Chapter 6

Baculovirus Display: A Novel Tool for Vaccination

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1. Introduction

Baculoviruses are enveloped viruses that infect insect larvae mainly from the order Lepidoptera. Their genomes are circular double-stranded DNA molecules of about 80 to 180 kbp and are packed in rod-shaped nucleocapsids with a typical size of 40-50 nm in diameter and 200-400 nm in length.

Among the numerous baculoviruses, *Autographa californica* multiplenucleopolyhedrovirus (AcMNPV) is the most widely studied and used in biotechnology.

During its infection cycle it produces two phenotypes. Occlusion derived viruses (ODV) initiate the infection at the larvae midgut. After this primary infection, the viral progeny consists of budded viruses (BV) that carry on the systemic infection in larvae. These types of virions differ in their efficiencies of infection for different cell types; ODV infect midgut epithelial cells up to 10,000 fold more efficiently than BV. In contrast, BV are up to 1,000-fold more efficient at infecting cultured cells than ODV. As the viral propagation in cell culture is mediated by BV phenotype (Rohrmann, 2011), most of the knowledge regarding baculovirus infection cycle is based on studies performed in insect cells infected by BV (Figure 1).

Cell entry is mediated by a class III viral glycoprotein located at the virion surface, Gp64, which interacts with an unknown cell receptor (Backovic & Jardetzky, 2009). This interaction triggers clathrin-dependent endosomal internalization. This internalized vesicle becomes subsequently acidified. This causes a conformational change in Gp64 that result in the fusion of the viral envelope with the endosome membrane. Thus the nucleocapsid is released in the cytoplasm and migrates to the nucleus. Once in the nucleus, DNA is uncoated and the transcriptional cascade begins (Figure 2).

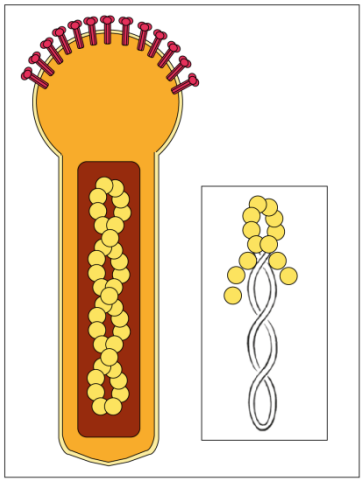

Figure 1. Structure of the budded virus.

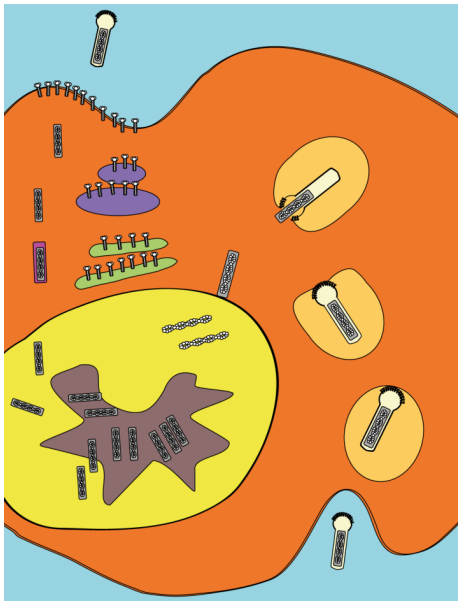

Figure 2. Baculovirus replication cycle. Infection cycle initiates when a budded virus (BV) interacts with the cell membrane and is endocytosed. When the endocytic vesicle is acidified, GP64 fusion protein, located at the BV membrane, trigger the fusion of the plasma membrane and the BV envelope releasing the nucleocapsid in the cytoplasm. The nucleocapsid is then transported to the nuclus where it transcribes its genes, replicates its DNA in the virogenic stroma where new nucleocapsids are assembled. Nucleocapsids then egress from the nucleus, travel to the cytoplasmic membrane and bud through aquiring an envelope containing the surface protein GP64.

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AcMNPV genome encodes about 150 genes which are transcribed in a temporal fashion. Firstly, immediate early genes are transcribed by the host RNA-polymerase II. These genes generally encode for transcription factors, like Ie1, that aid the subsequent transcription of genes. After this early phase DNA replication occurs. Immediately after DNA replication there may be a transient period when proteins are not bound to the DNA and this might expose late promoters and facilitate their activation (Rohrmann, 2011). Baculoviruses also encode a novel RNA polymerase that transcribes late and very late genes and that recognizes the unique baculoviral promoter consensus sequence DTAAG. During the systemic infection nucleocapsids are assembled in the virogenic stroma. The envelope proteins are synthesized, translated in association with the endoplasmic reticulum, glycosylated and transported to and incorporated into the cytoplasmic membrane via the Golgi apparatus. Nucleocapsids destined to become BV exit the nucleus. They move to the cytoplasmic membrane at the site where envelope proteins (Gp64 and F protein) concentrate, and bud through obtaining their envelopes. Early in the systemic infection more BV are produced which spread the infection throughout the insect. Finally, late in infection, occluded virions are produced, and the cell dies releasing the occlusion bodies.

There are many biotechnological uses for baculoviruses. One of the most widespread is their use as insecticide agents. There have been much work on the development of baculoviruses to control insects but the acceptance and use of viruses for insect control has been limited. This can be attributed to their slow speed of kill and their limited host range. At present many research groups are working with the aim of overcoming these limitations developing novel strategies such as baculovirus-mediated expression of toxic proteins for insects. Moreover, recombinant baculoviruses have been extensively used as expression vectors in insect cell cultures. A variety of technological improvements have eliminated the tedious procedures to isolate the recombinant viruses turning the baculovirus-based expression system in a safe, easy to use and scale up system. (Kost et al., 2005).

Another application of baculovirus is their use as expression vectors for eukariotic proteins. Their ability to include quite large DNA extra fragments in their genomes and the possibility to use their very strong polihedryn promoter, which activates upon infection, make baculoviruses a very useful tool in biotechnology for the production of recombinant proteins in insect cells.

In addition, protein expression in larvae or cell culture is not the only application of baculoviruses. In fact, baculoviruses are widely used in the development of strategies for displaying foreign peptides and proteins on the virus surface as well as mammalian cell transduction using different mammalian expression cassettes. Baculovirus display consists of the expression of proteins or peptides in the surface of a baculovirus. This is achieved by fusing the protein of interest with the major baculoviral envelope glycoprotein Gp64, resulting in the localization of the chimeric protein on the viral envelope and the plasmatic membraneof infected cells. The surface displaying of antigenic epitopes make baculoviruses efficient vaccine vehicles capable of mounting a strong specific immune response.

The aim of this chapter will be to describe the biotechnological utilities of baculovirus display. Particularly, it will describe this technique for vaccination and gene delivery. It will discuss

the adjuvant effects of baculoviruses and the immunity response of recombinant viruses. Moreover, other applications of baculovirus display such as gene therapy and high throughput screening of antibodies and antigenic epitopes libraries will also be addressed.

2. Baculoviral fusion proteins

Entry of enveloped viruses into host cells requires fusion of the viral envelope with the cytoplasmic membrane by the action of viral envelope fusion proteins. If the fusion occurs at the cell surface, viral fusion proteins typically act at neutral pH. On the other hand, in receptor-mediated endocytosis the major fusion protein activity is most often observed at the acidic endosomal pH (Monsma & Blissard, 1995).

