S, Excler JL, Michael NL. HIV vaccines: lessons learned and the way forward. *Curr. Opin. HIV AIDS* 5, 428–434 (2010).
- 44 Marusic C, Vitale A, Pedrazzini E *et al.* Plant-based strategies aimed at expressing HIV antigens and neutralizing antibodies at high levels. Nef as a case study. *Transgenic Res.* 18, 499–512 (2009).
- 45 Scotti N, Buonaguro L, Tornesello ML, Cardi T, Buonaguro FM. Plant-based anti-HIV-1 strategies: vaccine molecules and antiviral approaches. *Expert Rev. Vaccines* 9, 925–936 (2010).
- 46 Kessans SA, Linhart MD, Matoba N, Mor T. Biological and biochemical characterization of HIV-1 Gag/dgp41 virus-like particles expressed in *Nicotiana benthamiana*. *Plant Biotechnol. J.* 11(6), 681–690 (2013).
- 47 Meyers A, Chakauya E, Shephard E *et al.* Expression of HIV-1 antigens in plants as potential subunit vaccines. *BMC Biotechnol.* 8, 53 (2008).
- 48 Scotti N, Alagna F, Ferraiolo E *et al.* High-level expression of the HIV-1 Pr55^{gag} polyprotein in transgenic tobacco chloroplasts. *Planta* 229, 1109–1122 (2009).
- 49 Yu CI, Chiang BL. A new insight into hepatitis C vaccine development. *J. Biomed. Biotechnol.* 2010, 548280 (2010).
- 50 Natilla A, Piazzolla G, Nuzzaci M *et al.* Cucumber

- mosaic virus as carrier of a hepatitis C virus-derived epitope. *Arch. Virol.* 149, 137–154 (2004).
- 51 Nemchinov LG, Liang TJ, Rifaat MM, Mazyad HM, Hadidi A, Keith JM. Development of a plant-derived subunit vaccine candidate against hepatitis C virus. *Arch. Virol.* 145, 2557–2573 (2000).
- 52 Nuzzaci M, Piazzolla G, Vitti A *et al.* Cucumber mosaic virus as a presentation system for a double hepatitis C virus-derived epitope. *Arch. Virol.* 152, 915–928 (2007).
- 53 Piazzolla G, Nuzzaci M, Tortorella C *et al.* Immunogenic properties of a chimeric plant virus expressing a hepatitis C virus (HCV)-derived epitope: new prospects for an HCV vaccine. *J. Clin. Immunol.* 25, 142–152 (2005).
- 54 Uhde-Holzem K, Fischer R, Commandeur U. Characterization and diagnostic potential of foreign epitope-presenting Ty1 virus-like particles expressed in *Escherichia coli* and *Pichia pastoris*. *J. Mol. Microbiol. Biotechnol.* 18, 52–62 (2010).
- 55 Nianiou I, Kalantidis K, Madesis P, Georgopoulou U, Mavromara P, Tsafaris A. Expression of an HCV core antigen coding gene in tobacco (*N. tabacum* L.). *Prep. Biochem. Biotechnol.* 38, 411–421 (2008).
- 56 Mahy BW. Introduction and history of foot-and-mouth disease virus. *Curr. Top. Microbiol. Immunol.* 288, 1–8 (2005).
- 57 Animal and Plant Health Inspection Service. *Foot-and-*
- Mouth Disease Vaccine.* United States Department of Agriculture, Washington DC, USA (2007).
- 58 Doel TR. Natural and vaccine-induced immunity to foot and mouth disease: the prospects for improved vaccines. *Rev. Sci. Tech.* 15, 883–911 (1996).
- 59 Porta C, Kotecha A, Burman A *et al.* Rational engineering of recombinant picornavirus capsids to produce safe, protective vaccine antigen. *PLoS Pathog.* 9, e1003255 (2013).
- 60 Usha R, Rohll JB, Spall VE *et al.* Expression of an animal virus antigenic site on the surface of a plant virus particle. *Virology* 197, 366–374 (1993).
- 61 Carrillo C, Wigdorovitz A, Oliveros JC *et al.* Protective immune response to foot-and-mouth disease virus with VP1 expressed in transgenic plants. *J. Virol.* 72, 1688–1690 (1998).
- 62 Wigdorovitz A, Carrillo C, Dus Santos MJ *et al.* Induction of a protective antibody response to foot and mouth disease virus in mice following oral or parenteral immunization with alfalfa transgenic plants expressing the viral structural protein VP1. *Virology* 255, 347–353 (1999).
- 63 Wigdorovitz A, Pérez Filgueira DM, Robertson N *et al.* Protection of mice against challenge with foot and mouth disease virus (FMDV) by immunization with foliar extracts from plants infected with recombinant tobacco mosaic virus expressing the FMDV structural protein VP1. *Virology* 264, 85–91 (1999).
- 64 Carrillo C, Wigdorovitz A, Trono K *et al.* Induction of a virus-specific antibody response to foot and mouth disease virus using the structural protein VP1 expressed in transgenic potato plants. *Viral Immunol.* 14, 49–57 (2001).
- 65 Dus Santos MJ, Carrillo C, Ardila F *et al.* Development of transgenic alfalfa plants containing the foot and mouth disease virus structural polyprotein gene P1 and its utilization as an experimental immunogen. *Vaccine* 23, 1838–1843 (2005).
- 66 Pan L, Zhang Y, Wang Y *et al.* Foliar extracts from transgenic tomato plants expressing the structural polyprotein, P1-2A, and protease, 3C, from foot-and-mouth disease virus elicit a protective response in guinea pigs. *Vet. Immunol. Immunopathol.* 121, 83–90 (2008).
- 67 Wu L, Jiang L, Zhou Z *et al.* Expression of foot-and-mouth disease virus epitopes in tobacco by a tobacco mosaic virus-based vector. *Vaccine* 21, 4390–4398 (2003).
- 68 Yang CD, Liao JT, Lai CY *et al.* Induction of protective immunity in swine by recombinant bamboo mosaic virus expressing foot-and-mouth disease virus epitopes. *BMC Biotechnol.* 7, 62 (2007).
- 69 Zhang Y, Li J, Pu H *et al.* Development of tobacco necrosis virus A as a vector for efficient and stable expression of FMDV VP1 peptides. *Plant Biotechnol. J.* 8, 506–523 (2010).
- 70 Parashar UD, Hummelman EG, Bresee JS, Miller MA, Glass RI. Global illness and deaths

