

Baculoviruses as Vectors for Gene Therapy against Human Prostate Cancer

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Current curative strategies for prostate cancer are restricted to the primary tumour, and the effect of treatments to control metastatic disease is not sustained. Therefore, the application of gene therapy to prostate cancer is an attractive alternative. Baculoviruses are highly restricted insect viruses, which can enter, but not replicate in mammalian cells. Baculoviruses can incorporate large amounts of extra genetic material, and will express transgenes in mammalian cells when under the control of a mammalian or strong viral promoter. Successful gene delivery has been achieved both *in vitro* and *in vivo* and into both dividing and nondividing cells, which is important since prostate cancers divide relatively slowly. In addition, the envelope protein gp64 is sufficiently mutable to allow targeted transduction of particular cell types. In this review, the advantages of using baculoviruses for prostate cancer gene therapy are explored, and the mechanisms of viral entry and transgene expression are described.

WHY GENE THERAPY FOR PROSTATE CANCER?

The case for new therapies in prostate cancer is particularly strong, given the frequency of the disease in a western population that is increasingly elderly (Dijkman and Debruyne [1]). Conventional therapies, such as surgery and radiotherapy can be effective if early stage disease is detected and targeted for therapy, a strategy employed on a wide scale in the USA (Bubolz et al [2]; Pirskhalaishvili et al [3]). Even in the later stages of the disease, intervention to block the necessary supply of androgens is effective in the short term, although resistant tumours develop relatively rapidly, within 1–2 years (BJ Feldman and B Feldman [4]). Cytotoxic chemical therapies are rarely effective. Thus a therapeutic strategy, in which the genetic nature of the prostate tumour is turned against the cancer is very attractive. The mantra of successful gene therapy for prostate cancer has been repeated many times since the earliest reports of successful gene transfer were published (reviewed in Roth and Grammer [5]). In this respect, the prostate is both a good and bad target for specific therapy. On the credit side, the prostate itself, like most secretory organs, displays radically different patterns of gene expression from most other organs, and many of these “tissue-specific” products are retained in the tumours. Tissue specificity can be turned against the tumour, both at the cell surface level (attachment of therapeutic agents) and at the transcriptional level to direct expression of therapeutic genes. The range of candidates has been covered in an earlier review (Maitland [6]). In

addition, there are a number of tumour associated antigens, whose expression is upregulated in prostate tumours. It is in this respect that prostate tumours remain a poor candidate for strictly gene-based therapy. Firstly, the range of tumour antigens is small, but increasing in view of recent stimulation of research in this area (Liu [7]; Luo et al [8]; Ornstein et al [9]). Secondly, the natural history of the disease is relatively poorly understood, in comparison with breast cancer, a disease of similar incidence and mortality. Prostate tumours display genetic and antigenic heterogeneity (Macintosh et al [10]), and the ability to accurately predict the course of the disease (and therefore to identify patients for gene therapy regimes) remains rather primitive relative to breast cancers (Van't Veer and De Jong [11]), despite the application of gene array technology (Dhanasekaran et al [12]). Finally, prostate tumours display an ability to shift phenotype, probably by selectively activating or inactivating gene expression, for example, in the development of androgen-independent disease (Karan et al [13]; Tso et al [14]) and the inactivation at the transcriptional level of genes encoding carcinogen-inactivating enzymes (Lee et al [15]). This would seem to be the ideal mechanism to inactivate the expression of exogenous therapeutic genes.

It is also likely, given the genetic and clinical heterogeneity of prostate cancers, that a range or even a combination of gene therapy strategies with conventional treatments will be needed to achieve a substantial effect. This is particularly true with viral vectors, where an existing immune memory against human viruses (eg, adenoviruses)

could preclude their use in certain cases. Indeed, to optimise the therapeutic effects, simultaneous infection with a “cocktail” of therapeutic viruses (to overcome the initial tumour heterogeneity) or sequential inoculation with different virus types (to escape either preexisting or therapy-induced antiviral immunity) may be necessary. However, neither strategy will be clinically feasible unless all unacceptable risks of side effects can be eliminated. Lastly, and perhaps most importantly, the method of inoculation and dosage has to be optimised. For example, should the primary target for gene therapy be organ confined disease, where the conventional therapeutic strategies are moderately effective, or against metastatic disease?

So can baculoviruses provide an alternative means of delivering therapeutic genes into organ confined and metastatic prostate cancer?

WHAT ARE BACULOVIRUSES?

It is perhaps surprising that there are more than 500 different types of baculoviruses (Martignoni and Iwai [16]). They are widespread pathogens of insects and invertebrates, ranging from shrimps to moths and butterflies. However, the most studied types are those which cause disease in common insect pests. Research was driven initially by the intention to use them as a biological insecticide (Ignoffo [17, 18]; Martignoni [19]). The individual baculoviral strains have a limited host range, which is usually restricted to one species [20, 21]. Pioneering studies at Texas A&M University, where the effects of the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) on the fall army worm were first studied in molecular detail. MNPV's are a subgroup of baculoviruses that produce large polyhedral occlusion bodies as part of the viral life cycle [21]. Polyhedrin is a 246-amino acid (29 kd) protein, which forms a hard polyhedral protein matrix of between 0.15 to 15 μm in which Bv nucleocapsids, surrounded by a single unit membrane, are embedded. This provides the ultimate protection in the wild. These polyhedra, are however susceptible to the alkaline environment in the midgut of the host insect, releasing infectious virus, into susceptible host cells (reviewed in Harrap and Longworth [22]). Polyhedra can be visualised as refractive crystals in the nucleus of infected insect cells under light microscopy. The polyhedrin embedded occluded virus (OV) is formed very late in the baculoviral life cycle, in contrast to the budded virus (BuV) formed earlier in the infectious life cycle [20]. Both OV and BuV are rod shaped with a supercoiled dsDNA genome of 80–200 kilobasepairs (approximately 134 kbp in AcMNPV), which is condensed into a nucleoprotein core with proteins p39 and p87 [20, 23]. As a result of budding out through the cell plasma membrane, BuV acquire a loosely fitting viral envelope which has peplomers protruding around one end [20]. These peplomers are comprised of the major envelope glycoprotein gp64, which

