4

Use of the Baculovirus Expression System for the Generation of Virus-Like Particles

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Introduction

The study of viruses and virion structures or their use in vaccine production has been frequently impaired by difficulties in the production, recovery and purification of sufficient quantities of material in good condition. Many viruses do not grow properly in tissue culture and in some extreme cases adaptation to *in vitro* propagation is impossible. These limitations in the large-scale production of virus particles have also had a decisive influence on the development of a new generation of viral vaccines or proper reagents for viral diagnostics. The immunogenicity or antigenicity of a single protein or fragments of proteins are usually not comparable in quality or efficiency to those of a complete viral particle. Prokaryotic systems such as *E.coli* are not suitable for the production of correctly functional viral proteins. In contrast, the baculovirus expression vector system (BEVS) has been proven over the past few years to be very useful in the correct expression and regeneration of virus-like particles (VLPs).

Production of a virus-like particle is possible from the ability of one or more capsid proteins of a virus to self-assemble into multimeric structures that are morphologically and structurally identical to the original virus. They are extremely safe to handle because they do not contain the nucleic acids from the original virus. Only ten years ago this technology was thought to be restricted exclusively to the hepatitis B virus (Hep B), when it was found that some sub-viral particles were present in the plasma of infected patients. These particles were shown to be very effective as a vaccine

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Abbreviations: AHSV, African horse sickness virus; BEVS, baculovirus expression vector system; BTV, bluetongue virus; CPV, canine parvovirus; CTL, cytotoxic T lymphocytes; dsRNA, double-stranded RNA; HCV, hepatitis C virus; Hep B, hepatitis B virus; HbsAg, hepatitis B surface antigen; HEV, hepatitis E virus; HIV, human immunodeficiency virus; HPV, human papilloma virus; IBDV, infectious bursal disease virus; LCMV, lymphocytic choriomeningitis virus; PPV, porcine parvovirus; RHDV, rabbit haemorrhagic disease virus; Sf, *Spodoptera frugiperda*; SIV, simian immunodeficiency virus; ssRNA, single-stranded RNA; VLPs, virus-like particles; VP, virus protein.

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against hepatitis B (Stephenne, 1988). However, there were serious problems associated with its administration, arising from possible contamination with other infectious materials that could be present in the human plasma. A further characterization of these virus particles concluded that they were formed by the surface protein of the virus, called hepatitis B virus surface antigen (HBsAg). This 226-residue protein gives rise to a lipoproteic particle after budding from the infected cell. The gene S encoding for the HbsAg was subsequently expressed in *Saccharomices cerevisiae* (Valenzuela *et al.*, 1982). The expressed protein was able to self-assemble into HBsAg particles, forming the basis of the first recombinant vaccine ever made. Several billion doses of this vaccine have been produced already, indicating the success of this approach.

For a long time, it was generally assumed that this extraordinary property of the capsid proteins of Hep B was something unique for this system, and the technology was irreproducible for other virus systems. Indeed, it required the full development of a potent eukaryotic expression system such as baculovirus before it was clear that the phenomena observed with Hep B could be reproduced with other families of viruses.

Fundaments of the baculovirus expression vector system (BEVS)

The baculovirus *Autographa californica* nuclear polyhedrosis virus has been used to express foreign genes upon insect cell infection, generally from *Spodoptera frugiperda* or *Trichoplusia ni* (Summers and Smith, 1987). Baculovirus produce large amounts of extracellular virus particles, which can mediate infection of insect hosts or permissive cells in culture. The most abundantly expressed protein during the viral life cycle is the polyhedrin protein. Polyhedrin makes the matrix of the polyhedra, a crystalline array, which allows the survival of the virus under extreme environmental conditions. The polyhedrin gene is not essential for virus replication and can be replaced with a foreign gene such as viral structural genes. The polyhedrin promoter will drive the expression of the foreign gene. Other very late promoters such as the p10 promoter have been successfully used for this purpose. An advantage of the baculovirus system over bacterial expression is that BEVS-expressed proteins are often post-translationally modified, similar to mammalian cells. Approximately 95% of the expressed proteins are functionally active.