In general, baculovirus fusion proteins mediate the membrane fusion at the late endosomal phase. For this reason, the major fusogenic activity was observed at low pH. Although it has been possible to identify which are the proteins that build fusogenic function, which is the cell receptor that recognizes these proteins remains a mystery.

Baculoviruses can be divided into two different groups according to the surface glycoprotein they use to mediate the fusion between the endosomal membrane and the viral envelope. One group is composed by viruses that use Gp64 as its fusogenic protein whereas the other group uses the F protein to mediate membrane fusion. This division is coincident with a phylogenetic separation of lepidopteran NPVs into the two major Groups I and II. These two groups differ significantly in gene content, most notably Group I NPVs use GP64 as their BV fusion protein, whereas Group II NPVs lack gp64 and utilize F protein (Zanotto et al., 1993).

AcMNPV is one of the most widely described baculovirus and belongs to Group I. It presents on its surface the major glycoprotein Gp64 and the residual F protein. While the F protein does not develop any specific function, Gp64 has been identified as the glycoprotein responsible for membrane fusion.

In this section it will be described the structure and function of glycoprotein Gp64 as responsible for the fusion of membranes and its biotechnological applications for the presentation of foreign antigens.

2.1. Gp64: Structure and function

Three classes of viral membrane fusion proteins havebeen identified. Class I which contain N-terminalhydrophobic fusion peptides, Class II, which fusion peptides are located in internal loops, and Class III that exhibit distinctstructural features in their architectures as well as in theirmembrane interacting fusion loops. Gp64 belongs to this latter group.

The major envelope protein of the budded virions, GP64, has been shown to mediate acid-triggered membrane fusion both in virions and when expressed alone in transfected cells. The native GP64 is a phosphoglycoprotein fatty acid acylated near the transmembrane domain (Monsma & Blissard, 1995). The Gp64 open reading frame (ORF) of AcMNPV encodes a 512

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aminoacids polypeptide with 15 cysteine residues. The resulting disulfide bonds participate in the formation of the native structure.

As a member of the Class III fusion proteins, Gp64 is composed of five domains that result in a macromolecular structure very distinct from any reported class I or class II fusion protein. However, Gp64 conserves the typical characteristics of viral fusion proteins. It includes a fusion domain which mediates the fusion between the cell membrane and viral envelope; a transmembrane domain which anchors the protein in the lipidic bilayer and a multimerization domain that allows the protein to form trimmers. The detailed structure of AcMNPV Gp64 is shown in Figure 3 (Backovic & Jardetzky, 2009) Baculovirus gp64 also contains a seven residue C-terminal tail domain (CTD). Deletion of this domain does not significantly affect the ability to mediate fusion, but reduces the baculovirus titers to 50%. These data indicate that CTD is involved in virus budding (Figure 3).

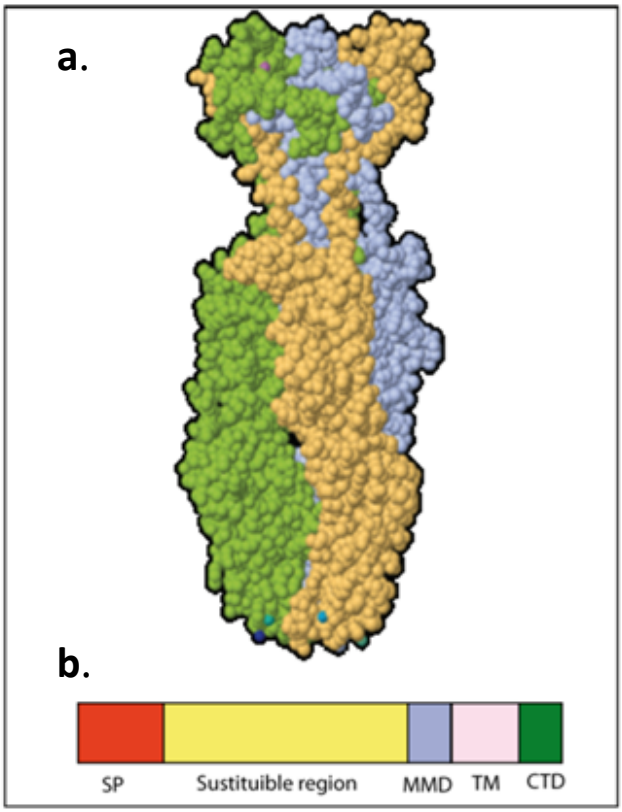

Figure 3. GP64 structure. a. Trimmeric structure of baculovirus major surface glycoprotein Gp64 obtained using the Expasy tool *Make multimer.py* in www.expasy.org. **b.** Gp64 polypeptide scheme showing different functional domains useful for antigen surface display.

Budded virions of baculoviruses enter cells by endocytosis. Gp64 is the major component of the viral envelope, and the unique protein with fusogenic activity in AcMNPV. Gp64 is triggered to induce the fusion at the low pH of endosomes. In addition Gp64 is distinguished from any other fusion protein in its ability of going through a reversible conformational change, unlike class I and class II fusion proteins, for which the post-fusion conformation is thermodynamically more stable and the conformational rearrangement is irreversible.

2.2. Gp64 for protein display

Gp64 is expressed early and late in the infection of an insect cell. It is a 64 kDa protein which forms trimmers and locates in the BV envelope with a polarized distribution. As Gp64 is a transmembrane protein that exposes an outer domain, it can be used to display a selected protein on the BV surface. A chimeric Gp64 can be constructed to contain the protein of interest allowing it to be incorporated in the BV structure upon infection of insect cells (Grabherr & Ernst, 2010).

In order to facilitate the construction of a chimeric protein it was shown that is not necessary to conserve the complete structure of Gp64. The signal peptide (SP), the multimerization domain, the transmembrane (TM) and the cytoplasmic tail domain (CTD)were shown to be enough for the surface display, whereas the rest of the protein can be eliminated. This strategy avoids the need of dealing with large transfer vectors as well as permitting to increase the number of displayed proteins.

3. Baculovirus as immunogens

The innate immune system provides the first line of host defense against infection. It is extremely important to mount a strong specific immune response by expressing co-stimulating factors necessary for the activation of adaptative immunity cells.

It was shown in previous articles that inoculation of a murine macrophage cell line with budded baculovirus induces the secretion of inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and IL-12 (Abe et al., 2005; Chimeno Zoth et al., 2012; Han et al., 2010; Hervas-Stubbs et al., 2007).

AcMNPV induces pro-inflammatory cytokines secretion through a MyD88/TLR9-dependent signaling pathway, while other signaling molecules may participate in IFN-α production in response to AcMNPV.

Toll-like receptors (TLRs) are a family of transmembrane proteins that recognize and bind endogenous and exogenous ligands. Signaling through TLR generally culminates in the production of pro-inflammatory cytokines resulting in modulation of several aspects of the innate immune response (Han et al., 2010). In the case of baculovirus, it has been reported that BVs could induce cytokine production through the TLR9 signaling pathway in mammals.