is responsible for cell to cell spread and secondary viral infection. In contrast, AcMNPVOV consists of multiple nucleocapsids surrounded by a *de novo* synthesised viral envelope which does not display gp64 peplomers [20]. This led to the development of the virus as a potential gene cloning vector, exploiting the readily available cell lines from the midgut of the moth, and the ability to recombine into the viral genome by cotransfection of intact viral DNA with a segment of the viral genome containing an exogenous gene, normally under the control of one of the very strong late promoters such as that for the polyhedrin gene. A diagrammatic version of the baculoviral infectious process is shown in Figure 1.

Many of the 151 recognised open reading frames (Genbank number NC_001623) encode proteins of as yet unknown function. However, like many larger DNA viruses, the Bv genome does contain a large number of proteins whose main function is to subvert both host cell and host organism defences. In many cases these are homologues of host cell proteins such as ubiquitin, PCNA, and viral DNA polymerase/RNA polymerase components (reviewed in Ayres et al [24]; Kool and Vlask [25]). There are also homologues of growth factors, intracellular signalling molecules, and perhaps most notably, a unique apoptosis suppressor p35, which is functional in both insect and human cells (Hsu et al [26]; Resnicoff et al [27]; Robertson et al [28]). The genomic location of some of these open reading frames is indicated in Figure 2. Clearly, the expression of some or all of these Bv functions, which are sufficiently closely related to human homologues, would be undesirable in a human gene therapy vector, particularly one designed to kill cancer cells.

GENERATION OF RECOMBINANT BACULOVIRUSES

When the first baculoviral protein expression vectors were generated, the selection system was based on the loss of the polyhedrin gene, by recombination from a transfer plasmid, which disrupted the PH coding sequence. This resulted in infected cells lacking the characteristic occlusion bodies, composed of enveloped nucleocapsids embedded in a polyhedrin matrix. In practice it took some time to become experienced in identifying nonoccluded viral plaques, and the recombinants were frequently contaminated by nonrecombinant wild-type virus, which in a large scale culture could outgrow the recombinants, particularly when the recombinant protein expressed had cytopathic properties.

More recent developments have used cotransfection of multiply-deleted viral genomes (eg, the commercially available BacVector 1000 series) together with the transfer vector (generated in a bacterial plasmid) which contains recombination sequences, normally from the nonessential polyhedrin gene. Capacity for recombinant inserts is at least 40 kbp in this rod form virus, although in practice this could be difficult to maintain in the transfer vector.