Manipulation of baculovirus genomes is difficult due to their large size (130 kb). To address this problem, baculovirus transfer vectors have been developed in which the foreign gene is introduced into a cassette comprising the promoter and termination sequences and flanked by the viral DNA sequences, which flank the point at which it is to be inserted. A variety of transfection methods based on cationic liposomes have been developed for the regeneration of the recombinant baculoviruses. Initially, recombinant baculoviruses were identified by their lack of visible polyhedra in plaque-forming cell assays. Later on, reporter genes such as the lacZ cassette have been introduced in the transfer vectors to facilitate the screening of the recombinant baculoviruses. Since the efficiency of this approach was very low (1%), many improvements have been accomplished in the past years. Linearization of the baculovirus genome by introduction of a single Bsu36I site (Kitts *et al.*, 1990) or two further sites (Kitts and Possee, 1993) have been shown to greatly facilitate the production and screening of recombinant baculoviruses. As a consequence of these

developments many commercial systems for direct cloning and expression in the baculovirus system are now available.

Use of the baculovirus expression system for the production of VLPs in different viral families

In this review, the discovery and production of VLPs in insect cells for different viral families will be summarized (*Figure 4.1*). The focus will only be on animal viruses, although there are several plant viruses that also share this property of forming VLPs with one or more structural proteins.

PARVOVIRUS

Within the family *Parvoviridae* there are several genera. Here we will focus on two genera, parvovirus and erythrovirus. Most of the economically relevant veterinary parvoviruses belong to the genus parvovirus, for instance, porcine parvovirus (PPV), canine parvovirus (CPV) or mink enteritis virus. Human parvovirus B19 belongs to the genus erythrovirus. Parvoviruses are formed by a group of small (≈ 25 nm), non-enveloped, linear single-stranded DNA viruses. Since 1991, different groups have reported the production of parvovirus-like particles (see Casal, 1996 for a review). The capsid is formed by one or two proteins depending on the parvovirus. In the case of PPV, the virion is formed by 60 copies of the VP2 protein. Attempts to express the VP2 protein in *E.coli* were previously unsuccessful (Lopez de Turiso *et al.*, 1991). Then, the VP2 gene was cloned under the control of the p10 promoter and the new construction was used for the production of recombinant baculovirus (Martinez *et al.*, 1992). Infection of insect cells with this baculovirus led to the expression and self-assembly of large amounts of VP2 as particles with a size and morphology indistinguishable from the original virions. These particles have been shown to have high immunogenicity in the host animal and other animal species. Recently, a dose–response experiment was carried out in pregnant sows (Casal, 1996). As little as 1 μg of PPV VLPs was able to fully protect the animals against the infectious particles. All the foetuses from the vaccinated gilts were in a healthy state without any clinical symptoms of the disease. In contrast, foetuses from the non-vaccinated animals were either mummified or heavily infected.

Since the production costs are extremely low due to the high efficiency of the baculovirus/insect cell system, these PPV VLPs are an excellent candidate for the development of a new subunit vaccine which is safer and cheaper than the current vaccine based on inactivated viruses grown in conventional cell lines. These VLPs can be used also for structural analysis by X-ray crystallography, as has been shown recently for human parvovirus B19 or CPV (Agbandje *et al.*, 1994; Hernando *et al.*, 2000).

PPV VLPs are also being used as a vehicle for the induction of other branches of the immune system, namely as CD4 and cytotoxic T lymphocyte (CTL) responses, by foreign epitopes. Of particular interest is the ability of these non-replicating particles to induce protective CTL responses. The insertion of a single CD8 epitope from the nucleocapsid protein of the lymphocytic choriomeningitis virus (LCMV) in the amino terminus of the PPV VP2 has been shown to allow the recovery of chimeric

