Poxviruses
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Recombinant poxvirus vaccines in biomedical research

Barbara S. Schnierle, Yasemin Suezer and Gerd Sutter

Paul-Ehrlich-Institut, Department of Virology, Paul-Ehrlich-Straße 51-59, 63225 Langen, Germany

Abstract

In biomedical research recombinant poxviruses are investigated as important candidate medicines to derive advanced options for prevention and/or treatment of infectious diseases or cancer. Genetically engineered viruses can readily synthesize biologically active heterologous proteins, serve to determine relevant targets of cell-mediated and humoral immunity, and identify types of immune responses needed for protection against a multitude of different specific diseases. Substantial progress in vaccine development is based on the availability of exceptionally safe but efficient carrier viruses, on increasingly versatile vector technologies and on the feasibility of large scale manufacturing. Moreover, advances in deciphering the molecular pathways regulating poxvirus-host interactions will provide additional means to potently activate innate immune stimulation upon vaccination and to derive vectors with specifically targeted replicative capacity for experimental tumor therapy.

Introduction

Poxviruses engineered to express foreign genes have been established as extremely valuable tools in modern biotechnology and for vaccine development in medical and veterinary research (for review see [1]). Compared to currently marketed vaccines, viral vectors appear still as futuristic option in vaccine development. Yet, many of today's health problems where vaccines are believed to become key medicines are likely not to be solved with existing technologies. Adequate biological and clinical safety, large packaging capacity for recombinant DNA, precise virus-specific control of target gene expression, high-level immunogenicity, lack of persistence or genomic integration in the host, and ease of vector and vaccine production are important features supporting the use of recombinant poxviruses as advanced tools for immunization. While making a multivalent poxvirus vector vaccine has been proposed as a particularly desirable medicinal product [2], recombinant poxviruses are primarily investigated as novel vaccines against major

human and animal diseases that still lack effective intervention strategies. A major achievement has been the application of vector viruses providing extraordinary levels of safety with regard to protection of the non-target environment and use in possibly immunocompromised target populations [3, 4] (for review see [5, 6]). The substantial recent progress in conducting clinical research with candidate vaccines against AIDS, tuberculosis, malaria or tumor diseases may serve as an example. Poxviruses engineered as vector vaccines include viruses from multiple genera with the *Orthopoxvirus Vaccinia virus* (VACV) [7, 8] and *Avipoxvirus*es [9, 10] being the first and most frequently developed for applications in human and veterinary medicine. Other promising candidate vaccines against animal diseases are derived from *Parapox*- [11, 12], *Suipox*- [13], *Capripox*- [14], or *Leporipoxvirus*es [15, 16]. In this review, we attempt to provide an update on the state-of-the-art in poxvirus vector technologies and to sum up the recent progress in the development of prophylactic and therapeutic recombinant vaccines.

Generation of recombinant poxviruses

Poxviruses replicate within the cytoplasm of the infected cell and therefore their genome is not transcribed by cellular enzymes. The virus encodes its own transcription and replication machinery and its DNA is not infectious. The currently most frequently practiced strategy to generate recombinant poxviruses employs homologous DNA recombination in infected cells, a relatively frequent event during poxviral replication (0.1%). Recombination is typically directed by a plasmid-based transfer vector, containing the following features: an expression cassette, including a poxvirus-specific promoter, usually followed by a multiple cloning site to allow the insertion of the foreign gene of choice. In addition, selection or screening procedures are quite useful to ease the clonal isolation of recombinant viruses by plaque purification, which requires the additional insertion of selection marker gene expression cassettes. These heterologous DNA sequences are flanked by poxvirus DNA sequences that direct the recombination to a desired locus in a non-essential region of the poxviral genome (Fig. 1). A large variety of different natural and synthetic virus-specific promoters that are transcribed at early, intermediate or late times of VACV infection are available (for review see [17]). For vector construction tandem early and late promoters are commonly used to allow for moderate to strong target gene expression during the whole virus life cycle [18–20].