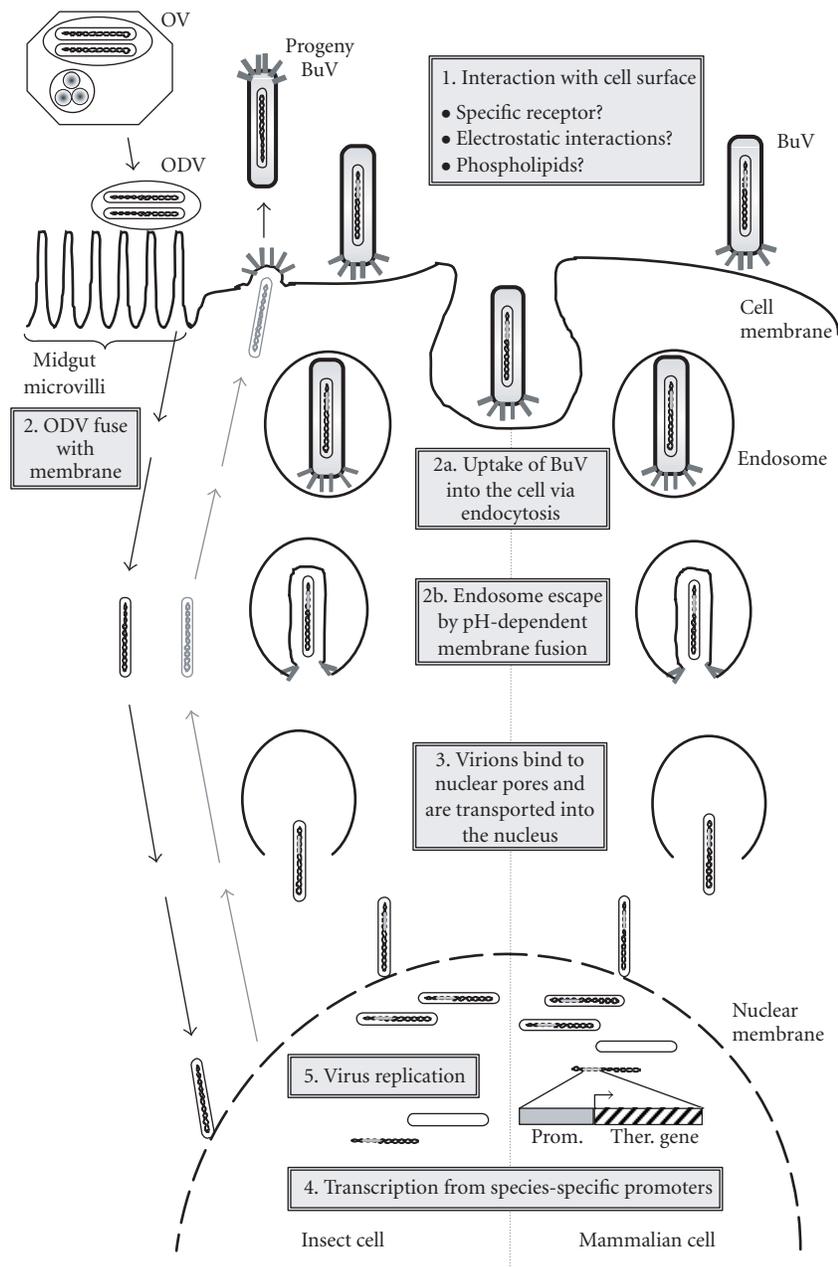


FIGURE 1. Baculovirus infection of insect and mammalian cells. (1) Budded virus (BuV) particles interact with the mammalian cell surface via the surface gp64 protein in both insect and mammalian cells (described in the text). The occluded virus (OV) polyhedrin matrix is digested in the insect midgut lumen releasing occlusion derived virions (ODV), which consist of multiple nucleocapsids surrounded by a membrane. (2) ODV particles fuse with the microvilli membranes of midgut epithelial cells, releasing nucleocapsids into the cytoplasm. (2a) BuV particles are taken up into endosomes from which they escape by endosome acidification and membrane fusion (2b). (3) Virus particles are transported to the nucleus where they bind the nuclear pores and whole virions containing the genome are actively transported through the pores into the nucleus. Viral DNA is released from the nucleocapsid. (4) In insect cells, the baculoviral genes are transcribed whereas in mammalian cells, only genes under the control of a mammalian promoter are transcribed. (5) In insect cells, viral DNA is replicated and packaged into nucleocapsids. During the late phase of infection, nucleocapsids exit the nucleus and bud from the cell membrane to form BuV. During the very late stage of infection, nucleocapsids remain in the nucleus where they are enveloped and embedded in a polyhedrin matrix to produce OV. In mammalian cells, virus replication does not take place and no progeny virus is produced.

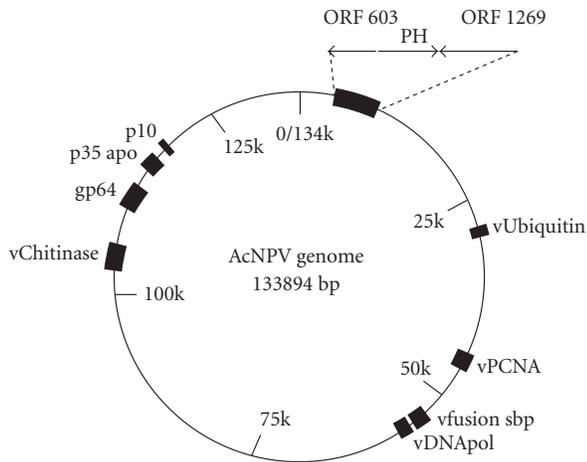


FIGURE 2. Genomic map of Bv (AcNPV) genome. Some significant open reading frames are indicated. Detail is the polyhedrin (PH) cloning sites for recombinants.

Recombinant viral yields from the BacVector (Novagen Inc, Madison, Wis, USA) system are high and wild-type viral yields are virtually eliminated, by use of the “triple cut” system, which uses Bv DNA in which the PH gene has been disrupted by insertion of the β gal gene, leading to blue nonrecombinant Bv plaques in the presence of Xgal indicator. The second and more important step is the introduction of a site for the rare cutting restriction endonuclease Bsu36I within the essential (for replication) Bv gene ORF1629. Further Bsu36I sites are located in the β gal and in the other Bv ORF flanking PH ORF: ORF603. The complementing ORF1629 protein is supplied by the recombination transfer vector, and the triple cut Bv DNA cannot produce viable virus without addition of the ORF1629 (see Figure 2). Thus the production of wild-type virus and isolation of recombinants is not a serious problem in this system.

The recent development of the BAC to BAC vector system (Invitrogen), in which the entire Bv genome has been cloned into a bacterial artificial chromosome, enables the recombination to be carried out with higher efficiency and control in bacteria. This results in higher yields and more rapid recombinant generation (Luckow et al [29]) as with similar adenoviral systems (eg, AdEasy from Q-Biogene, Illkirch, France).

ADVANTAGES OF BACULOVIRUSES AS A GENE THERAPY VECTOR

Firstly, Bv is a rod form, and limitations to the amount of extra genetic information inserted into the recombinants such as those imposed by the defined adenovirus capsid are not appropriate. It has been estimated that standard Bv, without further deletions, can accommodate more than 38 kilobases of extra genetic information (Cheshenko et al [30]).

Baculovirus does not express its own genes or replicate in human cells. As long ago as 1983, Tjia et al showed that there was an absence of Bv gene transcription in infected HeLa cells (Tjia et al [31]). In another study, no detectable expression from the polyhedrin promoter was demonstrated in Huh7 cells (Hofmann et al [32]). In a recent study in our laboratory (A. Jones et al, manuscript in preparation), using the most sensitive methods currently available (ie, RT-PCR) the expression of a number of the potentially pathogenic Bv genes was assessed after successful infection of human and insect cells. Whereas the appropriate PCR products were detected in the infected insect cells, they were absent in Bv infected human cells, which did however express a marker gene under human promoter control. Even extending PCR cycles up to 40 did not produce a Bv gene product in the human cells. These results are not entirely unexpected, particularly as the late and very late Bv genes are transcribed by an alpha-amanitin resistant RNA polymerase (BvAARP) (Huh and Weaver [33]), which is at least partly encoded within the Bv genome (Passarelli et al [34]). In addition, the absolute requirement for BvAARP mediated expression of the late structural proteins in the assembly and production of progeny virus provides another “fire wall” to prevent the generation of replicating virus in human cells and tissues.