- caused by rotavirus disease in children. *Emerg. Infect. Dis.* 9, 565–572 (2003).
- 71 Martella V, Banyai K, Matthijnssens J, Buonavoglia C, Ciarlet M. Zoonotic aspects of rotaviruses. *Vet. Microbiol.* 140, 246–255 (2010).
- 72 Todd S, Page NA, Duncan Steele A, Peenye I, Cunliffe NA. Rotavirus strain types circulating in Africa: review of studies published during 1997–2006. *J. Infect. Dis.* 202(Suppl.), S34–S42 (2010).
- 73 Li Z, Baker ML, Jiang W, Estes MK, Prasad BV. Rotavirus architecture at subnanometer resolution. *J. Virol.* 83, 1754–1766 (2009).
- 74 Matsui SM, Offit PA, Vo PT *et al.* Passive protection against rotavirus-induced diarrhea by monoclonal antibodies to the heterotypic neutralization domain of VP7 and the VP8 fragment of VP4. *J. Clin. Microbiol.* 27, 780–782 (1989).
- 75 Gilbert JM, Greenberg HB. Virus-like particle-induced fusion from without in tissue culture cells: role of outer-layer proteins VP4 and VP7. *J. Virol.* 71, 4555–4563 (1997).
- 76 Peixoto C, Sousa MF, Silva AC, Carrondo MJ, Alves PM. Downstream processing of triple layered rotavirus like particles. *J. Biotechnol.* 127, 452–461 (2007).
- 77 Rodriguez-Limas WA, Tyo KE, Nielsen J, Ramirez OT, Palomares LA. Molecular and process design for rotavirus-like particle production in *Saccharomyces cerevisiae*. *Microb. Cell. Fact.* 10, 33 (2011).
- 78 Shoja Z, Tagliamonte M, Jalilvand S *et al.* Development of a stable insect cell line constitutively expressing rotavirus VP2. *Virus Res.* 172, 66–74 (2013).
- 79 Saldana S, Esquivel Guadarrama F, Olivera Flores Tde J *et al.* Production of rotavirus-like particles in tomato (*Lycopersicon esculentum* L.) fruit by expression of capsid proteins VP2 and VP6 and immunological studies. *Viral Immunol.* 19, 42–53 (2006).
- 80 Yang Y, Li X, Yang H *et al.* Immunogenicity and virus-like particle formation of rotavirus capsid proteins produced in transgenic plants. *Sci. China Life Sci.* 54, 82–89 (2011).
- 81 Mutepfa DL. Expression of rotavirus capsid proteins in *N. benthamiana* leaves using an agrobacterium-mediated transient expression system. [Master of Science thesis]. University of Cape Town, South Africa (2011).
- 82 Rybicki EP, Hitzeroth II, Meyers A, das Santos M, Wigdorovitz A. Developing country applications of molecular farming: case studies in South Africa and Argentina. *Curr. Pharm. Design* 19(31), 5612–5621 (2013).
- 83 Maan NS, Maan S, Belaganahalli MN *et al.* Identification and differentiation of the twenty six bluetongue virus serotypes by RT-PCR amplification of the serotype-specific genome segment 2. *PLoS ONE* 7, e32601 (2012).
- 84 Perez de Diego AC, Athmaram TN, Stewart M *et al.* Characterization of protection afforded by a bivalent virus-like particle vaccine against bluetongue virus serotypes 1 and 4 in sheep. *PLoS ONE* 6, e26666 (2011).
- 85 Purse BV, Brown HE, Harrup L, Mertens PP, Rogers DJ. Invasion of bluetongue and other orbivirus infections into Europe: the role of biological and climatic processes. *Rev. Sci. Tech.* 27, 427–442 (2008).
- 86 Savini G, MacLachlan NJ, Sanchez-Vizcaino JM, Zientara S. Vaccines against bluetongue in Europe. *Comp. Immunol. Microbiol. Infect. Dis.* 31, 101–120 (2008).
- 87 Roy P, Noad R. Bluetongue virus assembly and morphogenesis. *Curr. Top. Microbiol. Immunol.* 309, 87–116 (2006).
- 88 Roy P, Bishop DH, LeBlois H, Erasmus BJ. Long-lasting protection of sheep against bluetongue challenge after vaccination with virus-like particles: evidence for homologous and partial heterologous protection. *Vaccine* 12, 805–811 (1994).
- 89 Thuenemann EC, Lenzi P, Love AJ *et al.* The use of transient expression systems for the rapid production of virus-like particles in plants. *Curr. Pharm. Des.* 19(31), 5564–5573 (2013).
- 90 Thuenemann EC, Meyers AE, Verweij J, Rybicki EP, Lomonosoff GP. A method for rapid production of heteromultimeric protein complexes in plants: assembly of protective bluetongue virus-like particles. *Plant Biotechnol. J.* 11(7), 839–846 (2013).

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Chapter

8

Production of complex virus-like particles in insect cells

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Fabiana Fernandes, Ana P Teixeira, Nuno Carinhas, Manuel JT Carrondo & Paula M Alves

Virus-like particles (VLPs) are multiprotein structures that resemble the conformation of native viruses but lack a viral genome, potentiating their application as safer and cheaper vaccines. The production of VLPs has been strongly linked with the use of insect cells and the baculovirus expression vector system, especially those particles composed of two or more structural viral proteins. In fact, this expression platform has been extensively improved over the years to address the challenges of coexpression of multiple proteins and their proper assembly into complexes in the same cell. Herein, the role of insect cell technology in the development and production of complex VLPs will be overviewed. Recent achievements, current bottlenecks and future trends are also highlighted.