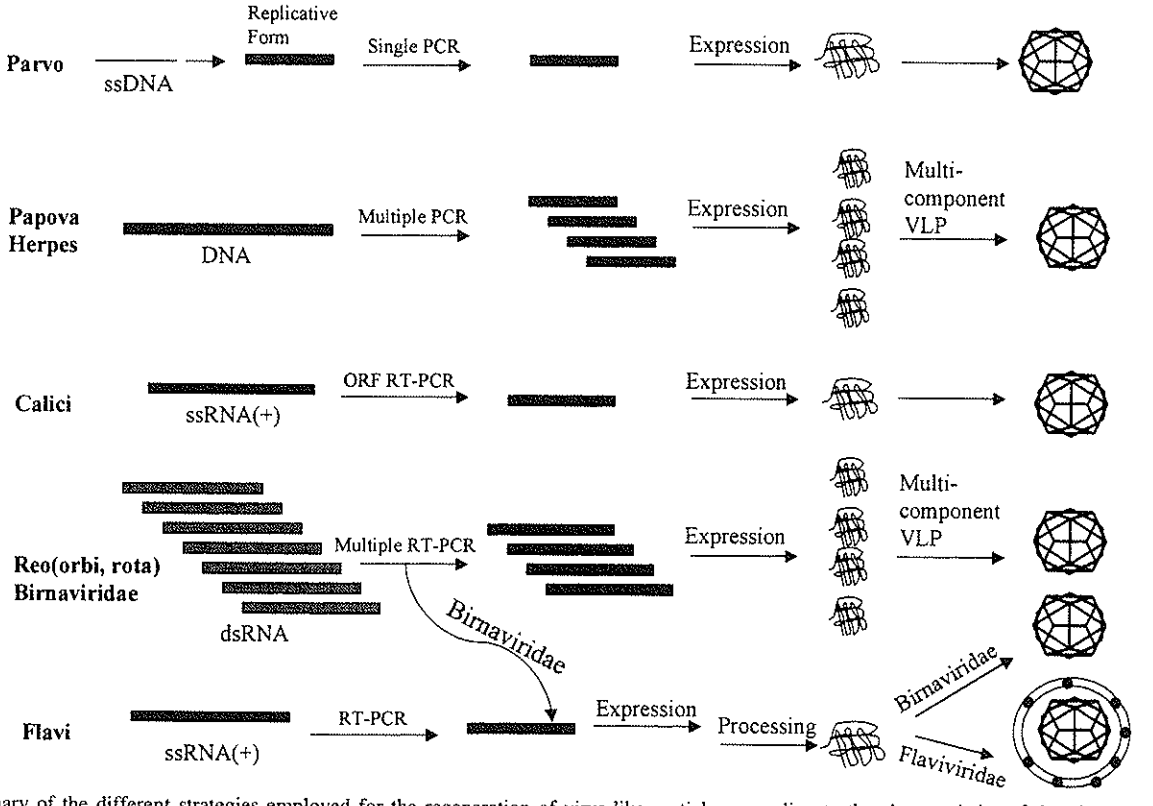

Figure 4.1. Summary of the different strategies employed for the regeneration of virus-like particles according to the characteristics of the viral genome. Based on the characteristics of the viral genome, different amplification steps will be required for the isolation and cloning of the gene(s) encoding for the viral capsid. Single or multiple transfer vectors will be used depending on the composition of the VLPs. In some cases, such as parvovirus and calicivirus, the production of VLPs will require a single protein. In other cases, such as herpesvirus or reovirus, VLPs will require a multicomponent assembly. Other viruses, such as birnavirus or flavivirus, will require the correct processing of the polyprotein by the insect cells in order to synthesize the corresponding VLPs. Abbreviations as in the text.

PPV VLPs. Immunization of Balb/c mice with 10 μg of LCMV-PPV:VLPs led to the complete protection of the mice against an intracranial challenge with virulent virus (Sedlik *et al.*, 1997). Moreover, subsequent experiments have demonstrated the efficacy of these particles in the induction of protective responses after intranasal immunization, leading to an effective generation of responses at the mucosal level (Sedlik *et al.*, 1999; see Casal, 1999 for a review).

PAPILLOMAVIRUS

Human papillomaviruses (HPV) are associated with the development of cervical cancer, one of the most common cancers of women worldwide. It is not possible to propagate HPV *in vitro*, therefore alternative methods of production have been necessary for the production and characterization of viral particles, as well as for the development of vaccine and diagnostics candidates. Expression of the L1 major capsid protein of HPV in the baculovirus system was sufficient for the formation of VLPs (Kirnbauer *et al.*, 1992; Rose *et al.*, 1993). By contrast, expression in other systems such as vaccinia (Gao *et al.*, 1994) required the presence of the two proteins L1 and L2. In a later study, these researchers demonstrated that the co-expression of L1 and L2 in Sf9 insect cells provoked a fourfold increase in particle yield (Kirnbauer *et al.*, 1993). This enhancement could be raised to as high as a 100-fold in other serotypes such as HPV1 (Hagensee *et al.*, 1993). It was observed in addition that a single amino acid substitution (Asp → His) was able to significantly inhibit particle formation. These VLPs have been used abundantly for diagnostic purposes. Moreover, they were able to elicit high-titre neutralizing antibodies (Kirnbauer *et al.*, 1992) and protection in model animals (Breitburd *et al.*, 1995). However, their efficacy as vaccines for humans remains to be shown.