Mammalian viruses, and in particular those currently employed in gene therapy trials are critically dependent on host cell functions to complete their life cycle. The viral proteins can interact with host cell proteins and nucleic acids, often perturbing the host cell cycle and viability. As the Bv genes, which perform these functions in insect cells are not expressed in mammalian cells, Bv infection is unlikely to affect the target cells. This is particularly important for gene therapy protocols involving correction of a cell gene defect rather than killing of the target cells, as proposed for cancer gene therapy. Encouragingly, Bv infection of primary pancreatic islet β -cells did not affect normal cellular calcium responses to glucose, which has important implications for gene therapy of diabetes (Ma et al [35]).

Baculovirus does not recombine with preexisting genetic material: a potential drawback of the mammalian viruses, where endogenous virus is widespread in the human population, and the potential for interspecific recombination could produce new replication-competent viruses with a new pathogenicity, or cell tropism. Baculovirus also cannot “help” replication of endogenous viruses in humans, such as adenovirus and adeno-associated viruses.

The BV gp64 envelope protein is sufficiently mutable to allow the rapid insertion of new and more specific attachment sequences, much more readily than those described recently for AdV fibre protein (Krasnykh et al [36]), without perturbing its function as the principal attachment protein. This technique has already been exploited to produce antigen display in the membrane of Bv (Ernst et al [37]). However, to be bifunctional in human

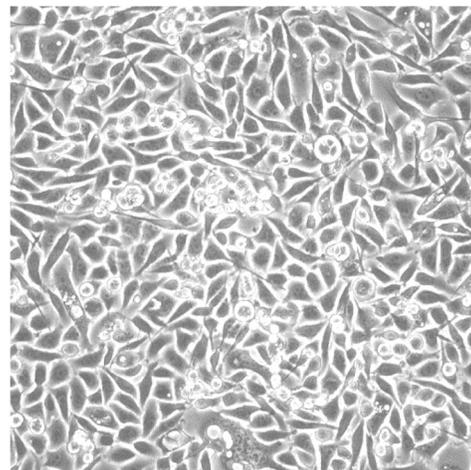
and insect cells, a mosaic envelope with wild-type and recombinant gp64 is required, which cuts down on infection efficiency in both cell types. However, effective retargeting has already been demonstrated.

In contrast to many of the other therapeutic viruses, Bv can be grown in serum-free culture media and in large quantities. In our hands, the viruses are completely stable and their production can be readily scaled up to industrial levels (currently 2–3 litres can be cultured without loss of viral viability or selection of mutant Bv). The industrial scale culture of Bv is also possible in serum-free culture conditions, which removes the potential hazard of serum contamination of the therapeutic agent with viral and prion agents from the donating animal. There is however a tendency in high level production for the virus particles (like many recombinant enveloped viruses) to aggregate, which could limit the dosages applied clinically.

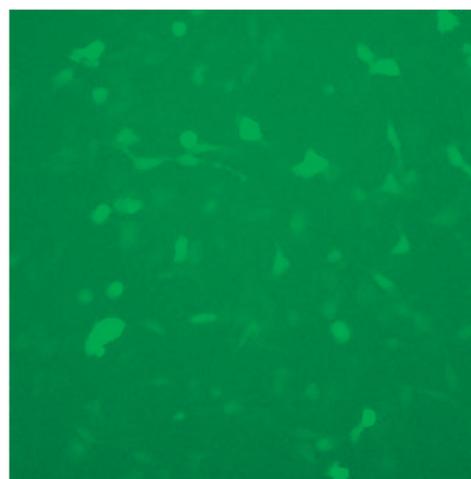
Lastly, for in vivo use, there is no preexisting immune response against Bv in humans (a common problem associated with all human viral vectors, including adenoviruses). However a complement response has been demonstrated, although a number of investigations have produced inhibition strategies in animal models which prevent rapid elimination of intravenous injected Bv. This is dealt with later in more detail.

IN VITRO TRANSDUCTION OF MAMMALIAN CELL LINES WITH BACULOVIRAL VECTORS

Expression of a transgene under the control of a mammalian promoter in human cells following transduction with a recombinant baculovirus was first shown by Hofmann et al in 1995 (Hofmann et al [32]) and Boyce and Bucher in 1996 (Boyce and Bucher [38]). These studies and others have reported that hepatic cells, such as the human liver tumour cell lines HepG2 and Huh7, are generally the most susceptible mammalian cell type to infection by baculoviruses in vitro (Sandig et al [39]). Following infection with a recombinant baculovirus at an MOI of 100 pfu per cell, approximately 25–50% of HepG2 cells were shown to be positive for *LacZ* transgene expression whereas transduction of COS-7 (monkey kidney), A549 (human lung), and 293 (human kidney) cells was 10 to 100-fold less efficient (Boyce and Bucher [38]). However, there remains some controversy about the best target for Bv transduction in comparative tests of Cos-1 (SV40-transformed green monkey kidney epithelial cells), T47-D (mammary ductal carcinoma), A549, CHO (Chinese hamster ovary), HeLa (cervical carcinoma), HaCaT (keratinocyte), NIH 3T3 (fibroblasts), and COS-7 (Hofmann et al [32]; Sarkis et al [40]). The human osteogenic sarcoma cell line SAOS-2 cells expressed a baculoviral-mediated *LacZ* transgene at levels almost 20-times greater than in HepG2 cells (Song and Boyce [41]). Our own studies show that both 293 (human embryonic kidney) and PC3 (prostate cancer) cells can be transduced with approximately equal efficiency



(a)



(b)

FIGURE 3. Infection of PC3 prostate cancer cells with a humanised Bv expressing EGFP from a hybrid CAG promoter. Phase image of infected cells (a) after 24 hours indicates confluency of cultures while fluorescent image (b) confirms high infectivity (at > 100 particles/cell) of the tumour cells. After a further 24–48 hours more than 90% of the tumour cells express EGFP.