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Virus-like particles (VLPs) are viral proteins that self-assemble into structures resembling the conformation of the native virus but are devoid of viral genetic material [48]. Such structures have the ability to enhance immunogenicity and are widely used for the development of subunit vaccines to be administered against many diseases.

The insect cell/baculovirus expression vector system (BEVS) has been one of the leading platforms for the production of multiprotein virus-like particles (VLPs). Advantages include the high protein-expression levels attained with eukaryotic post-translational processing and the

straightforward construction of recombinant baculoviruses (rBV), which have a large packaging capacity allowing expression of multiple proteins simultaneously in a rapid and efficient manner [1]. The most utilized insect cell lines are Sf9 (derived from *Spodoptera frugiperda*) and BTI-Tn5B1-4 (or High Five™, Invitrogen, Paisley, UK; derived from *Trichoplusia ni*). Sf9 cells are preferentially used for the generation and propagation of rBV, while High Five cells have been reported to provide higher recombinant protein yields [2].

Despite the advances in this insect cell platform during the past 10 years, in some cases, it still fails in the delivery of baculovirus-free vaccines, requiring further improvements in both upstream and downstream processing towards meeting the quality of the final product. In this work, we will focus on the emergence and progress of complex VLP vaccine candidates that use insect cell technology as the manufacturing platform, further exploring its inherent flexibility, current bottlenecks and future trends.

VLPs produced in insect cells

Different types of viruses with single or multiple capsid proteins, with or without envelope, have been simulated by VLPs using insect cells as the production platform [3]. This structural diversity implies different production challenges where a correct assembly of the different proteins is required. **Table 8.1** lists key VLPs targeting both human and veterinary vaccine candidates, composed of two or more viral structural proteins (involved in capsid and/or envelope formation), which have been expressed in insect cells.

Nonenveloped VLPs composed by multiple structural proteins

Nonenveloped VLPs composed by multiple structural proteins have been proposed for both structurally simple viruses with a single capsid layer and those composed by multiple concentric shells. Parvovirus B19 has a single layer composed of two proteins (5% VP1 and 95% VP2); VLPs that mimic the native ratio of VP1 and VP2 are less effective in inducing an immune response than VLPs where the percentage of VP1 is increased (from 25 up to 40%) [4]. This is an example of a VLP where the use of

Production of complex virus-like particles in insect cells

Table 8.1. Complex virus-like particles produced in insect cells using baculovirus expression vector systems.

Virus/family (nonenveloped/ enveloped VLP)	VLP proteins	Promoter(s)	Production strategy	Ref.
Parvovirus/ <i>Parvoviridae</i> (nonenveloped VLP)	■ VP1 and VP2	■ p10 (VP1) and polh (VP2) ■ polh (VP1) and polh (VP2)	■ Coexpression using a bicistronic rBV ■ Coinfection using two monocistronic rBVs	[5–7]
Poliovirus/ <i>Picornaviridae</i> (nonenveloped VLP)	■ VP0, VP1 and VP3	■ All polh ■ polh (VP0), p10 (VP1) and polh (VP3) ■ polh (the entire poliovirus coding region)	■ Coinfection using three monocistronic rBVs ■ Coinfection using mono- and bicistronic rBVs ■ Coexpression from a single rBV	[8,9]
Enterovirus/ <i>Picornaviridae</i> (nonenveloped VLP)	■ P1 (VP0, VP1 and VP3) and CD3	■ polh (P1) and p10 (CD3)	■ Coinfection using two monocistronic rBVs ■ Coexpression using a bicistronic rBV	[10,11]
Infectious bursal disease virus/ <i>Birnaviridae</i> (nonenveloped VLP)	■ VP2, VP3 and VP4	■ All polh	■ Coinfection using a monocistronic and a bicistronic rBVs	[12]
Bluetongue virus/ <i>Reoviridae</i> (nonenveloped VLP)	■ VP2, VP3, VP5 and VP7	■ All polh ■ polh (VP2), p10 (VP3), polh (VP5) and p10 (VP7)	■ Coinfection and coexpression using two rBVs ■ Coexpression using quadruple-cistronic rBV	[13,14]
Rotavirus/ <i>Reoviridae</i> (nonenveloped VLP)	■ VP2, VP4, VP6 and VP7	■ polh (VP2, VP4 and VP6) or polh (VP2, VP6 and VP7) ■ All polh ■ polh (VP2), polh (VP6) and p10 (VP7)	■ Coinfection using three monocistronic rBVs ■ Coinfection using four monocistronic rBVs ■ Coexpression using a tricistronic rBV	[42–45]

GP: Glycoprotein; E: Envelope; HA: Hemagglutinin; M: Membrane; M1: Matrix protein from influenza virus; M2: Envelope protein of influenza virus; NA: Neuraminidase; rBV: Recombinant baculovirus; S: Spike; VLP: Virus-like particle.

Table 8.1. Complex virus-like particles produced in insect cells using baculovirus expression vector systems.