The standard insertion locus for generating recombinant VACV is the thymidine kinase (TK) locus, which allows the selection of recombinant virus by its TK-negative phenotype due to the insertional inactivation of TK in TK-deficient cells [18]. Recently, an improved dominant negative selection procedure has been developed. A recombinant VACV with an inserted *E. coli* TK/thymidylate kinase (tk/tmk) fusion gene, which converts 3'-azido-

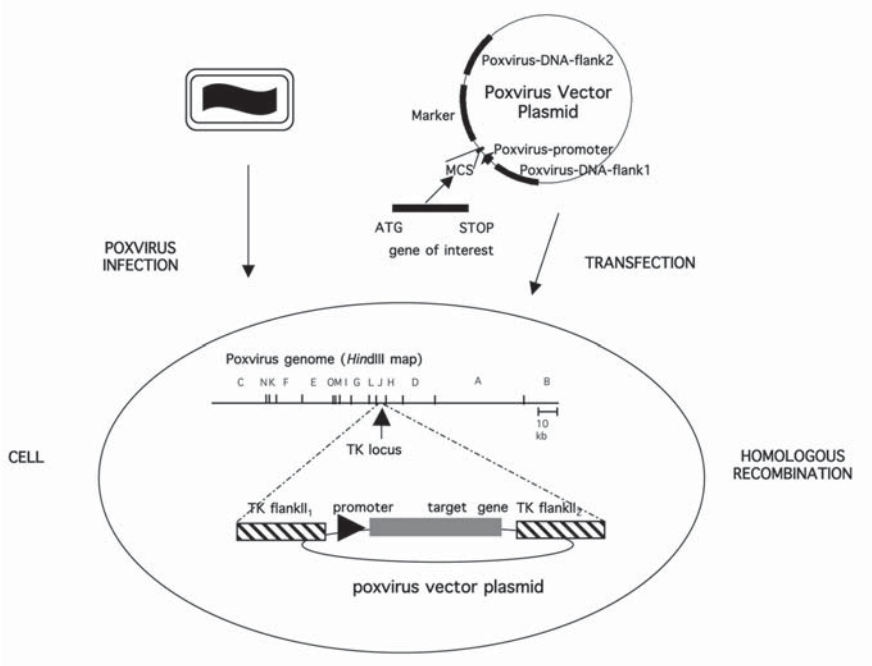

Figure 1. Generation of recombinant poxviruses by homologous recombination. Upper panel: A virus particle is shown on the left; on the right a schematic representation of a poxvirus vector plasmid is depicted. Viral DNA sequences adjacent to the genomic insertion site (flank1, flank2) are cloned in the plasmid and target genes are inserted between these sequences and placed under transcriptional control of poxvirus-specific promoters. Recombinant viruses are generated by infection and simultaneous transfection of cells with vector plasmid DNA, resulting in recombination between homologous DNA sequences of plasmid and virus. Lower panel: Poxvirus-infected, transfected cell. Schematic map of the viral genome and a plasmid designed for insertion of foreign DNA at the locus of the thymidine kinase (TK) gene. Sites of the restriction endonuclease *Hind*III within the virus genome are indicated at the top. The position of the TK gene is marked by an arrow. Virus DNA sequences adjacent to TK insertion locus (TK flank1, TK flank2) are contained in the plasmid.

2',3'-dideoxythymidine (AZT) into a toxic compound, has been used to construct recombinants. Inactivation of the tk/tmk gene by insertion of the transfer vector conveniently allows selection by AZT without the requirements of using TK-deficient cells [21].

Alternatively, the transfer vector contains an antibiotic selection marker or a reporter gene allowing the screening due to a change in phenotype such as co-expression of the *E. coli* β-galactosidase [22] and β-glucuronidase [23]. Among the co-expressed antibiotics, the *E. coli* gpt gene encoding the enzyme xanthine guanine phosphoribosyl transferase is frequently used for

purification of recombinant viruses by dominant positive selection for resistance against mycophenolic acid [24].