TLR9 was shown to be responsible in vivo for immune system stimulation by oligodeoxynucleotides containing unmethylated CpG motifs. Like bacteria, AcMNPV contains a significant number of potentially bioactive CpG motifs. Indeed, a number of studies demonstrate that AcMNPV can stimulate professional Antigen Presenting Cells (APCs) by this pathway. Furthermore, Abe et al. demonstrated that internalization and endosomal maturation are required for TLR9 activation by CpG-rich DNA. They showed that the inhibition of endosomal maturation abolishes the immune system activation of AcMNPV in a dose-dependent manner. These results imply that immune system activation by AcMNPV through TLR9 requires membrane fusion via Gp64 as well as the liberation of the viral genome into cytoplasmic TLR9-containing vesicles (Figure 4.a)

On the other hand, despite BVs cannot replicate in mammalian or other vertebrate animal cells (Via et al., 1983), recent studies showed that BVs have strong adjuvant properties in mice, promoting potent humoral and CD8+ T cell adaptive responses (Abe et al., 2003; Gronowski et al., 1999). In addition, BVs induce the production of inflammatory cytokines by the *in vivo* maturation of dendritic cells (Figura 4.c).

Zoth et al. evaluated the effect of baculovirus administration on the innate immune response of chickens. They found an upregulation of IFN-γ and IL-6 in the baculovirustreated chicken spleens and a decrease of the TGF-β gene expression. These facts indicated a strong pro-inflammatory immune response. Moreover, they demonstrated that BV induced modifications in the mononuclear cells pattern of different organs using flow cytometry.

The duration of the BV-induced response is very limited. This fact represents one of the many interesting benefits of the use of baculovirus for stimulating innate immunity, because the potential damage for a strong inflammatory immune response on an extended time period could be avoided (Chimeno Zoth et al., 2012)

On the other hand, it could be presumed that baculovirus inoculation produced an indirect effect on monocytes/macrophages. Zoth et al. also showed an increase of both the mRNA and the protein levels of IFN-γ, and a priming effect of Nitric Oxyde (NO) response in splenocytes of chickens treated with baculoviruses. NO acts as a multi-functional mediator with diverse physiological and pathological roles in host defense, (MacMicking et al., 1997). The production of NO by activated monocytes/macrophages is an important innate immune response sign of cellular antiviral and bactericidal activity.

Moreover, Kitajima et al. demonstrated that AcMNPV inoculation of mice induced NK cells activation. They observed that in AcMNPV inoculated animals there was up to fourfold increase in the number of NK cells in spleen, liver, bone marrow and thymus. Furthermore, it was analyzedt he antitumor ability of AcMNPV-induced NK cells and they concluded that AcMNPV injection induces a NKT cell and IFN-γ independent NK cell cytotoxicity against tumor cells in mice (Kitajima et al., 2007) These findings will be approached in section 7.

In conclusion, the strong immune response induced by AcMNPV makes it a promising candidate for a novel, adjuvant- containing vaccine vehicle against infectious diseases (Abe et al., 2005).

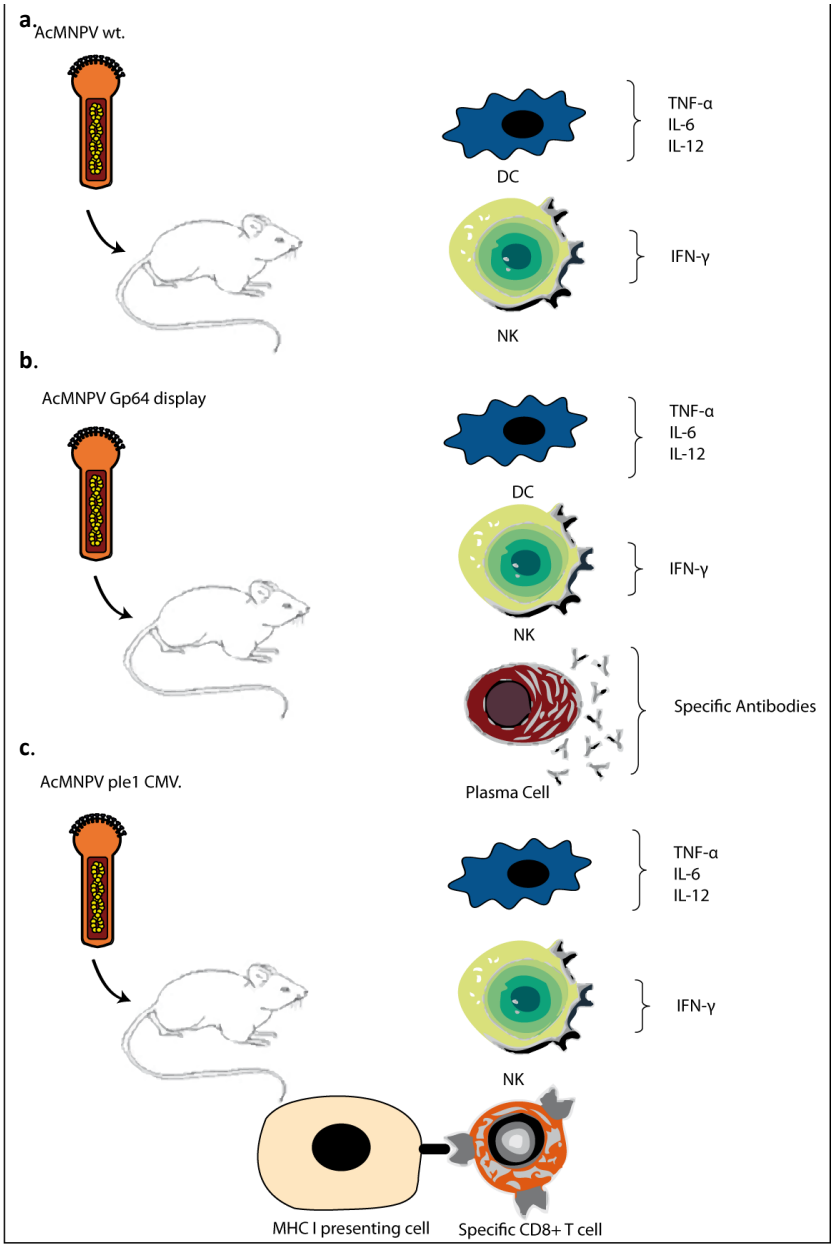

Figure 4. Immune response induced by baculovirus summarized. a. Activation of immune cells by inoculation with AcMNPV wild type. **b.** Immune response triggered by AcMNPV displaying a Gp64 fused antigen. **c.** Immune response generated by antigen coding AcMNPV under the control of CMV Ie1 promoter.

4. Baculovirus display

Eukaryotic systems represent a highly interesting model for the study of higher eukaryotic structures and interaction mechanisms because they provide posttranslational modifications and complex protein folding, in contrast to prokaryotic systems. Moreover, displaying a protein on the surface of a cell or a virus is a very successful strategy, for recreating and maturing binding properties such as antigenic recognition (Grabherr & Ernst, 2010).

Several strategies have been developed for displaying heterologous peptides or proteins on the baculovirus envelope by fusing the peptide or protein to gp64. In most instances the vector is designed with the aim of obtaining baculovirus particles that contain both wild-type gp64 and chimeric gp64 molecules. Furthermore, baculoviruses displaying proteins fused to Gp64 have proven to be very effective immunogens and they have been used successfully to generate antibody responses to a variety of displayed proteins (Kost et al., 2005).