Virus/family (nonenveloped/ enveloped VLP)	VLP proteins	Promoter(s)	Production strategy	Ref.
Hepatitis C/ <i>Flaviridae</i> (enveloped VLP)	▪ Core, E1 and E2	▪ All polh	▪ Coexpression using a tricistronic rBV	[16]
SARS/ <i>Coronaviridae</i> (enveloped VLP)	▪ S, E and M proteins of the SARS virus	▪ All polh	▪ Coinfection using three monocistronic rBVs ▪ Coexpression using a tricistronic rBV	[18,19]
Ebola/ <i>Filoviridae</i> (enveloped VLP)	▪ VP40 and GP	▪ polh/pcap (VP40 and GP)	▪ Coinfection using two monocistronic rBVs	[22]
Influenza A/ <i>Orthomyxoviridae</i> (enveloped VLP)	<ul style="list-style-type: none"> ▪ HA, NA, M1 and M2 ▪ HA and M1 ▪ HA, NA and M1 ▪ HA, NA and M1 ▪ HA, NA and M1 	<ul style="list-style-type: none"> ▪ polh (HA), p10 (NA and M2), polh (M1) ▪ pcap/polh (HA) and pcap/polh (M1) ▪ All polh ▪ All polh ▪ polh (HA), p10 (NA) and polh (M1) 	<ul style="list-style-type: none"> ▪ Coexpression using a quadruple rBV ▪ Coinfection using two monocistronic rBVs ▪ Coexpression using a tricistronic rBV ▪ Coinfection using three monocistronic rBVs ▪ Coinfection with monocistronic and bicistronic rBVs 	[17,24, 25,46, 47]

GP: Glycoprotein; E: Envelope; HA: Hemagglutinin; M: Membrane; M1: Matrix protein from influenza virus; M2: Envelope protein of influenza virus; NA: Neuraminidase; rBV: Recombinant baculovirus; S: Spike; VLP: Virus-like particle.

multiple baculoviruses has allowed flexibility in tuning particle composition [5–7].

VLPs composed of multiple interacting capsid proteins are more challenging to produce. Each protein must be expressed in stoichiometric amounts, otherwise cellular resources will be wasted to synthesize monomers that do not contribute to the complete particle yield. Examples of viruses with multiple concentric shells whose VLPs have been produced in insect cells using the BEVS are: poliovirus [8,9] and enterovirus 71 [10,11] from *Picornaviridae*, infectious bursal disease virus from *Birnaviridae* [12], and bluetongue virus and rotavirus from *Reoviridae*. Bluetongue virus VLPs have been produced by coinfection with two bicistronic rBVs [13] or by using a strategy of single infection with a multicistronic rBV encoding four structural proteins (VP2, VP3, VP5 and

VP7) [14]; the latter methodology providing better results. The suitability of rRBVs for production of rotavirus-like particles (RLPs) has also been demonstrated in several works. RLPs are formed by the simultaneous expression of up to four viral proteins: VP2 protein in the core, a middle layer of the polymorphic VP6 protein, and an external layer formed by the VP7 glycoprotein where spikes of VP4 can be found. Different double- and triple-layered configurations of RLPs have been produced using BEVS [14,15] and data from immunogenicity studies have encouraged their development as vaccine candidates against rotavirus infection [15].



The production of multiprotein VLPs using baculovirus expression vector systems can be accomplished using either recombinant baculoviruses (rRBVs) that carry a single recombinant gene (monocistronic rRBVs) or rRBVs encoding more than one recombinant gene (polycistronic rRBVs). Therefore, two infection schemes can be employed: coinfection with multiple monocistronic/polycistronic rRBVs (depending on the number of proteins); or infection with a single polycistronic rRBV encoding all proteins. The use of polycistronic vectors has become popular for expressing different protein subunits in the same infected cell as the use of multiple vectors can result in an uneven distribution of viruses entering each cell.

VLPs from enveloped viruses

A vast number of pathogenic viruses (e.g., hepatitis C virus [HCV], HIV and influenza) possess a lipidic envelope composed of viral proteins and residual proteins derived from the host cell. The envelope proteins constitute the main target of neutralizing antibodies of a vaccine. Successful cases of enveloped VLP production leveraging the BEVS technology are those from HCV, SARS coronavirus and influenza virus [16–18]. Enveloped VLPs have irregular size and structure in contrast to the defined shape of nonenveloped VLPs.

In 1998, Baumert *et al.* demonstrated for the first time the production of a HCV VLP in insect cells where the core protein and both envelope proteins (E1 and E2) were properly assembled [16]. These HCV VLPs were tested in animal studies stimulating strong cellular and humoral immune responses. Despite the BEVS's success in producing active HCV VLPs, particularly considering the demanding glycosylated E1 protein, there will be further advances in this area aiming at a consistent product that could be considered an effective vaccine candidate.

Similar efforts were directed towards the construction of a SARS coronavirus VLP vaccine, mainly due to the global outbreak of the disease [19]. The production strategy of a trivalent particle involved the coinfection with a bicistronic baculovirus coding for two structural proteins (membrane [M] and envelope [E] proteins), and a monocistronic baculovirus coding for a third structural protein (Spike [S] protein), which increases the immunogenicity of the particle and is specific to each coronavirus strain [20]. The production of HIV VLPs, described in **Chapter 1** of this book [21] and

Ebola [22] and Marburg VLPs are also among the successful applications of the BEVS for the production of VLPs, resulting in yields significantly higher (five- to 20-fold increase) than those obtained with mammalian cell expression systems [23].

The potential advantages of the BEVS over other production systems are even more evident in the case of influenza vaccines where annual replacement of the viral proteins is required to match emerging strains, as well as assuring the capacity to respond to a pandemic scenario. Vector construction, virus amplification and large-scale production of recombinant influenza vaccines can be accomplished much faster (~3 months) than using the traditional egg-based platform. In fact, the US FDA has just approved the first BEVS-derived influenza vaccine, FluBlok®, a trivalent hemagglutinin (HA) vaccine manufactured by Protein Sciences Co. (CT, USA). Concerning influenza VLPs, several particle configurations composed of two (HA and NA), three (HA, neuraminidase [NA] and M1) or four (HA, NA, M1 and M2) structural proteins have been attempted: the transmembrane glycoproteins HA and NA, the capsid protein M1 and the transmembrane ion channel protein M2. Although the simpler combination of HA and M1 is sufficient for particle assembly and results in efficient immunological activity [24], trivalent VLPs composed of HA, NA and M1 have also been studied [25]. The production of these VLPs in insect cells has been accomplished either by coinfection with different rBVs [24,25], each expressing one or more structural influenza proteins or infecting with a single rBV encoding all proteins [17,25]. For instance, Novavax (MD, USA) produces influenza VLPs by infecting Sf9 cells with a tricistronic baculovirus encoding HA, NA and M1, and one of its VLP vaccines (targeting H1N1) has recently shown encouraging results in a Phase II trial [26]. Finally, several enveloped chimeric VLPs have been proposed in the last few years as part of the continuous effort to improve the safety, efficacy and cost-effectiveness of immunogens [27].