CALICIVIRUS

Caliciviruses are non-enveloped RNA viruses with a capsid of icosahedral symmetry and 40 nm in diameter. They infect humans and other animals. Several cases of VLP formation have been described. Here, rabbit haemorrhagic disease virus (RHDV), Norwalk virus and hepatitis E virus will be used as examples. The genome consists of a single-stranded RNA (ssRNA) molecule of positive polarity of 8 kb with a short 3' poly (A) tail. RHDV is one of the most characteristic viruses of the family *Caliciviridae*. It causes a devastating disease in young rabbits, with mortality close to 90%. The viral capsid is formed by 180 copies of a single VP60 protein. The RHDV VP60 gene has been cloned under the control of different baculovirus promoters: polyhedrin (Laurent *et al.*, 1994) and p10 (Plana-Duran *et al.*, 1996). In all cases, the recombinant protein self-assembled to form VLPs that were easily purified. However, the expression of VP60 under the control of the p10 promoter was 5–10 times higher than that using the polyhedrin promoter. As happened in papilloma, a single change in the VP60 amino acid sequence is enough to abolish capsid assembly. RHDV VLPs have been used successfully to protect rabbits against the disease even at doses as low as 0.5 μg per animal or, even more remarkably, at 3 μg when administered by the oral route (Plana-Duran *et al.*, 1996). This intriguing finding could be due to the presence of attachment motifs in the viral capsid that may interact with the mucosal tissue, which is the

normal route of entry of the virus into the organism. These motifs can only be regenerated via formation of VLPs. This result, together with similar results found in Norwalk virus (Ball *et al.*, 1998), paved the way for the use of the calicivirus VLPs for oral delivery.

Norwalk virus is a human pathogen that causes diarrhoea and gastroenteritis. Expression of the second open reading frame rendered a protein of 58 kDa and this protein was shown to self-assemble into VLPs similar to the native capsids (Jiang *et al.*, 1992). The VLPs elicited high levels of virus-specific antibodies and have been used to set up reliable tests for the diagnosis of Norwalk virus infection. These VLPs have been used among other things to solve the 3D structure of the non-cultivable Norwalk virus (Prasad *et al.*, 1996, 1999). Interestingly enough, it was discovered subsequently that the expression of the ORF2 of Norwalk produces two sizes of particles (White *et al.*, 1997). The large particles would assemble according to a T=3 lattice, whereas the small particles (23 nm) would assemble in a T=1 symmetry. This result opens interesting questions about the mechanisms driving the morphogenesis and assembly process in icosahedral viruses.

Hepatitis E virus

Hepatitis E virus (HEV) shares many structural features in common with the caliciviruses and, for this reason, it belongs to the family *Caliciviridae*. However, due to some peculiar characteristics, the International Committee for the Taxonomy of Viruses has recently assigned a new genus exclusively for HEV (Hepatitis E-like viruses). As with the case for other hepatitis viruses, HEV cannot be grown *in vitro*. As for other caliciviruses, expression of the coat protein was supposed to be able to render VLPs. Unexpectedly, this was not the case, and only a truncated form comprising 111 amino-terminal residues, corresponding to a 50 kDa protein, self-assembled to form empty VLPs, although with a smaller diameter (23.7 vs 27 nm) than the regular virions (Li *et al.*, 1997). A remarkable further observation was made: only expression in Tn5 insect cells led to the release of VLPs in the supernatant of the cultures. By contrast, regular Sf9 cells were unable to make VLPs. The reason for this surprising result still remains unclear and it is, to the best of the author's knowledge, the only reported description of such phenomena. As in other cases, HEV VLPs have revealed their usefulness as diagnostic reagents. However, as far as the author is aware, no further data are available about its utility as a candidate vaccine for hepatitis E.

Baculovirus-derived particles have been used for the reconstruction of the 3D structure of the virus by cryo-electron microscopy and image reconstruction (Xing *et al.*, 1999). The particles assembled in a T=1 symmetry, whereas the virion is supposed to have a T=3 lattice. This important difference is probably due to the removal of the 111 amino-terminal residues from the capsid protein.