Given that baculoviruses are able to mount a robust innate immune response by activating professional APCs, it is expected that baculovirus expressing an heterologous antigen on its surface could generate a specific response against this antigen. In fact, several works showed that baculoviruses expressing chimeric Gp64 on its surface were able to mount a very strong humoral response against the antigen displayed (Figure 4.b).

Xu et al. demonstrated in several works that baculovirus surface display of different proteins of Japanese Encephalitis Virus and swine fever virus generated high titers of specific antibodies useful for the protection against the disease. More specifically, they found that inoculation with recombinant baculoviruses produced a specific IgG response comparable with the response mounted by the preexistent attenuated vaccine and high neutralizing antibody titers against the virus (Xu et al., 2008; Xu & Liu, 2008; Xu et al., 2009; Xu et al., 2011).

Furthermore, numerous studies used baculovirus display for the development of new generation vaccines and obtained similar results to those showed by Xu et al. In this context, baculovirus surface display conferred protection and induced a strong humoral response against avian reovirus (Lin et al., 2008), human enterovirus (Meng et al., 2011), influenza (Jin et al., 2008; Prabakaran et al., 2010), malaria (Yoshida et al., 2009), etc.

In the next sub-sections the different strategies for efficient baculovirus display will be discussed. These include baculovirus display using the entire Gp64 for the generation of the chimeric proteins, baculovirus display based on single peptide insertion in Gp64 and a truncated Gp64 system with several cloning advantages will be considered (Figure 5).

Baculovirus display strategies have also been used for modification of the viral surface to command baculovirus mediated transduction of mammalian cells. In addition, capsid modifications may allow novel approaches for enhancing baculovirus mediated gene delivery. These studies will be discussedlater.

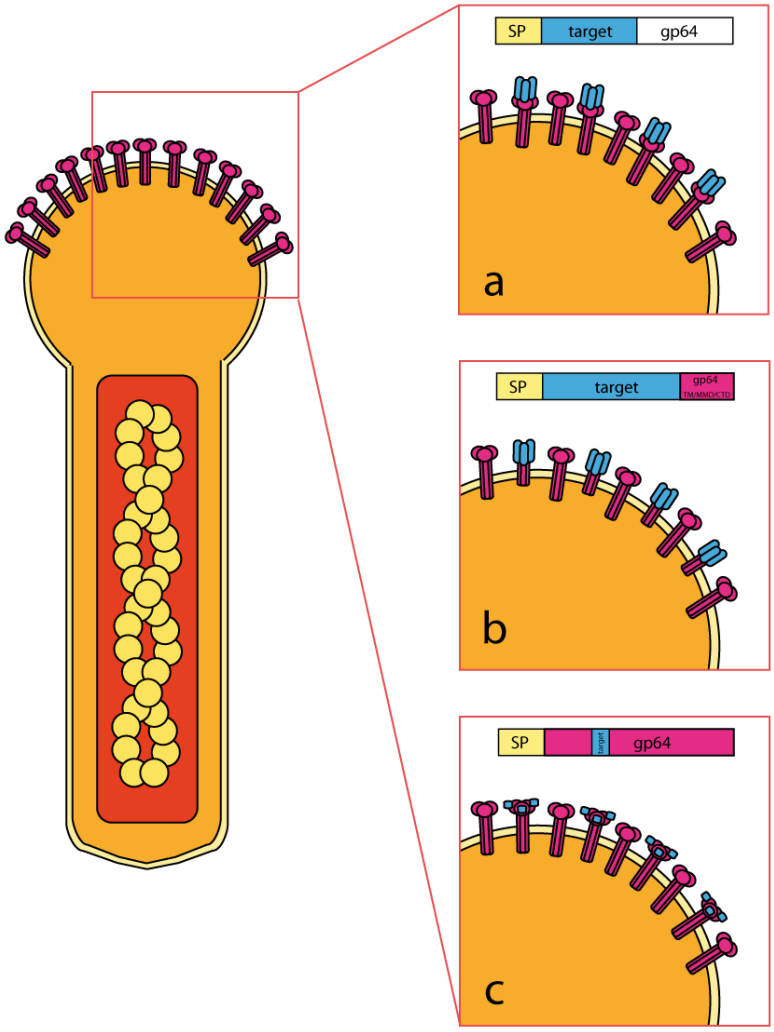

Figure 5. Different kinds of baculovirus display. a. Baculovirus surface display using the entire Gp64. **b.** Baculovirus surface display using only TM, MMD and CTD as fusion partner of the antigenic target. c. Baculovirus display using recombinant Gp64 expressing a small peptide.

4.1. Chimeric proteins using the entire Gp64

Gp64 can serve as a fusion partner that together with a chosen target protein gets incorporated into the cell membrane and into budded virions. In the first reports of baculovirus display proteins were fused to the complete gp64. In these works the target proteins were cloned into a vector providing N-terminal fusion with the gp64 signal peptide and C-terminal fusion with

the full length gp64 coding region. (Boublik et al., 1995; Grabherr & Ernst, 2010). The conservation of the biological function of several proteins when they were expressed by the baculovirus display system, e.g HIV gp120, indicated that large, complex proteins could be displayed on the surface of baculovirus particles in a functional form.

The mechanism of incorporation into the viral particle was proposed to be due to oligomerization of the chimeric Gp64 with wild-type Gp64. In addition the CTD of the chimeric Gp64 may play an important role in the nucleocapsid recognition for budding process (Figure 5.a)

For the purpose of antigen display various epitopes were presented and shown to induce immune response in mice.

The advantages of this method reside in that all needed sequences for glycoprotein transport and maturation are present in the entire sequence of Gp64. Complete Gp64 fused antigens will be synthetized through the glycoprotein synthesis pathway and will be directed to plasmatic membrane and also budded virus envelope.

However, the utilization of entire Gp64 may cause some problems in the cloning process due to the length of the subsequent transfer vector.

4.2. Peptide insertion on Gp64

Another strategy consists in peptides directly engineered into the native Gp64 of AcMNPV in order to increase the avidity of the displayed target. In this case a short peptide is inserted into the sequence of the wild type Gp64, being this protein the only variant expressed in the virion, in contrast to the previous approach where both wt and the modified versions coexisted in the BV surface. It has been reported that this method resulted very efficient to mount a robust specific antibody response against the inserted peptide with a significantly increased avidity.

However, manipulating the native gp64 envelope protein may cause some problems. Given that no wild-type gp64 exists in order to guarantee functional cell fusion and virus budding, it is possible that the overall incorporation of the recombinant protein into cell membrane or viral envelope as well as viral titers decrease considerably. For this reason, insertion sites for foreign fragments must be chosen carefully. Moreover, the size of the peptides for insertion results in a limiting condition. Indeed, only small peptides have been inserted into the native gp64 with a maximum size of 23 amino acids (Figure 5.c).

Alternatively, expressing a second copy of gp64 displaying the target peptide in addition to the wild type Gp64 represents an effective solution (Grabherr & Ernst, 2010; Spenger et al., 2002).

4.3. SP, TM and CTD display systems

More recently, several reports demonstrated that using only the signal peptide region (SP),transmembrane region (TM) and the cytoplasmic tail domain (CTD) was enough for surface display on the insect cell surface as well as on the budded virions. The resulting smaller transfer vectors represented a significant improvement for the increased cloning efficiencies

and the number of displayed chimeric proteins (Grabherr & Ernst, 2010; Spenger et al., 2002; Xu et al., 2009).