BEVS & insect cell technology: advances & bottlenecks

The insect cell/baculovirus system has been continuously improved over the years to cope with upstream and downstream challenges, both in terms of expression vector design as well as rational approaches to understand the infection process, protein expression and assembly.

Baculovirus vector design

The stability of polycistronic vectors is often affected by promoter sequence repetitions; to avoid juxtaposition of the same promoters, they have been segregated into different transcriptional directions [14].

Improved stability has also been achieved by coupling the expression of the recombinant gene to baculovirus essential genes (such as *gp64*) using an internal ribosome entry site element to achieve a single bicistronic transcript [28]. Targeting improved product quality and quantity, deletions to the baculovirus genome have been performed, namely of DNA sequences coding for proteases (e.g., v-cathepsin) or baculovirus proteins, which are thought to obstruct the secretory pathway (e.g., *chiA* chitinase), as well as other nonessential genes such as *p10*, *p26* and *p74* [29]. Furthermore, to improve the fraction of correctly assembled proteins, coexpression of chaperones or foldases have been pursued. In some cases, the use of baculovirus early promoters (e.g., *ie-1* and *ie-2*) have been shown to produce higher quantities of biologically active proteins, as they are expressed at earlier stages in infection when the cellular protein processing machinery is fully operational. Although these promoters are intrinsically weaker, the addition of baculovirus regulatory elements can be a solution to enhance the expression rate. An additional alternative is activation of transcription at earlier times postinfection with the use of synthetic versions of very late promoters; this strategy allowed the production of enveloped VLPs with yields superior to those obtained using very late promoters [22].

In order to address the presence of rRBVs within the VLP bulk, which still represents a safety concern adding costs to the downstream processing, a novel nonreplicative baculovirus vector was engineered by deleting a gene (*vp80*) that is essential for viral protein cleavage, maturation and assembly as well as release from infected cells [30]. Hence, this strategy requires a *vp80* trans-complementing cell line for the efficient propagation of the deficient virus.

Insect cell culture engineering & infection parameters

The multiplicity of infection (MOI), cell concentration at infection (CCI) and time of harvest are key bioprocess parameters in baculovirus infection with influence on productivity and quality of protein targets. Often, the infection strategy at low CCIs with low MOIs (≤ 1) is employed to produce recombinant proteins, allowing multiple infection cycles to occur and being characterized by longer bioreaction times [31]. However, long bioreaction times can compromise the integrity of the final protein products due to increased exposure to proteases. By contrast, high MOIs



The *Autographa californica* multiple nucleopolyhedrovirus is the prototype baculovirus generally used as a vector to produce recombinant proteins. The strong, very late promoters *polh* and *p10* are the first choices in assisting the expression in the majority of baculovirus vectors, and are normally repeated to express multiple genes in the same vector. Several commercial baculovirus vectors are readily available to handle simultaneous expression of multiple recombinant proteins.

demand large viral stocks and are associated with shorter productive periods and lower volumetric yields [32].

In order to improve the cellular environment, fed-batch cultures have allowed more productive infections by supplementing nutrients such as glucose, amino acid sources, lipids and vitamins that could otherwise become limiting. Perfusion allows pushing even further the CCI and the total product yield given the continued renewal of 'exhausted medium' [33]. On a different ground, models describing the elaborate process of baculovirus infection and protein expression have been reported for over 20 years and represent a useful tool to systematically handle the complexity of the system and predict experimental outcomes. However, future efforts should be directed at explicitly accounting for coinfection strategies, integrating the expression rates from different promoters and further detailing the mechanistic processes of intracellular assembly of individual units into multiprotein complexes [34].

Downstream processing of VLPs

One of the critical aspects of using the BEVS for VLP manufacture is the coproduction of high titers of rBVs. Conventional ultracentrifugation techniques based on density gradients (e.g., sucrose) are unable to provide VLPs with acceptable rBV removal as their densities are very similar. Moreover, these techniques are time- and labor-intensive, which hamper straightforward scale-up. An example of a scalable purification process was developed by Novavax for BEVS-derived influenza VLPs composed of HA, NA and M1. It includes tangential flow filtration for cell removal followed by concentration/diafiltration to remove media components and cell debris. Based on charge differences between VLPs, rBVs and cell host contaminant DNA, ion exchange chromatography was used for their separation. Residual rBVs are inactivated by treatment with β -propiolactone and the final polishing is performed with size-exclusion chromatography to remove residual host contaminants [35].

To achieve uniform particle morphology and maximum stability of VLP vaccines, the reprocessing of these particles after purification is common practice; both insect cell- and yeast-derived human papillomavirus VLPs approved for human use include *in vitro* disassembly and reassembly as postpurification steps [36]. It also allows the elimination of eventual contaminating baculovirus and/or cellular DNA fragments that are incorporated during intracellular assembly of VLPs.

Insect cell platforms bypassing baculovirus infection

Despite the popularity of BEVS for the production of VLPs and the efforts/advances described above, other insect cell-based alternatives have been

proposed to bypass drawbacks of baculovirus infection, such as virus stock maintenance and rBV removal. Stable expression in insect cells is being increasingly explored for continuous expression of the desired proteins in a virus-free process. Two different stable cell

lines were recently established that allowed similar amounts of VLP production to those using the BEVS [37,38]. In another application, a bicistronic expression system using an internal ribosome entry site element was stably integrated in the *Drosophila S2* cell line, resulting in efficient production of VP2-VP6 double-layered RLPs [39]. More recently, Japanese encephalitis complex VLPs were expressed with relevant yields (~30 mg/l) in stably transformed lepidopteran cells [40].