REOVIRUS

The family *Reoviridae* contains several different genera pathogenic for humans and animals. Here we will focus on two of the genera: orbivirus and rotavirus.

Orbivirus

Bluetongue virus (BTV) is the prototype member of the orbivirus genus of the *Reoviridae* family. It was one of the first cases of baculovirus-derived VLP production (French and Roy, 1990; French *et al.*, 1990). Orbiviruses cause economically important diseases in animals (BTV in sheep and cattle, African horsesickness virus (AHSV) in equids). The genome is comprised of 10 double-stranded RNA (dsRNA) segments. The capsid contains seven structural proteins (VP1–7), which are organized into two concentric layers. The outer capsid consists of two major proteins, VP2 and VP5. The inner layer is formed mainly by two proteins, VP3 and VP7. In order to study the process of virus assembly, the baculovirus system was used first to co-express VP3 and VP7, which would form the core of the particles (French and Roy, 1990). For this reason, a dual baculovirus expression vector was constructed (Emery and Bishop, 1987). This dual vector contains two polyhedrin promoters placed in opposite directions to minimize homologous sequence recombination. Using the same approach, a dual vector has been constructed to regenerate the outer layer formed by VP2 and VP5. However, since these proteins need the scaffolding of VP3 and VP7, two strategies were used: (i) to use both dual expression vectors to co-infect the insect cells, or (ii) to prepare a quadruple expression vector (Belyaev and Roy, 1993) that is capable of expressing four different proteins at the same time. Both systems were able to render complete VLPs with the double layer protein. These particles were used for cryo-electron microscopy reconstruction studies and 3D analysis of the orbivirus structure (see Roy, 1996, for a review).

Although successful in the regeneration of particles, this process of formation of VLPs by co-infection or by using quadruple vectors suffers from several drawbacks. First, the efficiency of the co-infection is generally low and needs a fine tuning of the multiplicity of infections of both recombinant baculoviruses in order to get a correct expression and stoichiometry of the four proteins. Any problem in the expression of one of the proteins is enough to stop the whole assembly process, making the regeneration of this type of particle an extremely delicate process. An example of this limitation is the impossibility of regenerating VLPs for AHSV serotype 4. In this virus, all the attempts to express the core protein VP3 were unsuccessful, impeding the production of CLPs (core-like particles, formed by VP3 and VP7) and, as a consequence, also the VLPs. Second, the stability of quadruple transfer vectors is rather low due to the large size of the vectors, making the simultaneous production of the four proteins very inefficient.

In any case, BTV VLPs were the first example, other than the Hep B system, of the efficacy of VLPs as vaccines in the natural host. Several vaccination trials were made in Merino sheep (Roy *et al.*, 1992). All the sheep that had been given VLPs in combination with commercial adjuvants developed a strong antibody response. The smallest dosage (10 μg) was still sufficient to confer protection against the challenge.

Rotavirus

Rotavirus infection is one of the leading worldwide causes of diarrhoea in children. Rotavirus particles are approximately 102 nm in diameter and consist of two protein capsids surrounding a central protein core that contains the genome (this is designated as a triple-layer particle). The genome is formed by 11 dsRNA fragments. The innermost layer is composed of VP1, VP2 and VP3 and the genome, the middle layer

is composed of VP6 and the outer layer is composed of the glycoprotein VP7 and spikes of VP4 dimers. VP4 and VP7 possess distinct antigenic activities, defining P serotypes and G serotypes, respectively. VP4 and VP7 independently elicit antibodies capable of neutralizing rotavirus infectivity and inducing protective immunity.

Rotavirus genes encoding the rotavirus structural proteins VP2, VP6, VP4 and VP7 have been cloned in baculovirus and the recombinant rotavirus proteins have been co-expressed in the baculovirus expression system. Stable VLPs were shown to self-assemble following expression of VP2 alone (Labbe *et al.*, 1991). These particles showed structural polymorphism including elongated bristly structures, helix-like structures, and sheet-like helix structures (Zeng *et al.*, 1994). Co-expression of VP2 and VP6, alone or with VP4, was shown to result in the production of double-layered 2/6- or 2/4/6-VLPs, respectively (Redmond *et al.*, 1993). Co-expression of VP2, VP6 and VP7, with or without VP4, results in triple-layered 2/6/7- or 2/4/6/7-VLPs (Crawford *et al.*, 1994). All VLPs maintained the structural and functional characteristics of native particles. Preliminary results for rabbits have indicated that VLPs administered parenterally induce active protective immunity (Conner *et al.*, 1996; Ciarlet *et al.*, 1998). Also, rotavirus VLPs were immunogenic and protective when administered mucosally in combination with cholera toxin subunit B (O'Neal *et al.*, 1997). This result is particularly important since the route of entry, as well as the pathogenesis of the virus, is mucosa-related.