This method conserves the advantages of the baculovirus surface display using the entire Gp64, reducing significantly possible cloning troubles (Figure 5.b).

5. Baculovirus and cellular immunity

Apart from infecting insect cells, baculoviruses are able to transduce different types of animal cells such as human, rodent, rabbit, porcine, bovine, fish and avian cells (Hu, 2005; Hu, 2006) In addition, baculovirus can transduce embryonic stem cells, adult stem cells and induced pluripotent stem cells (Chen et al., 2011).

Baculovirus are safer than other transduction vectors because they don´t integrate its DNA into host genome, nor replicates it inside the transduced cells (Chen et al., 2011; Merrihew et al., 2001). It has been demonstrated that humans do not possess pre-existing antibodies and specific T-cells against baculoviruses (Strauss et al., 2007). For this reason, baculoviruses may avoid the pre-existing immunity problem caused by other viral vectors.

Thus, the coding sequence of a protein of interest can be cloned into the viral genome under the control of a suitable promoter. Then, the inoculation of an animal with the recombinant virus results in the expression of the heterologous protein inside different cell types. The expression of a foreign protein in the cytoplasm trigger the MHC class I antigen presentation of proteasome processed peptides of the recombinant protein. In this way, joined to the adjuvancy showed by baculoviruses, transduction of animal cells may induce a strong cellular immune response (Figure 4.c).

Yoshida et al. have developed a baculovirus based dual expression system, with the aim to develop multifunctional vaccines capable of inducing strong humoral and cellular immune responses. In this study a chimeric protein was constructed with the display necessary sequences of Gp64 and the entire open reading frame of the*Plasmodium berghei* circumsporozoite protein (PbCSP) under the control of polyhedron and CMV Ie1 promoters. ELISPOT assays with splenocytes from immunized mice with the recombinant baculovirus showed significative IFN-γ secretion compared with the results for immunization with a recombinant AcMNPV without the CMV Ie1 promoter when the splenocytes was stimulated with a PbCSP synthetic peptide. In addition, this baculovirus based dual system showed to be more protective than the simple baculovirus display system (Yoshida et al., 2009)

On the other hand, Hervas-Stubbs et al. demonstrated that baculoviruses induced strong humoral and cellular immune responses by co-administration of AcMNPV wt. and a purified antigen.They showed that budded baculoviruses had strong adjuvant properties, promoting humoral and CTL responses against coadministered antigen. They observed also that baculovirus could induced DC maturation, and the production of inflammatory mediators through mechanisms primarily mediated by IFN-α and IFN-β.It has been shown previously that type

I IFNs act directly on naive B cells and CD4+ and CD8+ T cells, promoting clonal expansion and differentiation (Curtinsger 2005; (Bon & Lucchetti, 2006).

5.1. Baculovirus and mammalian cell transduction

Baculovirus entry into mammalian cells represents an important goal for immune response induction and most recently for different genic therapies. It was initially suggested that baculovirus entry depended on electrostatic interactions, heparin sulfate and phospholipids (Duisit et al., 1999; Tani et al., 2001), but the exact cell surface molecules and the involved mechanism remained unknown. Based on the mechanism of Gp64 mediated membrane fusion and the entry pathway of baculoviruses in insect cells, it was also proposed that clathrin-mediated endocytosis and macropinocytosis play roles in baculovirus entry (Long et al., 2006; Matilainen et al., 2005) In contrast, Laakkonen et al.(2008) discovered that baculovirus could enter some types of mammal cells, such as hepatic cells, by a pathway independent of clathrin-mediated endocytosis and macropinocytosis suggesting that phagocytosis might play a role (Chen et al., 2011).

These data suggest that baculovirus entry pathway varies with cell types and will be necessary more studies to elucidate the complete mechanisms. Nevertheless, all studies determined that baculovirus envelope protein gp64 is pivotal for entry and for the activation of dendritic cells (DCs) (Abe et al., 2005; Niu et al., 2008; Schutz et al., 2006).

Once inside the cells, baculovirus is transported to the endosome. Then, virions are released by the acid-triggered gp64 fusion (Kukkonen et al., 2003) and subsequently transported into the nucleus (Laakkonen et al., 2008; van Loo et al., 2001) reorganizing the actin cytoskeleton (Matilainen et al., 2005); Salminen et al., 2005). A major component of type III intermediate filaments, vimentin, also participates in intracellular trafficking (Mahonen et al., 2010). Inside the nucleus, baculoviral DNA could be recognized by the cellular transcription machinery and recombinant proteins could be expressed.

5.2. Baculovirus capsid display

As was described before in this section, several authors reported strategies in which the coding sequence of an antigen was cloned driven by the cytomegalovirus (CMV) promoter to obtain antigen specific T cell immune responses, resulting in high levels of protection against parasitic diseases.

In addition to the baculovirus surface display on the envelope, heterologous protein has been displayed on the capsid by fusion with the major capsid protein VP39 without any interference in the virus assembly (Molinari et al., 2011). Kukkonen et al. fused the enhanced green fluorescent protein (EGFP) with VP39 with the aim to improve the nuclear traffic of BV in mammalian cells, and shown no interference with virus titer (Kukkonen et al., 2003). This finding suggested the possibility of performing insertions into the inner capsid of the BV particle. VP39 is the most abundant protein of the nucleocapsid and consist in a 39 KDa polypeptide with monomers arranged in stacked rings around the nucleoprotein core (Molinari et al., 2011).

In the section 5.1 it were described the possible mechanisms of entry of baculovirus in mammalian cells. Besides the complete mechanism diverge in different cell types, endosome trafficking and Gp64 mediated fusion are always involved. Under these circumstances, it seems unlikely that the antigen displayed on the BV envelope would be able to efficiently reach the cytoplasm and consequently would be preferentially presented by MHC class II pathway. For this reason, antigen displayed on the envelope of baculovirus failed to produce a robust CD8+ T cell response, but was very effective to induce a CD4+ T and B cell responses.

However, antigen capsid display should be able to reach the cytosol and preferentially trigger MHC class I presentation pathway and mount a strong CD8+ T cell response (Molinari et al., 2011).

In this context, Molinari et al. developed a capsid display system and probed it fusing OVA with VP39 (BV-OVA) and showed that OVA could enter into the MHC class I pathway. Consequently, it was observed that inoculation of an animal model with the recombinant baculovirus triggered the activation of naive CD8+ T cells inducing an OVA-specific cytotoxic response. Though the mechanism involved in OVA MHC class I presentation was not elucidated, all these data suggest that capsid display is more convenient over envelope surface display for CTL activation. One of the proposed hypothesis consists of the possibility of the entire baculovirus capsid digestion by proteasome generating MHC class I binding peptides.

In summary, baculovirus are internalized by DCs and induce their maturation and the production of the pro-inflammatory cytokines IL-6 and IL-12 and are able to mount a type I IFN response (Section 3). Finally, Molinari et al. also examined the efficacy of the strong CTL and innate immune response elicited by baculovirus by the capacity of BV-OVA to confer protection against the classical MO5 melanoma tumor model. It was observed that inoculation with the BV-OVA protect against this tumor model.