(25–50%) when infected with a CAG-EGFP baculovirus at an MOI of >100 (A. Jones, unpublished results, 2000). A typical result is shown in Figure 3.

INFECTION OF PRIMARY CELL CULTURES WITH BACULOVIRUSES

Keratinocytes and bone marrow fibroblasts are among the primary human cell types to be successfully transduced with a baculoviral vector (Condreay et al [42]). Approximately 70% of primary hepatocytes were shown to express β -galactosidase following transduction with

an RSV-*LacZ* baculovirus at an MOI of 430 (Boyce and Bucher [38]). A much lower MOI of 25 was reportedly sufficient to transduce approximately 30% of undifferentiated primary human neural progenitor cells and approximately 55% of differentiated primary human neural cells with a CMV-EGFP baculovirus (Sarkis et al [40]).

BACULOVIRUS INFECTION OF EPITHELIAL CELLS IS INDEPENDENT OF THE CELL CYCLE

One unusual feature of prostate cancers is that the tumour cells multiply at an apparently slower rate than other cancers. Recombinant baculoviruses are however able to transduce both nondividing and actively dividing cells. For example, a G1/S arrested epithelial pig kidney cell line was infected as efficiently as dividing cells of the same type (van Loo et al [43]).

EFFICIENCY OF BACULOVIRAL-MEDIATED GENE TRANSFER IN VITRO

At a MOI of 10, comparing both levels of transgene expression and percentage of cells expressing the transgene, baculoviruses are comparable to lipofectamine and calcium phosphate precipitation as gene delivery vehicles for Huh7 cells (Hofmann et al [32]). At an MOI of 100, baculoviruses are much more efficient than both nonviral methods. A comparison with adenoviruses shows that at an MOI of both 10 and 100, transduction by a baculovirus vector results in higher β -galactosidase activity, than when the same expression cassette was transduced by an adenovirus. However, the overall percentage of cells expressing the *LacZ* gene following transduction with the adenoviral vector was approximately twice that obtained when transduced with the baculovirus vector (Hofmann et al [32]). The number of primary pancreatic islet β -cells expressing GFP following transduction with a CMV-EGFP baculovirus were comparable to that reported for lentiviral and adenoviral vectors (Ma et al [35]).

IN VIVO TRANSDUCTION OF MAMMALIAN CELLS WITH A BACULOVIRAL VECTOR

Initial attempts to use baculovirus vectors as gene delivery vehicles in vivo failed because the virus particles were inactivated by the complement immune response (Sandig et al [39]). However, in vivo gene delivery protocols that bypass the complement system have shown encouraging results. A CMV-*LacZ* baculovirus was administered to rabbit carotid arteries in vivo via a silastic collar fitted directly onto the artery (to sequester the Bv from exposure to blood). This resulted in expression of β -galactosidase in a comparable number of cells as achieved by administration with a CMV-*LacZ* adenovirus (Airenne et al [44]). Direct injection of a CMV-EGFP baculovirus into rat and mouse brain striatum resulted in

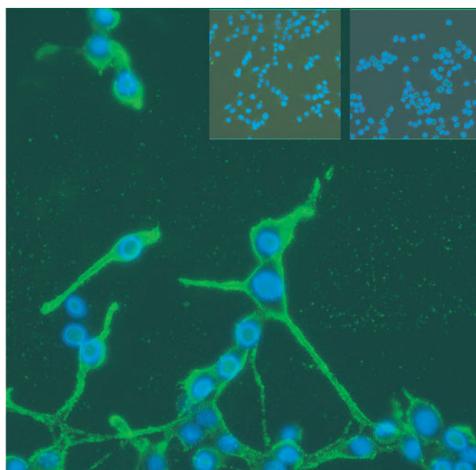
transduction of neural cells in vivo, within a millimetre of the injection site (Sarkis et al [40]). The brain may represent a privileged site for Bv infection as the complement response did not abrogate transduction. In vivo gene transfer by direct injection into mouse skeletal muscle was achieved in the presence of complement with a baculoviral vector pseudotyped with VSVG, which has been shown to protect viral vectors from complement (Pieroni et al [45]).

MECHANISM OF MAMMALIAN CELL TRANSDUCTION BY BACULOVIRAL VECTORS

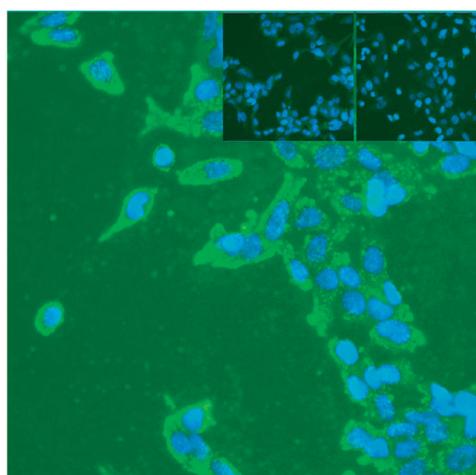
For infection of both insect and mammalian cells, baculoviruses are required to interact with the cell surface via its surface gp64 protein (Hefferon et al [46]; Tani et al [47]). It has been suggested that the cell binding and uptake mechanism may be via a specific receptor since permissiveness varies widely between different cell types. For example, the rat hepatoma cell line H35, may be nonpermissive (van Loo et al [43]). This could reflect differences in receptor expression levels but to date, no such receptor has been identified. The asialoglycoprotein receptor was initially suggested as a candidate but it was subsequently shown that a cell line expressing the cloned receptor did not show significant viral uptake (Hofmann et al [32]) and van Loo et al demonstrated efficient transduction of an epithelial pig kidney cell line (Pk1) that did not express this receptor (van Loo et al [43]). Electrostatic interactions between the viral and cellular membranes have been shown to be critical for baculovirus transduction, probably via negatively charged cell surface epitopes such as heparan sulphate (Duisit et al [48]).