BIRNAVIRUS

Birnaviruses are non-enveloped viruses that infect mainly fish and avian species, causing major diseases as infectious bursal disease (IBD) in chickens or infectious pancreatic necrosis in salmonids. Birnaviruses have icosahedral symmetry and contain bisegmented dsRNA genomes. The large RNA segment A encodes a precursor polyprotein in the order 5'-VPX-VP4-VP3-3'. The precursor polyprotein is processed co-transductionally through different proteolytic steps to yield mature virion capsid proteins VP2 (41 kDa) and VP3 (32 kDa), and the viral protease VP4 (28 kDa). VP2 is formed after proteolytic processing of the precursor VPX in a later stage. VP2 and VP3 are the major structural components of the IBDV virion, while VPX is relatively minor. Here we will comment on some reports of data that use infectious bursal disease virus (IBDV) as a model.

Expression of IBDV polyprotein has been reported in various expression systems such as vaccinia, which led to the production of IBDV VLPs (Fernandez-Arias *et al.*, 1998). However, production of VLPs in insect cells has been shown to be quite inefficient and the processing of VPX to VP2 was highly variable or absent (Vakharia *et al.*, 1993; Kibenge *et al.*, 1999). Recently, we have expressed the polyprotein using two different baculovirus transfer vectors, pFastBac and pAcYM1 (Martinez-Torrecuadrada *et al.*, 2000). Surprisingly, the expression of the capsid proteins led to a structural polymorphism (similar to that observed for rotavirus VP2 particles, see above), as observed by electron microscopy and immunofluorescence, dependent on the transfer vector used. FastBac expression led to the production of only rigid tubular structures. These tubules revealed a hexagonal arrangement of units that are trimer clustered, similar to those observed in IBDV virions. By contrast, pAcYM1 expression led to the assembly of VLPs into flexible tubules and intermediate assembly

products formed by icosahedral caps elongated in tubes, suggesting an aberrant morphogenesis. Processing of VPX to VP2 seems to be a crucial requirement for the proper morphogenesis and assembly of IBDV particles. Immunoelectron microscopy studies of tubules and VLPs allowed the determination of the position of VPX/VP2 on the surface of tubules and VLPs. Assembly of VLPs appears to require the internal scaffolding of VP3, which seems to induce the closing of the tubular architecture into VLPs and, thereafter, the subsequent processing of VPX to VP2. Structural and protection studies in chicken with the different types of particles are currently in progress. VP2 seems to be particularly effective (in preparation).

FLAVIVIRUS: HEPATITIS C VIRUS

Hepatitis C virus (HCV) is responsible for a major human disease that can progress eventually to liver cirrhosis and hepatocellular carcinoma. HCV is the only member of the genus hepacivirus within the virus family *Flaviviridae*. Flaviviruses are enveloped ssRNA viruses. The genome consists of a large open reading frame that is translated into a polyprotein of 3000 residues, which is then subsequently processed into the nucelocapsid protein C and, among others, two envelope glycoproteins, E1 and E2. It is not possible to propagate the virus in tissue culture; thus, any alternative for the production of VLPs of this virus has been the subject of an intense search.

A recombinant baculovirus expressing the core, E1, E2 and p7 proteins plus 21 residues of the NS2 protein render a polyprotein that can be processed efficiently to the individual structural proteins (Baumert *et al.*, 1998). These recombinant proteins assemble into enveloped VLPs (40–60 nm in diameter) that display similar properties to those of virions isolated from infected humans. Immunization of mice with these HCV VLPs elicited a humoral response identical to those of HCV virions, suggesting the potential of these VLPs as a vaccine candidate (Baumert *et al.*, 1999). This has been one of the first successful approaches in the assembly of VLPs with a lipid bilayer envelope containing both envelope glycoproteins. This result opens an interesting route for the production of enveloped VLPs, which automatically would allow the inclusion of many other families of viruses. However, this has been the only successful report of formation of VLPs in flavivirus. This result could be of particular interest for the application to pestivirus, another genus of the *Flaviviridae* family, which comprise some economically relevant veterinary viruses, such as classical swine fever virus and bovine viral diarrhoea virus.