Other researchers used capsid display as an alternative for mammalian cells transduction. In the work presented by Song et al. the ZnO binding peptide has been fused to the N-terminus of VP39 while retaining the viral infectivity and conferring the ability to bind nanosizedZnO powders (Chen et al., 2011; Song et al., 2010).

In conclusion, capsid display results in a very attractive alternative for cells transduction and for triggering MHC class I presentation of antigenic peptides. In this way, capsid display showed to be strongly effective to mount a robust cellular response against heterologous proteins promoting both IFN secretion and cytotoxicCD8+ T cells activation.

6. Baculovirus and complement

Complement is an important component of the innate immune system and plays an important role in the recognition and elimination of pathogens. Complement can be activated by three separate pathways: the classical, alternative, and lectin pathways (Ricklin et al., 2010). The classical activation pathway begins with the binding of the complement protein C1q to the pathogen surface or to antibody-antigen complex. The alternative complement activation

pathway is initiated by spontaneous hydrolysis of the C3 protein into C3a and C3b and the subsequently attaching of C3b to amine and carbohydrate groups on the target surface. Finally, the lectin pathway is activated by the recognition of specific carbohydrate patterns on the pathogen surface by mannose-binding proteins. Once complement was activated, a cascade of proteolysis events of complement proteins leads to the recruitment of the membrane attack complex (MAC) and the subsequently target membrane perforation (Kaikkonen et al., 2011) (Figure 6.a.b).

On the other hand, complement must be regulated. There are two different types of complement regulators: Surface-bound regulators, and soluble regulators. Surface-bound regulators consists in a group of molecules integratedby factors that accelerate decay of the convertases (complement receptor 1, CR1; decay accelerating factor, DAF), act as a cofactor for the factor I-mediated degradation of C3b and C4b (CR1; membrane cofactor protein, MCP), or prevent the formation of the membrane attack complex (CD59) (Hourcade et al., 2000; Ricklin et al., 2010). Soluble regulators also mediate the first two functions of surface-bound regulators. C4bbinding protein (C4BP), factor H (FH) and FH like protein-1 (FHL-1) are examples of the members of this group (Kaikkonen et al., 2011) (Figure 6.c).

In this context, baculovirus engineering with the aim to confer it resistance to complement inactivation results very attractive to improve the efficiency of baculoviruses for gene delivery.

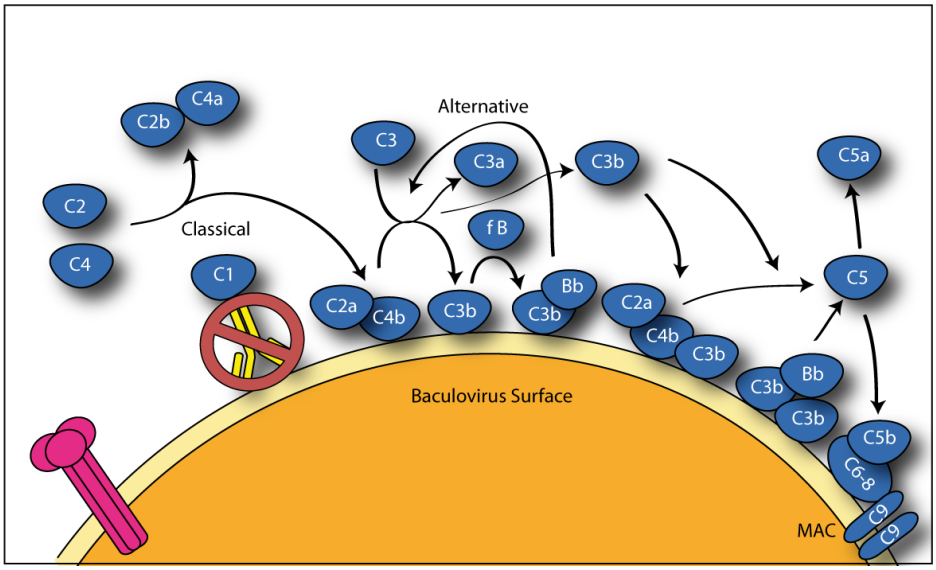

Figure 6. The two major complement activation pathways in baculoviruses: The classical pathway is triggered by the binding of C1 to antigen-bound antibody molecules. The classical pathway utilize C2 and C4 to generate the C3-convertase C4b2a. The alternative pathway is initiated by the spontaneous hydrolysis of C3. Then, the complete complement cascade (from C3 proteolysis to formation of membran attack complex (MAC)) proceeds. Adapted from Kaikkonnen et al. 2011.

6.1. Complement activation by baculoviruses

As particulated antigens, baculoviruses are vulnerable to the action of the complement. This fact was observed in several studies which demonstrate that baculovirus-mediated gene transfer into hepatocytes is strongly reduced in the presence of untreated human serum.

The complement cascade is usually activated to protect the host from foreign elements. The complement activating properties of various gene transfer vectors was demonstrated. The mechanism of complement activation by liposomes and synthetic DNA complexes depends mainly on the formulation, charge and size. Murine retroviruses are effectively lysed by primate complement triggered by the classical pathway, involving direct binding of C1q and C1s to the envelope and/or to antibody-antigen complexes. Comparatively, Hoffmann et al. found baculovirus survival in C1q-depleted human serum indicating baculovirus-mediated activation of the complement cascade through the classical pathway.

Given that there is no evidence of pre-existing anti baculovirus antibodies in human sera, this data suggests that baculoviruses activate the complement cascade by an antibody-independent activation of the classical pathway (Hofmann & Strauss, 1998).

6.2. Strategies for complement inactivation

At present there are different strategies which help to avoid complement attack during baculovirus treatment (Huser et al., 2001; Kaikkonen et al., 2010). As noted previously, the surface of baculovirus particles can easily be engineered. As an example,desired peptides or proteins can be displayed as fusion proteins (Boublik et al., 1995; Makela & Oker-Blom, 2008; Oker-Blom et al., (2003). The most widely used technique for surface engineering makes use of the trimeric major baculoviral envelope glycoprotein GP64 as a fusion partner (Kadlec et al., 2008). In this section, diverse strategies for complement inactivation mediated by baculoviruses will be discussed. In particular, the discussion will be focused in the use of polymers for baculovirus surface coating, pseudotyping of baculoviruses by the expression on VSV-G protein and surface display of eukaryotic complement inhibitors.

6.2.1. Polymer coating

With the aim to protect baculoviral vectors against complement inactivation, using polymers should be appropriated. The coating is based on the electrostatic interaction between the virus particle and the polymer. In the case of baculovirus, the negative charge of its surface allows coating with positively charged polymers such aspolyethylenimine (PEI) (Yang et al., 2009). It was observed that the 25 kDa PEI protected the virions against complement destruction resulting in a 10% to nearly 100% of vector survival in samples treated with human and rat serum, respectively. In addition,Kim et al. observed thatafter intraportal delivery the PEI-treated viruses exhibited improved transduction of liver and spleen compared to non-coated virions(Kim et al., 2009).

Additionally, another polymer, PEG (Mw 5000), has also been reported to increasebaculovirus transduction efficiency in vitro and in mouse brain and lung (Kim et al., 2007;

Kim et al., 2010; Kim et al., 2006). Although serum stability of the PEG-coatedbaculoviruses was not directly studied, these results support the notion that PEG coating can be used to protect baculovirus vectors against the immune system and prolong its survival time in circulation (Jevsevar et al., 2010).