At high MOI, Bv particles can be seen to completely coat mammalian cells. For example, in Figure 4a, a 3T3 cell has been infected at 4°C with an excess of Bv, and the extracellular gp64 stained with a monoclonal antibody. The fluorescent ring follows the murine cell membrane, indicating high efficiency attachment. A similar result is obtained with prostate epithelial cells (PNT1A), which contract after 4°C exposure.

Evidence that baculoviruses are taken up by endocytosis has been provided by (i) electron microscopy of CHO cells (Condreay et al [42]), and (ii) the observed reduction of virus transduction in the presence of chloroquine (Boyce and Bucher [38]; Hofmann et al [32]). As with other viruses (eg, influenza and adenoviruses), endosome acidification is required for release of the baculoviruses from the endocytotic pathway into the cytoplasm and subsequent transport, probably involving actin filaments, to the nucleus (Blissard and Wenz [49]; Boyce and Bucher [38]; van Loo et al [43]). Unlike many other viruses, both nucleocapsids and viral genomes can be detected inside the nucleus of infected cells (van Loo et al [43]). Electron microscopy images indicate that the baculoviral nucleocapsids dock onto nuclear pores in infected cells, before being transported through into the nucleus.



(a)



(b)

FIGURE 4. Specific attachment of recombinant Bv to mammalian cells. 3T3 cells (a) and prostate epithelial cells PNT1A (b) were infected with > 100 particles/cell with a humanised Bv for 1 hour at 4°C to permit attachment but not penetration. The cells were then fixed and stained with an antibody against the Bv gp64 protein. Controls are shown in the inserts (no virus infection and no primary antibody). Note the even coating of the 3T3 cells and the patchy but strong staining of the PNT1A cells.

This is observed in mitotic and nonmitotic cells. Therefore, it is likely that the capsids are transported through nuclear pores rather than taken up during mitosis (van Loo et al [43]).

The lack of transgene expression in cell lines less permissive to baculoviral transduction is more likely to be due to a block at the level of viral uncoating or transcription rather than virus entry, since viral DNA can be detected at approximately equal amounts in highly permissive (HepG2) and less permissive (Sk-Hep-1) cells

24 hours postinfection (Boyce and Bucher [38]). Also, RNA transcribed from a mammalian promoter-gene cassette can only be detected in transduced HepG2 cells and not in Sk-Hep-1 cells (Boyce and Bucher [38]). Barsoum et al (Barsoum et al [50]) demonstrated that in the highly permissive HepG2 cells, baculovirus DNA was present in the nucleus 24 hours after infection and that the DNA was packaged into chromatin as determined by digestion with staphylococcal nuclease. Conversely, in HeLa cells, DNA was not detected in the nucleus, and EM analysis supports the theory that much of the virus infected into HeLa cells is trapped inside intracellular vesicles. Effective escape from endosomes is thus a critical step in baculoviral transduction of mammalian cells.

BACULOVIRUS-MEDIATED TRANSGENE EXPRESSION IN MAMMALIAN CELLS

The onset of transgene expression has been shown as early as 6 hours posttransduction with a recombinant baculovirus and can reach peak expression levels after 12 to 24 hours (Boyce and Bucher [38]). Expression has consistently been shown to persist at approximately peak levels for at least a week both in vitro (Hofmann et al [32]; Ma et al [35]) and in vivo (Airenne et al [44]; Haeseleer et al [51]; Sarkis et al [40]). In the absence of complement, transgene expression has been detected for up to 178 days in vivo (Pieroni et al [45]).

It is possible to generate stable cell lines from cultured cells by selection of baculovirus transduced cells with G418 when a neomycin resistance cassette is included in the baculoviral transfer vector (Condreay et al [42]). Following infection of CHO cells at an MOI of 1 pfu per cell, approximately 1–2% of cells that had been transduced by the virus went on to form G418 resistant colonies (Condreay et al [42]). This is relatively inefficient and is even less likely to occur in vivo since there will be no selective pressure for integration to become a selective advantage. Further analysis revealed that fragments of the baculoviral genome ranging in size from 5 to 18 kb had integrated into the CHO cell genome (Merrihew et al [52]). The breakpoints in the virus genome were randomly located and with little homology between baculovirus and CHO cell DNA at recombination sites, suggests a mechanism of illegitimate recombination (Merrihew et al [52]). If stable integration is required for a particular gene therapy protocol, site specific integration should be safer since the risk of insertional inactivation is much lower. This has been approached by creation of a hybrid baculovirus-AAV (adeno-associated virus) vector resulting in Chromosome 19-specific integration in mammalian cells (Palombo et al [53]).

HDAC inhibitors such as trichostatin A (TCA) and butyrate have been shown to increase expression levels of a baculovirus encoded mammalian transgene in a wide variety of cultured cells including HeLa, Huh7, CHO, COS7, and 293 in addition to primary cultures of human keratinocytes, bone marrow fibroblasts, and neural

cells (Airenne et al [44]; Condreay et al [42]; Sarkis et al [40]). Although probably not feasible to use for in vivo gene therapy protocols, this could be applied to *ex vivo* gene transfer systems.