RETROVIRUS: HUMAN IMMUNODEFICIENCY VIRUS

Last, but not least, we have to mention the development of retrovirus-derived particles. We will use as an example the human immunodeficiency virus (HIV), although the results reviewed here can be extrapolated to other human and animal retroviruses. The virion of HIV consists of a membrane-enveloped, nucleoprotein core containing the genomic RNA and nucleocapsid protein (p15gag) surrounded by the capsid proteins (p24gag). The p24 gag comes from the proteolytic cleavage of the 55 kDa precursor (gag Pr55). As early as 1989, the implications of the gag precursor polyprotein of simian immunodeficiency virus (SIV) and HIV in morphogenesis and assembly had been considered (Delchambre *et al.*, 1989; Gheysen *et al.*, 1989). The

unprocessed gag precursor was expressed in insect cells. Electron microscopy analysis showed that the gag protein was able to assemble into 100–120 nm particles that were targeted to the plasma membrane, budding from the cell surface and released into the culture supernatant. It is debatable if these particles can be properly called VLPs, since there is no authentic reconstruction of the viral particle as the envelope glycoproteins are missing as well as other proteins of the capsid. Nonetheless, VLPs have been the internationally accepted terminology for these gag particles. However, since they do not really mimic the virion, the efficacy of these 'VLPs' as vaccines has to date been inadequate.

After those early findings there have been many attempts to try to incorporate the env proteins in the gag particles. Different strategies have been applied in trying to solve this crucial problem. Moreover, many studies have been conducted for the use of gag particles as carriers and delivery of other epitopes of interest. I would like to mention one of the most recent approaches to find dispensable regions in gag that are not essential for assembly in insect cells. Kang *et al.* (1999) have recently found that the deletion of 143 amino acids at the C terminus did not affect particle formation. These researchers then used these mutants for insertion of three tandem copies of V3 domains from different HIV isolates. V3 contains one of the most neutralizing epitopes of the env protein of HIV. Immunization of rabbits with these chimeric gag–V3 particles led to the production of neutralizing antibodies to HIV. In addition, these gag particles were able to induce a strong CTL response against V3 peptide-treated target cells (Kang *et al.*, 1999).

Major applications of VLPs

TOOLS FOR MOLECULAR BIOLOGY

Synthesis of sub-viral particles in eukaryotic cells opens up new possibilities for defining mechanisms of protein–protein assembly under natural intracellular conditions, important domains or essential residues for assembly (as has been shown, for instance, in HPV). In this way, by manipulating the sequence of the different proteins involved it will be possible to define experimentally the essential morphogenic interactions required for the assembly process. Baculovirus-driven expression has been very useful for studying the capsid assembly process of complex viruses such as herpes simplex virus type 1 in great detail (Newcomb *et al.*, 1996, 1999; Spencer *et al.*, 1998). A whole panel of recombinant baculoviruses expressing the different capsid proteins has been used to infect insect cells and to analyse the assembly intermediates *in vitro*. A similar approach has been followed for polyoma virus (An *et al.*, 1999). It is interesting to note that these particles were able to package fragments of DNA up to 5 kb, which indicates a new avenue of using the VLPs as delivery systems. For instance, polyoma VLPs (Goldmann *et al.*, 1999) have been shown to be able to package a 4.5 kb pCMV-β-galactosidase plasmid into VP1 VLPs and the DNA was efficiently transferred into COS-7 cells, as demonstrated by the expression of β-galactosidase. This result may represent a potential new transporter system for human gene therapy.

VACCINATION

It is clear from the studies so far that this technology has much to offer for the

development of vaccines for human and veterinary use. Most of the VLPs have been demonstrated to be highly immunogenic and effective in the induction of protection. Some of them, such as those for human papillomavirus, Norwalk virus and human rotavirus, are already in clinical trials and they are probably only the beginning of a long list. For veterinary viruses the situation is more complicated because most of the VLPs already have a conventional counterpart (inactivated or live-modified vaccine) and it is not clear that the savings in cost of production or the improvements in safety may convince the current manufacturers to make further investments in the registration of these new vaccines. The registration phase, due to the complexity of the regulatory issues, which implies huge economical investments, continues to be the major deterrent to translating the innovation in vaccinology to the market. There are no indications that this situation is going to change in the near future.