Nevertheless, it is necessary adjust the ratiosof PEI or PEG and virus particles, and the polymer sizeto preserve virus infectivity and minimize cytotoxicity.

6.2.2. Pseudotyping

Pseudotyping consists in a process in which the natural envelope proteins of the virus are replaced with surface proteins from another virus. This strategy has been shown to mitigate the problem of complement attack (Tani et al., 2003). Unlikechemical engineering which is limited and requires extensive optimization to retain virus infectivity, pseudotyping conserves virus infectivity and allows virusevasion ofcomplement-mediated destruction.The most widely used method of pseudotyping of baculoviruses relies on the employ of the VSV-Gprotein. Several researches have shown that VSV-G is capable to improve transduction efficiency of baculovirus in vertebrate cells (Barsoum et al., 1997; Pieroni et al., 2001; Tani et al., 2003; Tani et al., 2001). VSV-G can alsoreplace GP64 and allow productive infection, replication, and propagation of thevirus in Sf9 insect cells (Kitagawa et al., 2005; Mangor et al., 2001). However, pseudotyping is typically performed by co-expressing both the desired molecule and Gp64.

Other reportsalso demonstrated increased gene delivery into mouse after direct intramuscular injection of VSV-Gpseudotypedbaculovirus (Pieroni & La Monica, 2001; Pieroni et al., 2001). Additionall, Tani et al. found that the VSV-Gmodified baculovirusexhibited greater resistance to human, rabbit, guinea pig, rat, hamster and mouse serum inactivation compared to the unmodified control baculovirus (Tani et al., 2003). Furthermore, co-display of a short transmembrane fragment of VSV-G was found to give similar complement protection as intact VSV-G (Kaikkonen et al., 2010). These results suggest that envelope modification of the baculovirus can change its immunogenic properties and protect them for complement inactivation.

6.2.3. Display of complement inhibitors

The last strategy for complement inactivation that will be discussed in this section consists in the baculovirus surface display of eukaryotic complement inhibitors. Several reports showed that genetic modificatedviruses expressing complement regulators presented an improved survival rate, unlike wild type controls. In this context, the most promising results to increase the serum stability of baculovirus vectors by genetic means have been attained by displaying complement regulating proteins fused to Gp64 on the virion surface (Huser et al., 2001; Kaikkonen et al., 2010). The first described research generated a recombinant baculovirus which expressed on its surface the DAF complement regulator. Kaikkonen et al. have recently verified the protective nature of DAF-display and studied the efficacy of other complement regulatory proteins (FHL-1, C4BP and MCP) and their combinations for complement inactivation and consequently baculovirus survival rates. (Kaikkonen et al., 2010). Their resultscon-

cluded that serum stability was dependent on the displayed complement regulatory protein and the source of serum.

In general, the complement regulators DAF and MCP gave the best results. Conversely, simultaneous co-display of soluble complement regulatory proteins did not provide further benefit.Best protection was gained in mouse serum (70%), while the worst protection rate was obtained with rat serum (13%). In the case of human serum, about 30% of the viral particles were still competent to transduce mammalian cells after 1 h preincubation with serum (Kaikkonen et al., 2010).

All these data suggest that engineering of baculoviral vectors for complement inactivation result very convenient not only to reduce the number of necessary inoculations for an efficient transduction, but also to avoid the undesired mortality induced by high doses of non-modified vectors.

7. Other applications

The use of baculoviruses as vectors for the generation of immunity is not the only possible application for these viruses. Their ability to transduce mammalian cells and their capacity to allow the introduction of large amounts of heterologous DNA in their genomes represent remarkable advantages. In addition to the biosafety benefits of baculovirus in comparison with other viral vectors, these features make baculoviruses as adequate vectors for in vivo animal transduction. The absence of preliminary immune cells against baculoviruses makes them a promising tool for human treatment.

In this section, two different novel applications for baculoviruses will be discussed. In first place, the use of baculoviruses for gene therapy and the goals and limitations of this practice will be analyzed. Then, the construction of displaying libraries using baculovirus display system will be exemplified.

7.1. Gene therapy

There are two different categories in which gene therapy vectors can be classified: nonviral and viral vectors. Non-viral vectors consist in polycation conjugated polymers that allow delivery of the DNA. Positively charged liposomes are one example of this type of vectors. Although these vectors are advantageous in biosafety, its application is restricted by the low efficiency in the delivery and expression of transgenes. (Verma & Somia, 1997). On the other hand, viral vectors, such as retroviral, lentiviral, adenoviral, and adeno-associated viral (AAV) vectors, have a higher efficiency in cell entry and transduction by expressing different transgenes. Advantages and disadvantages depend on each particular viral vector. The mechanism used for replication and protein expression, and the biological hazard inherent in its use are some of the features to be analyzed at the moment in which a viral vector is chosen for gene therapy. In comparison with these common viral vectors, baculoviruses possess a number of advantages.

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In first place, baculovirus-mediated transduction does not present any toxic effect against mammalian cells and does not disturb cell growth even at high MOI (Gao et al., 2002; Hofmann et al., 1995). In contrast, cell proliferation may be retarded by transgene products because they could be toxic and even induce apoptosis in some cells (Detrait et al., 2002; Liu & Carstens, 1999). Furthermore, baculoviruses do not replicate in transduced mammalian cells (Kost & Condreay, 2002). These features of baculoviruses are particularly important because other viral vectors are human pathogens, and consequently represent a biological risk.

Another advantage of baculoviruses as gene therapy vectors consists in its large cloning capacity. The baculovirus (AcMNPV) genome is a large circularized DNA molecule with 130 kb of length and a maximum cloning capacity of at least 38 kb. This flexibility results particularly advantageous in contrast to retroviral and AAV vectors whose cloning capacities are limited (Hu, 2008).

In comparison with other viral vectors, baculoviruses are easy to produce. Retroviral, lentiviral, and AAV vectors require transfection of plasmids encoding essential genes into packaging cells for its production. In contrast, baculovirus can be easily propagated by infecting insect cells in suspension culture or monolayer and harvesting the supernatant 3–4 days postinfection. In addition, the construction, propagation, and handling of baculoviruses can be performed in Biosafety Level 1 laboratories without the need for specialized equipment.

Finally, one of the most important advantages is that baculoviruses do not present preexisting immunity in mammalian. One of the problems associated with other viral vectors is that most people are exposed to these viruses and develop specific humoral response. Circulating antibodies can significantly reduce the efficiency of transduction with the viral vector. The use of baculovirus vectors in gene therapy, therefore, may avoid the problem of preexisting immunity (Hu, 2008).

However, baculoviruses have a number of disadvantages as gene therapy vectors. One of these is that baculovirus induce a transient expression in mammalian cells. In vivo, transgene expression typically declines by day 7 and disappears by day 14 (Airenne et al., 2000; Lehtolainen et al., 2002). The duration of in vitro transgene expression using baculoviruses is significantly shorter than expression mediated by retroviral, lentiviral, and AAV vectors.