Despite these efforts, the establishment of stable insect cell lines does not go along with industry preference, since it normally has longer development times and final costs compared with BEVS. Taking that into consideration, the company Aldevron (ND, USA) and partner Altravax (CA, USA) have adopted a transient transfection method for rapid expression of influenza VLPs in Sf9 cells. This methodology has been proven to possess some benefits in comparison to the BEVS in terms of time and effort without compromising the quality of the final product [101]. In a step further, we recently established a recombinase-mediated cassette exchange system in Sf9 cells [41] as a reusable platform for stable protein expression that significantly obviates the conventional laborious and time-consuming cell line development process (**Figure 8.1**). The feasibility of flipase-FRT recombination in Sf9 cells was demonstrated and production titers in small-scale batch cultures were similar to those achieved using the BEVS.

Conclusion

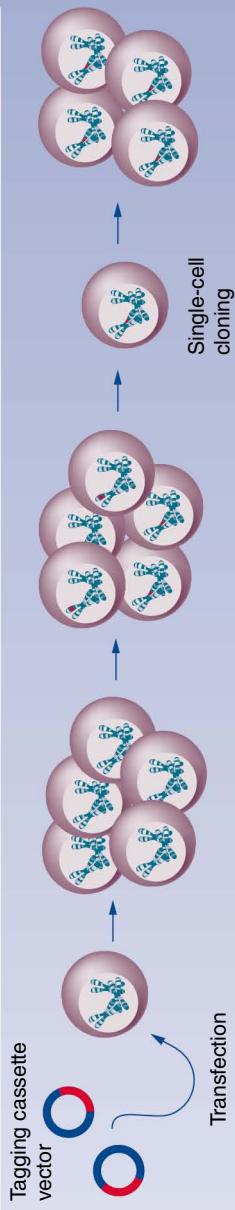
A cost-effective manufacturing process yielding sufficient quantity and quality of a multigenic product is generally not an easy task to accomplish due to product complexity. Despite the success of the BEVS in producing VLP-based vaccines for both veterinary and human use, its performance regarding the expression of complex VLPs has room for improvement. The yields of correctly assembled VLPs and associated levels of baculovirus contamination are still not satisfactory according to commercial requirements. Significant efforts to address these issues continue to be directed towards ameliorating molecular design for the construction of more efficient and reliable expression systems. Nonreplicative rBVs



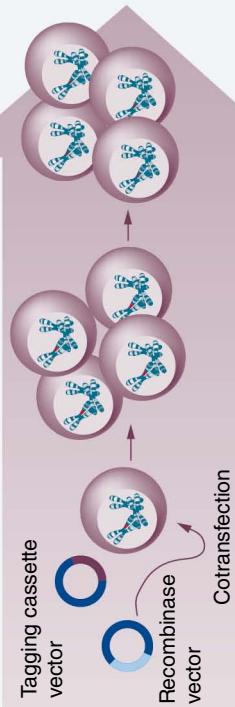
The recombinase-mediated cassette exchange technology makes use of a recombinase to mediate the integration of a gene-of-interest in a pre-characterized chromosome locus flanked by recombinase recognition target sites, allowing re-using the same locus for expression of different proteins.

Figure 8.1. Development of a master cell line through recombinase-mediated cassette exchange.

Step 1: tagging the insect cell's genome



Step 2: site-specific integration of target recombinant gene



RMCE

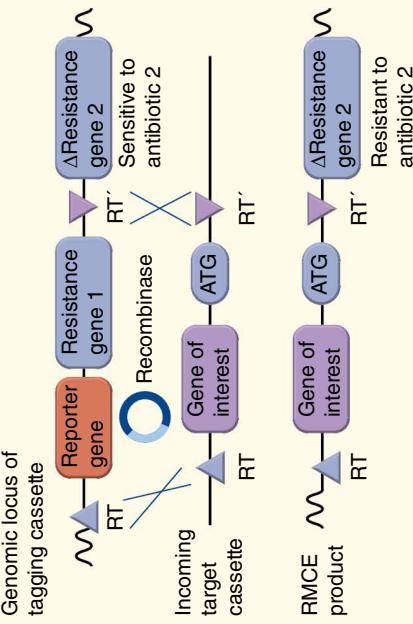


Figure 8.1 (cont.); see previous page. Step 1 is similar to the conventional cell line development process (time span of 3–6 months). A tagging cassette vector containing recombinase RT sites is stably integrated into the cell genome by means of selective pressure of marker 1. Single-cell cloning is performed to screen for high protein-producing clones. Selected clones are expanded and stored. To express a desired target protein (step 2), the initial tagging cassette is replaced by the target cassette containing the gene of interest (flanked by the same pair of RT sites), through a recombinase-mediated process. A defective selection marker within the integrated tagging cassette is activated by an ATG complementary sequence present in the target vector. Upon 3 weeks in antibiotic selection (marker 2), a resulting stable cell line producing the target protein is established with similar expression properties to the tagged cell line, thus avoiding repetition of the laborious and time-consuming Step 1.

RMCE: Recombinase-mediated cassette exchange; RT: Recognition target.

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represent one advance that aims at debottlenecking the problem of contamination of the VLP bulk with viral material. Stable protein expression through engineered insect cell lines constitutes another alternative since it does not rely on viral infection for protein production, thus minimizing downstream challenges. Still, VLP production will benefit from improvements in downstream processes that could improve recovery yields without compromising quality. Further work should also be conducted towards tuning the expression levels of each protein subunit to improve the assembly efficiency of complex VLPs and also the consistency of the final product composition. Ultimately, advances in the fields of systems biology and genetic engineering will complement each other towards the development of faster, robust and simpler production processes based on effective biological hosts and vectors. In the end, all will contribute to the sustainability of the insect cell technology and reinforce its credibility as a platform for the production of complex VLP vaccines.