It is interesting to remark on the possible application of these VLPs in the induction of alternative immune responses. General interest in mucosal immunization stimulated new efforts and ideas about how to optimize delivery of vaccines to protect against infections that replicate on mucosal surfaces. Previous dogma had stated that induction of mucosal immunity required the use of live attenuated vaccines, but this idea is slowly changing as new results of efficient oral immunization appear, such as those shown for calicivirus VLPs. New methods for delivery of non-replicating vaccines by increasing doses, investigating new immunization protocols or by delivery of virus in microencapsulated or other forms offer the possibility of inducing mucosal immunity to non-replicating antigens. These new delivery systems may protect the antigen from other non-interfering substances and deliver it directly to the Peyer's patches for uptake and processing.

DIAGNOSTICS

The use of VLPs in diagnostics has been much more immediate and widely accepted. Since they mimic outstandingly well the original virions in antigenic structure, VLPs are the most improved alternative to develop new diagnostic kits, replacing the need for infectious virus in the production process. VLPs can be used in all the conventional technologies, such as ELISA, agglutination, immunochromatography, immunoelectrophoresis, etc. There are many advantages to the use of VLPs: for example, there is no need to propagate the infectious virus (which in some cases is not possible), there is no risk of virus transmission or infection, production levels are much higher, production is cheaper and, generally, VLPs are more stable.

VLPs can also be used in combination with marker vaccines to distinguish recent infections from vaccinal immunity. Another possible application is to distinguish acute phase (<2 weeks after viraemia) and convalescent sera (weeks to months after infection) based on the ability to recognize linear determinants unique to capsid-forming proteins.

General remarks

It is amazing to look back on the fast pace of discovery in this area. In less than 10 years more than thirty different viral VLPs have been produced and the number

continues to increase at great speed. It is anticipated that VLPs will provide an increasingly powerful tool in the study of virus structure and function and will form the basis for the development of a new generation of safer vaccines, diagnostics and gene delivery systems.

Looking at the different experiences accumulated in the past years for the different viral families, it is possible to enumerate a few predictive rules about the chances of success in the recovery or expression of VLPs. First, non-enveloped icosahedral viruses are the most suitable candidates for the successful production of VLPs, as shown in *Table 4.1*. Most of the non-enveloped viruses are easily reconstituted by expression of one or more proteins using the baculovirus system. Second, a capsid made by the assembly of a single protein will have many more chances to produce large amounts of high-quality VLPs, as for instance parvovirus, calicivirus, papillomavirus. As a consequence, an increase in the number of proteins to be expressed and to assemble will be proportional to the difficulties in correct assembly and, therefore, will lead to poorer recovery of VLPs, i.e. orbivirus, herpesvirus. Third, capsid proteins that need to be processed from a polyprotein precursor in a timely fashion (monocystronic RNAs) are also less efficiently assembled, namely picornavirus, birnavirus, flavivirus, due to relatively inefficient processing of the precursors in insect cells. However, it is interesting to note the progress that is being made in the production of VLPs for enveloped viruses such as hepatitis C virus or retroviruses (HIV) in general. These findings indicate that new strategies could be developed in the near future to overcome the problems derived from the presence of lipidic envelopes in the virions, or new ways found to enhance the efficiency of processing of the polyproteins in insect cells.

Table 4.1. Summary of the effect of the presence of viral envelope on VLPs formation in different viral families

Viral family	Envelope	VLPs formation
DNA viruses		
Parvoviridae	No	Yes
Papovaviridae	No	Yes
Adenoviridae	No	ND
Herpesviridae	Yes	Yes
Poxviridae	Yes	No
RNA viruses		
Picornaviridae	No	No
Caliciviridae	No	Yes
Reoviridae	No	Yes
Flaviviridae	Yes	+/-
Rhabdoviridae	Yes	No
Filoviridae	Yes	No
Orthomyxoviridae	Yes	No
Bunyaviridae	Yes	No
Arenaviridae	Yes	No
Birnaviridae	No	Yes
Retroviridae	Yes	Yes

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