ENGINEERING PROSTATE SPECIFICITY FOR THERAPEUTIC GENE EXPRESSION IN Bv VECTORS

Although most studies of baculoviral-mediated gene transfer to date have employed the use of strong, virus derived promoters such as the CMV immediate early promoter or RSV, expression from a tissue specific promoter has also been demonstrated. The α -fetoprotein (AFP) promoter was successfully used to direct expression of a transgene specifically in AFP-expressing hepatic cells in vitro (Park et al [54]). This has important implications for gene therapy in vivo, demonstrating that transcriptional targeting is a possibility for baculoviral gene therapy.

There are now numerous reports of successful use of prostate-specific gene promoters incorporated into other gene therapy vectors that have been tested in vivo, including a PSA promoter-based lentivirus (Yu et al [55]), a PSA promoter-based adenovirus (Li et al [56]), an osteocalcin promoter-based adenovirus (Matsubara et al [57]), a probasin promoter-based adenovirus (Lowe et al [58]; Martiniello-Wilks et al [59]) (Xie et al [60]), and a human kallikrein 2 promoter-based adenovirus (Xie et al [61]).

In addition, a number of "prostate-specific" promoters have been tested for specificity after transfection into cultured cells. Amongst the most promising are PSMA (O'Keefe et al [62]), DD3 (Verhaegh et al [63]), PART-1 (Lin et al [64]), prostate transglutaminase (Dubbink et al [65]), prostatic acid phosphatase (Zelivianski et al [66]), and NKX3.1 (Prescott et al [67]; Xu et al [68]). There are no good reasons to suspect that their enhanced activity in prostate cells will be compromised in any way by insertion into Bv vectors.

Also, prostate tumour cells in vitro are highly susceptible to Bv infection, as demonstrated not only by the attachment results shown in Figure 4, but also confirmed by the result shown in Figure 3, where the strong hybrid CAG promoter has been used to drive EGFP expression in PC3 prostatic carcinoma cells, after transduction by a recombinant Bv.

The additional genetic capacity of the recombinant Bv should also allow coexpression of transcriptional modulatory genes. The best example of this might be androgen receptor, whose activity is frequently depressed in androgen insensitive tumours. In addition, many of the prostate-specific gene promoters are positively regulated by male sex hormones, but could be inactive (mutated) or transcriptionally inactivated in hormone insensitive metastatic tumours. Therefore, coexpression of an intact or partial androgen receptor to stimulate expression from the androgen responsive promoters (Suzuki et al [69]) should be possible.

BACULOVIRUS VECTOR MODIFICATIONS FOR GENE THERAPY

Attachment targeting of baculoviruses to specific receptors on the surface of mammalian cells can be achieved by inserting attachment modifying sequences into the gp64 membrane protein, for example, insertion of a functional single chain antibody fragment specific for carcinoembryonic antigen (CEA) or two copies of a synthetic IgG binding domain of protein A (Ojala et al [70]). According to one study, addition of a modified gp64 coding region into the baculoviral genome resulted in an expression ratio of approximately 1 : 1 between wild-type and modified gp64 protein (Hüser et al [71]).

ELISA analysis indicated that the gp64 fusion proteins were capable of binding to their specific ligands and that the inserted coding region was located in an accessible part of the gp64 protein loop (Ojala et al [70]). The CEA fusion was incubated with PC-3 cells (previously shown to express CEA) and the IgG binding domain expressing baculovirus was bound to cells by preincubation of BHK cells with an anti- $\alpha 5\beta 1$ integrin polyclonal antibody and subsequent addition of the virus. Both methods result in detection of greater numbers of baculovirus particles bound to the cell surface as detected using an antibody against gp64. However, this increase in binding did not appear to enhance transduction of the cells as assessed by EGFP transgene expression, by both fluorescence microscopy and FACS (Ojala et al [70]). Thus the modification of the gp64 protein could be compromising normal functions such as endosomal escape. Thus if the rate-determining step of transduction is endosomal escape, increasing the number of bound viral particles would only have a limited effect on transduction efficiency. However, this result is still very important for gene therapy, since viral targeting could be utilised to reduce the number of viral particles required for a gene therapy regime in vivo; if the viruses can be engineered to bind more efficiently to a specific cell type (eg, prostate cancer cells) than to other cell types.

This is particularly relevant for liver, to where most intravenously injected virus will locate, as judged by studies with adenoviruses (Mizuguchi and Hayakawa [72]). By achieving retargeting, and by eliminating the liver cell tropism, the number of particles required to be administered systemically would be reduced to the benefit of the patient (and also co-incidentally increasing cost effectiveness). To date, there are no publications that assess whether a low MOI of targeted virus can achieve the same level of transduction as a nontargeted virus at a high MOI.

If the rate-limiting step of transduction is endosome escape, then modification of the baculoviral vector to mediate endosome lysis should further increase transduction efficiency. To overcome this block, the vesicular stomatitis virus G protein (VSVG) has been used. For example, efficiency of transduction and expression of the *LacZ*

transgene from a recombinant baculovirus in mammalian cells is increased by up to 200 fold by incorporation of VSVG on the virus surface (Barsoum et al [50]; Pieroni et al [45]). VSVG mediates escape from endosomes by membrane fusion (Eidelman et al [73]) but may also play a role in binding and entry of the baculovirus into mammalian cells (Tani et al [47]). Cell lines that are less permissive for baculovirus transduction, such as HeLa, A549, CHO, and NIH 3T3 cells, show the greatest difference in transgene expression between the nonpseudotyped and VSVG-expressing viruses, but even susceptible HepG2 cells show a 10-fold-increase in transgene expression (Barsoum et al [50]). Importantly, baculovirus-mediated transgene expression in HeLa cells, which are not very susceptible to baculovirus transduction, can be seen at an MOI of 1 with the VSVG-pseudotyped virus compared to an MOI of 100 with the nonpseudotyped virus (Barsoum et al [50]).