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Summary

- During the past years, virus-like particles (VLPs) have been exploited towards the development of safer and cheaper vaccines due to their immunological potency. Complex VLPs composed of two or more structural proteins, including envelope proteins, are emerging as vaccine candidates against major human health threats.
- Insect cells, together with the baculovirus expression vector system, are a well-known platform for the production of recombinant proteins. Cervarix™ (GlaxoSmithKline), a human papillomavirus VLP vaccine, has landmarked the use of this production technology in the clinical setting, further potentiating its application for the manufacture of complex VLPs for other diseases.
- Several improvements at the level of vector design, optimized culture conditions, infection strategies and purification schemes have culminated in a versatile technology suited to produce distinct VLPs with acceptable yields and quality. Nevertheless, the productive limitation associated with the lytic baculovirus infection cycle as well as additional downstream processing necessary to remove viral contaminants from final purified VLPs need yet to be de-bottlenecked.
- As an alternative to the baculovirus expression vector system, the development of stably-transformed insect cells allows bypassing the infection step while taking advantage of the *in vitro* growth properties of insect cells, and has the potential to stand out in the challenging production of complex VLPs.

References

- 1 Kost TA, Condreay JP, Jarvis DL. Baculovirus as versatile vectors for protein expression in insect and mammalian cells. *Nat. Biotechnol.* 23(5), 567–575 (2005).
- 2 Rhiel M, Mitchell-Logean CM, Murhammer DW. Comparison of *Trichoplusia ni* BTI-Tn-5B1-4 (High Five) and *Spodoptera frugiperda* Sf-9 insect cell line metabolism in suspension cultures. *Biotechnol. Bioeng.* 55(6), 909–920 (1997).
- 3 Roldao A, Mellado MC, Castilho LR, Carrondo MJ, Alves PM. Virus-like particles in vaccine development. *Expert Rev. Vaccines* 9(10), 1149–1176 (2010).
- 4 Bansal GP, Hatfield JA, Dunn FE *et al.* Candidate recombinant vaccine for human B19 parvovirus. *J. Infect. Dis.* 167(5), 1034–1044 (1993).
- 5 Brown CS, Van Lent JW, Vlak JM, Spaan WJ. Assembly of empty capsids by using baculovirus recombinants expressing human parvovirus B19 structural proteins. *J. Virol.* 65(5), 2702–2706 (1991).
- 6 Kajigaya S, Fujii H, Field A *et al.* Self-assembled B19 parvovirus capsids, produced in a baculovirus system, are antigenically and immunogenically similar to native virions. *Proc. Natl Acad. Sci. USA* 88(11), 4646–4650 (1991).
- 7 Tsao EI, Mason MR, Caciuttolo MA, Bowen SH, Folena-Wasserman G. Production of parvovirus B19 vaccine in insect cells co-infected with double baculoviruses. *Biotechnol. Bioeng.* 49(2), 130–138 (1996).
- 8 Brautigam S, Snezhkov E, Bishop DH. Formation of poliovirus-like particles by recombinant baculoviruses expressing the individual VP0, VP3, and VP1 proteins by comparison to particles derived from the expressed poliovirus polyprotein. *Virology* 192(2), 512–524 (1993).
- 9 Urakawa T, Ferguson M, Minor PD *et al.* Synthesis of immunogenic, but non-infectious, poliovirus particles in insect cells by a baculovirus expression vector. *J. Gen. Virol.* 70(Pt 6), 1453–1463 (1989).
- 10 Chung YC, Huang JH, Lai CW *et al.* Expression, purification and characterization of enterovirus-71 virus-like particles. *World J. Gastroenterol.* 12(6), 921–927 (2006).

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- 11 Hu YC, Hsu JT, Huang JH, Ho MS, Ho YC. Formation of enterovirus-like particle aggregates by recombinant baculoviruses co-expressing P1 and 3CD in insect cells. *Biotechnol. Lett.* 25(12), 919–925 (2003).
- 12 Hu YC, Bentley WE. Effect of MOI ratio on the composition and yield of chimeric infectious bursal disease virus-like particles by baculovirus co-infection: deterministic predictions and experimental results. *Biotechnol. Bioeng.* 75(1), 104–119 (2001).
- 13 French TJ, Marshall JJ, Roy P. Assembly of double-shelled, virus-like particles of bluetongue virus by the simultaneous expression of four structural proteins. *J. Virol.* 64(12), 5695–5700 (1990).
- 14 Belyaev AS, Roy P. Development of baculovirus triple and quadruple expression vectors: co-expression of three or four bluetongue virus proteins and the synthesis of bluetongue virus-like particles in insect cells. *Nucleic Acids Res.* 21(5), 1219–1223 (1993).
- 15 Perez N, Fourgeux C, Mohamed A et al. Rectal immunization with rotavirus virus-like particles induces systemic and mucosal humoral immune responses and protects mice against rotavirus infection. *J. Virol.* 80(4), 1752–1761 (2006).
- 16 Baumert TF, Ito S, Wong DT, Liang TJ. Hepatitis C virus structural proteins assemble into virus-like particles in insect cells. *J. Virol.* 72(5), 3827–3836 (1998).
- 17 Latham T, Galarza JM. Formation of wild-type and chimeric influenza virus-like particles following simultaneous expression of only four structural proteins. *J. Virol.* 75(13), 6154–6165 (2001).
- 18 Mortola E, Roy P. Efficient assembly and release of SARS coronavirus-like particles by a heterologous expression system. *FEBS Lett.* 576(1–2), 174–178 (2004).
- 19 Ho Y, Lin PH, Liu CY, Lee SP, Chao YC. Assembly of human severe acute respiratory syndrome coronavirus-like particles. *Biochem. Biophys. Res. Commun.* 318(4), 833–838 (2004).
- 20 Lu X, Chen Y, Bai B et al. Immune responses against severe acute respiratory syndrome coronavirus induced by virus-like particles in mice. *Immunology* 122(4), 496–502 (2007).
- 21 Buonaguro L, Tagliamonte M, Visciano ML, Tornesello ML, Buonaguro FM. Developments in VLP-based vaccines for HIV. *Expert Rev. Vaccines* 12(2), 119–127 (2013).
- 22 Ye L, Lin J, Sun Y et al. Ebola virus-like particles produced in insect cells exhibit dendritic cell stimulating activity and induce neutralizing antibodies. *Virology* 351(2), 260–270 (2006).
- 23 Yang C, Ye L, Compans RW. Protection against filovirus infection: virus-like particle vaccines. *Expert Rev. Vaccines* 7(3), 333–344 (2008).
- 24 Quan FS, Huang C, Compans RW, Kang SM. Virus-like particle vaccine induces protective immunity against homologous and heterologous strains of influenza virus. *J. Virol.* 81(7), 3514–3524 (2007).
- 25 Pushko P, Tumpey TM, Bu F, Knell J, Robinson R, Smith G. Influenza virus-like particles comprised of the HA, NA, and M1 proteins of H9N2 influenza virus induce protective immune responses in BALB/c mice. *Vaccine* 23(50), 5751–5759 (2005).
- 26 Lopez-Macias C, Ferat-Osorio E, Tenorio-Calvo A et al. Safety and immunogenicity of a virus-like particle pandemic influenza A (H1N1) 2009 vaccine in a blinded, randomized, placebo-controlled trial of adults in Mexico. *Vaccine* 29(44), 7826–7834 (2011).
- 27 Quan FS, Kim Y, Lee S et al. Virus-like particle vaccine induces protection against respiratory syncytial virus infection in mice. *J. Infect. Dis.* 204(7), 987–995 (2011).
- 28 Pijlman GP, Roode EC, Fan X et al. Stabilized baculovirus vector expressing a heterologous gene and GP64 from a single bicistronic transcript. *J. Biotechnol.* 123(1), 13–21 (2006).
- 29 Hitchman RB, Possee RD, Crombie AT et al. Genetic modification of a baculovirus vector for increased expression in insect cells. *Cell Biol. Toxicol.* 26(1), 57–68 (2010).
- 30 Marek M, van Oers MM, Devaraj FF, Vlak JM, Merten OW. Engineering of baculovirus vectors for the manufacture of virion-free biopharmaceuticals. *Biotechnol. Bioeng.* 108(5), 1056–1067 (2010).