Baculoviral vectors differ mainly than other viral vectors in the time that the carried genes can persist in the host nucleus. In the case of retroviral, lentiviral and adenoviral vectors, viral DNA can remain into the nucleus either in an integrated or episomal form, for a longer period. In fact, Tjia et al. demonstrated that baculoviral DNA persists in the nuclei of transduced mammalian cells for only 24–48 h (Tjia et al., 1983).

Another disadvantage of using baculovirus as gene therapy vector is the inactivation by complement. As described in previous sections, contact between baculoviruses and serum complement results in rapid inactivation of budded virions. There are need several modifications for reduce the negative effect of complement in baculovirus-mediated transduction. However, the complement system is not a problem only for baculovirus. It is also a potent barrier to in vivo administration of other gene delivery systems such as liposomes, murine retrovirus, and various synthetic DNA complexes (Hu, 2008).

Additionally, baculoviruses as enveloped virus are very fragile. The envelope structure is essential for virus infectivity because of the anchored Gp64, responsible of viral and cellular membrane fusion. (Blissard & Wenz, 1992). For this reason it renders virus vulnerable to mechanical force and results in relatively low virus stability, a common problem also observed for other enveloped viruses such as retrovirus. Ultracentrifugation is often necessary for budded virions purification, but also leads to significant loss of infectivity probably because of the viral envelopes damage. Labile thermal stability, in conjunction with the tendency to be inactivated by serum complement, may further restrict the in vivo application of baculovirus gene delivery vectors.

In vivo gene therapy

Due to their ability to transduce various cell types, baculoviruses have captured increasing interest as vectors for in vivo gene delivery. Baculovirus-mediated gene delivery was tested in different tissues that including rabbit carotid artery, rat liver, rat brain, mouse brain, mouse skeletal muscle, mouse cerebral cortex and testis, and mouse liver (Hu, 2006). However, for baculovirus-mediated in vivo gene therapy in all of these tissues the complement system appears to be a significant barrier.

Baculovirus vectors have also been injected into the rodent brain where complement proteins may be absent because of the blood–brain barrier (Hu, 2008; Lehtolainen et al., 2002). After injection into the brain, baculoviruses specifically transduced the epithelium of the choroids plexus in ventricles and the obtained transduction efficiency was very high.

As discussed in previous sections, baculoviruses can be alternatively pseudotyped by displaying VSVG on the envelope. This modified virus enhanced gene transfer efficiencies into mouse skeletal muscle and the transgene expression in mice. The VSVG-modified baculovirus also exhibited greater resistance to inactivation by the complement system present in animal sera.

Moreover, it has been shown that transduction of different cell lines with a baculovirus expressing shRNAs (short-hairpin RNAs) effectively knocked down expression of the target mRNA and protein (Nicholson et al., 2005). Additionally, baculoviruses have been used to mediate RNA interference (RNAi). The recombinant baculovirus encoding RNAi sequence was efficient in suppressing expression of the target gene by 95% in cultured cells and by 82% in vivo in rat brain. These data suggest that baculoviruses may be also used as delivery vectors for RNA interference therapies (Hu, 2008; Ong et al., (2005).

7.2. Libraries

Surface display libraries represent a very useful methodology for selecting binding proteins out of defined pools of protein variants. Although prokaryotic expression systems such as phage display technology or protein targeting to the cellular surface of Escherichia coli are widely used, they fail allowing the functional display of complex proteins such as eukaryotic glycoproteins which require a high degree of modification and processing. (Ernst 1998)

Eukaryotic expression libraries, in contrast, are a powerful tool for finding new ligands, identification of cellular interaction partners and affinity maturation of antibody and antibody fragments (Grabherr & Ernst, 2010).

As discussed before, the expression of foreign proteins on the surface of insect cells, in occlusion bodies and on the baculovirus surface make baculoviruses an important resource in biotechnology. Moreover, fusion proteins with the baculoviral envelope protein Gp64 as well as different foreign membrane proteins such as the influenza virus hemagglutinin or VSV-G protein have shown to be targeted to the surface of infected insect cells in several researches about baculovirus display. Then, it is possible take advantage of baculovirus display systems with the aim to generate a surface display library for high trhoughput screening.

Ernst et al. expressed a specific antibody epitope in the context of the influenza virus hemagglutinin, randomizing the adjacent amino acid. This procedure results in the construction of a baculovirus surface display library capable to allow the selection of the displayed peptide with optimal antigenicity. Furthermore, baculovirus surface display libraries served to identify MHC class I and II mimotopes (Grabherr & Ernst, 2010; Wang, 2005).

In comparison with bacterial phage display in which cross infection does not occur and every infected cell just propagates one individual phage, in baculovirus surface display cross infection is very probably. The situation may result advantageous or disadvantageous depending the aim of the library. For the assembly of a multisubunit protein, this fact is highly advantageous. However, when the library is performed to screening different proteins, these cross infections have to be considered (Grabherr & Ernst, 2010). Adjusting the multiplicity of infection (moi) usually result convenient for avoid the cross infection problem.

In conclusion, baculovirus insect cell system consists in a highly useful tool for constructing and screening of surface display libraries, specially for the expression of eukaryotic complex proteins (Ernst et al., 1998).

8. Perspectives and conclusions

There are many biotechnological uses for baculoviruses. One of the most widespread is the use of baculoviruses as insecticide agents. Moreover, recombinant baculoviruses have been extensively used as expression vectors in insect cell cultures. A variety of technological improvements have eliminated the tedious procedures to isolate the recombinant viruses turning the baculovirus-based expression system in a safe, easy to use and scale up system (Kost et al., 2005).

In addition, protein expression in larvae or cell culture is not the only application of baculoviruses. In fact, baculoviruses are widely used in the development of strategies for displaying foreign peptides and proteins on the virus surface as well as mammalian cell transduction using different mammalian expression cassettes.

As described in this chapter, baculovirus surface display based on the generation of Gp64 chimeric proteins result in a very efficient technology capable to induce a strong immune

response against specific antigens (Xu et al., 2009). The ability of baculoviruses to activate innate immune system cells guarantees the mount of a robust immune response and the generation of immunological memory. More specifically, AcMNPV induces pro-inflammatory cytokines secretion through a MyD88/TLR9-dependent signaling pathway (Abe et al., 2005; Chimeno Zoth et al., 2012).

It was showed by several authors that baculovirus surface display induced high specific antibody titers against various virus families and parasitic pathogens (Jordan et al., 2009; Meng et al., 2011; Prabakaran et al., 2010; Yoshida et al., 2009). Furthermore, it was demonstrated that many of these titers had neutralizing properties.

On the other hand, it was discussed before that baculoviruses could also transduce mammalian cells (Kost et al., 2005). This feature results very interesting because it allows intracellular expression of heterologous proteins and its subsequent presentation through the MHC class I pathway. In this context, several authors demonstrate that baculoviruses can also induce a specific cellular immune response either by cloning the desired antigen under the control of a suitable promoter, or through the capsid display technique. CTL activation and IFN-γ secretion was detected in all of these researches (Yoshida et al., 2009).

Finally, baculovirus were shown to be useful as gene therapy vectors so as to create libraries of binding proteins.

For all these reasons, we conclude that baculoviruses represent a very useful tool in biotechnology as vaccination vectors. Its adjuvant capacity makes baculoviruses in a promising alternative for the generation of immunological memory.

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