In vivo, VSVG pseudotyping may confer protection from the complement response since the transduction efficiency of mouse skeletal muscle cells was 5–10 times greater than transduction with nonpseudotyped virus. This improvement cannot be fully attributed to the enhanced transport into the cells, as the VSVG-pseudotyped virus only transduced twice as efficiently as a nonpseudotyped virus in complement deficient animals (Pieroni et al [45]). Furthermore, VSVG-pseudotyped retroviruses have been shown to be more resistant to complement than nonpseudotyped retroviruses (Ory et al [74]).

However, VSVG pseudotyping could compromise targeting strategies, since VSVG has also previously been shown to complement several functions of gp64 in a gp64 null baculovirus (Mangor et al [75]). In addition to mediating endosome escape, VSVG-enhances entry into mammalian cells, since competition with an anti-gp64 antibody did not completely inhibit cell transduction with a VSVG-pseudotyped virus. A non-VSVG-pseudotyped virus was inhibited to the normal extent (Tani et al [47]).

IMMUNE RESPONSES TO BACULOVIRUS VECTORS IN VIVO

The earliest attempts to achieve baculoviral-mediated gene transfer in vivo failed because of vector inactivation by serum components, most probably those involved in the complement response (Sandig et al [39]). The complement response is however also activated by other agents used for gene delivery such as liposomes (Szebeni [76]) and synthetic DNA complexes (Plank et al [77]). Although there is no preexisting humoral or cell mediated memory against Bv in humans and other mammals, repeated administration does give rise to neutralising antibodies. However, transgene expression has been shown to persist in the absence of complement (Pieroni et al [45]).

COMPLEMENT MANIPULATION TO POTENTIATE Bv TRANSDUCTION IN VIVO

Activation of the complement response following baculoviral infection has been investigated in more detail and it appears that complement is being activated via the classical pathway since serum depletion of C1q, unique to the classical pathway, allowed complete survival of the baculoviral vectors in vitro (Hofmann and Strauss [78]). Hofmann and Strauss (Hofmann and Strauss [78]) explored various strategies for complement inhibition to promote baculoviral vector survival in vivo. Incubation of human serum with an antibody against complement component C5, involved in both classical and alternative pathways, promoted vector survival in a dose dependent manner in vitro (Hofmann and Strauss [78]). In addition, treatment of human blood and plasma with cobra venom factor (CVF), an inhibitor of the complement component C3, also resulted in almost complete survival of baculovirus vectors as opposed to the 1% that survived in the absence of this factor (Hofmann and Strauss [78]). CVF has successfully been used to deplete the complement response in mammals in vivo, including monkeys (Chen et al [79]).

A recombinant soluble complement receptor type 1 (sCR1) lacking transmembrane and cytoplasmic domains has been shown to inhibit both classical and alternative pathways of complement activation. Presence of this factor promoted baculoviral vector survival following incubation with human serum and subsequent transduction of Huh7 cells was approximately 5 times more efficient than with baculoviruses incubated with serum in the absence of sCR1 (Hofmann et al [80]).

A further strategy designed to inhibit complement activation by baculoviruses has been to engineer expression of decay accelerating factor (DAF), a naturally occurring negative regulator of both classical and alternative complement pathways, on the virus surface as a fusion protein with gp64 (Hüser et al [71]). Following incubation with human serum, the DAF modified baculoviruses were shown to be able to transduce Huh7 cells in the presence of complement at a much higher frequency than unmodified viruses. This was shown to be due to increased survival of the vectors in the presence of complement (Hüser et al [71]). The same vectors were injected into rat livers in vivo and transgene expression, measured after 3 days, was five times higher in the livers treated with DAF-modified baculoviruses than the unmodified form (Hüser et al [71]).

Thus for cancer gene therapy, the complement response against Bv can be overcome by short term biochemical manipulation during viral inoculation. The optimal conditions have not been determined however, and a number of alternative inhibition systems, to those employed above can still be developed, particularly for prostate cancer patients. In the brain however, the complement response has no effect on Bv gene transduction, and it has been proposed as a safe and effective

agent (Sarkis et al [40]) for treatment of neural disorders.

CONCLUSIONS

Thus baculoviruses are a novel and sophisticated vector to carry therapeutic genes into human prostate cancers. They can be readily manipulated, using established and now commercially available technology, and perhaps most importantly, they have a vast capacity for exogenous DNA. This will allow larger control sequences, and even genes for transcriptional control proteins, which will offer greater independence from intracellular factors (which could simply be turned off, for example, by genome CpG methylation, as a defence mechanism) and provide greater control and specificity. The ability to target specific cell subtypes has been adequately demonstrated, although definitive data for prostate remains to be confirmed. The absence of preexisting humoral and cell-mediated immune memory against nonhuman viruses is well established, and if the complement inactivation can be overcome, their efficiency should exceed that of human viruses.

They are not without other serious problems, as industrial scale culture can be compounded by aggregation, although in our experience when employed for protein production, good virological practice prevents the build up of defective interfering particles. There is also the relatively high affinity of Bv for liver cells, which could produce undesirable hepatic side effects, unless other forms of targeting (eg, transcriptional/therapeutic gene targeting) have been included in the final vector construct. Repeated Bv inoculation will undoubtedly elicit a potent immune response, but use of Bv in combination with other viral (or nonviral) agents, could conceivably keep therapy ahead of the defence mechanisms of both the patient and his tumour, which have rendered prostate tumours so recalcitrant to normal anticancer therapy.

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