- 31 Wong KT, Peter CH, Greenfield PF, Reid S, Nielsen LK. Low multiplicity infection of insect cells with a recombinant baculovirus: the cell yield concept. *Biotechnol. Bioeng.* 49(6), 659–666 (1996).
- 32 Radford KM, Cavegn C, Bertrand M, Bernard AR, Reid S, Greenfield PF. The indirect effects of multiplicity of infection on baculovirus expressed proteins in insect cells: secreted and non-secreted products. *Cytotechnology* 24(1), 73–81 (1997).
- 33 Ikonomou L, Schneider YJ, Agathos SN. Insect cell culture for industrial production of recombinant proteins. *Appl. Microbiol. Biotechnol.* 62(1), 1–20 (2003).
- 34 Sokolenko S, George S, Wagner A, Tuladhar A, Andrich JM, Aucoin MG. Co-expression vs. co-infection using baculovirus expression vectors in insect cell culture: benefits and drawbacks. *Biotechnol. Adv.* 30(3), 766–781 (2012).
- 35 Pincus S, Boddapati S, Li J, Sadowski T. Release and stability testing programs for a novel virus-like particle vaccine. *Biopharm. Int.* 23(10 Suppl.), S26–S34 (2010).
- 36 Josefsberg JO, Buckland B. Vaccine process technology. *Biotechnol. Bioeng.* 109(6), 1443–1460 (2012).
- 37 Tagliamonte M, Visciano ML, Tornesello ML, De Stradis A,
- 38 Yang L, Song Y, Li X et al. HIV-1 virus-like particles produced by stably transfected *Drosophila S2* cells: a desirable vaccine component. *J. Virol.* 86(14), 7662–7676 (2012).
- 39 Lee JM, Chung HY, Kim KI et al. Synthesis of double-layered rotavirus-like particles using internal ribosome entry site vector system in stably-transformed *Drosophila melanogaster*. *Biotechnol. Lett.* 33(1), 41–46 (2011).
- 40 Yamaji H, Nakamura M, Kuwahara M, Takahashi Y, Katsuda T, Konishi E. Efficient production of Japanese encephalitis virus-like particles by recombinant lepidopteran insect cells. *Appl. Microbiol. Biotechnol.* 97(3), 1071–1079 (2012).
- 41 Fernandes F, Vidigal J, Dias MM et al. Lipase-mediated cassette exchange in Sf9 insect cells for stable gene expression. *Biotechnol. Bioeng.* 109(11), 2836–2844 (2012).
- 42 Conner ME, Zarley CD, Hu B et al. Virus-like particles as a rotavirus subunit vaccine. *J. Infect. Dis.* 174(Suppl. 1), S88–S92 (1996).
- 43 Crawford SE, Labbe M, Cohen J, Burroughs MH, Zhou YJ,
- 44 O'Neal CM, Crawford SE, Estes MK, Conner ME. Rotavirus virus-like particles administered mucosally induce protective immunity. *J. Virol.* 71(11), 8707–8717 (1997).
- 45 Vieira HL, Esteveao C, Roldao A et al. Triple layered rotavirus VLP production: kinetics of vector replication, mRNA stability and recombinant protein production. *J. Biotechnol.* 120(1), 72–82 (2005).
- 46 Kang SM, Yoo DG, Lipatov AS et al. Induction of long-term protective immune responses by influenza H5N1 virus-like particles. *PLoS ONE* 4(3), e4667 (2009).
- 47 Wen Z, Ye L, Gao Y et al. Immunization by influenza virus-like particles protects aged mice against lethal influenza virus challenge. *Antiviral Res.* 84(3), 215–224 (2009).
- 48 Chackerian B. Virus-like particles: flexible platforms for vaccine development. *Expert Rev. Vaccines* 6(3), 381–390 (2007).

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