Staining procedures require additional time of tissue culture, supplementation of agar overlays, and the use of chromogenic substrates and antibiotics. Complementation of a defect in virus production is a faster and more convenient method to obtain recombinant viruses. A first growth selection protocol was initiated using the VACV host range gene K1L to rescue mutant VACV replication in rabbit kidney RK-13 cells [25]. Blasco and colleagues [26, 27] introduced selection for plaque formation through co-insertion of the F13L gene. A VACV mutant and an appropriate complementing cell line enabled growth selection based on the essential D4R gene function [28]. Transient introduction of the K1L gene into the genome of severely growth-restricted modified VACV Ankara (MVA) is used for simple and efficient selection of recombinant MVA, because co-expression of K1L can also complement the defective MVA life cycle in RK-13 cells [29–31].

The large size and the covalently closed hairpin ends of the dsDNA poxvirus genome have been major hurdles for direct *in vitro* cloning of recombinant viruses. In addition, since poxviral DNA is not infectious, isolated poxvirus genomes require a helper poxvirus supplying essential enzymes that are needed to initiate transcription and replication of the recombinant virus. This helper virus should not recombine with the vector virus and not produce infectious progeny in the cells used for the generation of recombinant virus. Avian poxviruses or leporipoxviruses fulfill this requirement for reactivation of recombinant VACV [32, 33]. In one such approach, a unique restriction site was introduced into the VACV genome and the genome was cloned in two halves in lambda phages. Religation of the two halves together with the recombinant gene between them and direct transfection into helper virus-infected cells allowed the generation of recombinant poxviruses without cloning an intermediate DNA construct in *E. coli* [32, 34, 35]. Moreover, the efficient generation and reactivation of recombinant VACV from cells transfected with cloned DNA also enabled the construction of cDNA libraries [36].

Another elegant method to engineer poxvirus vectors has been pioneered recently [37]. The entire VACV genome was cloned into a bacterial artificial chromosome (BAC), which can be engineered in *E. coli* by homologous recombination with bacteriophage lambda-derived enzymes. The modified BAC clones can be used to produce pure recombinant poxvirus in mammalian cells with the initial assistance of a helper virus, but without further requirements for plaque purification.

Prophylactic recombinant poxvirus vaccines

Animal models for major viral diseases, such as influenza, hepatitis B, or rabies, have served to provide first strong proof-of-principle for protective

Table 1. Examples for infectious diseases in humans being targets of candidate poxvirus vector vaccines in recent clinical or preclinical research

Disease	Agent	Target antigens	Vector	Proposed use
AIDS	HIV-1, -2	Gag, Env, Nef, Tat, Rev	MVA, NYVAC, CPV, FPV	Prophylaxis / therapy
Hepatitis C	HCV	C, E1, E2, NS2, NS3, NS4, NS5	CPV, MVA	Prophylaxis / therapy
Cytomegalovirus infection	CMV	UL55, UL83, UL123	MVA	Prophylaxis / therapy
Tuberculosis	Mycobacteria	85A, Apa	MVA, FPV	Prophylaxis
Malaria	*P. falciparum*	TRAP, LSA-1, CS etc.	MVA, FPV, CPV, NYVAC	Prophylaxis
Leishmaniasis	*L. infantum*	LACK	VACV, MVA	Prophylaxis
Cervical carcinoma	HPV-16, 18	E2, E6, E7,L1	MVA	Prophylaxis / therapy

prophylactic vaccination with recombinant poxvirus vaccines [7, 8, 38, 39]. To prevent these diseases in humans, reasonably good and safe vaccines have been available, which has certainly contributed to a delay in the pharmaceutical development of poxvirus vectors for medical applications. In contrast, multiple poxvirus vector vaccines are already in use in veterinary medicine. Licensed products in Europe include vaccines based on recombinant VACV and recombinant *Canarypox virus* for prevention of rabies, equine influenza, and feline leukemia. Moreover, there is a steadily increasing interest to derive and test new vector vaccines, making veterinary medicine an important driving force in the development of advanced medicinal products [40–46]. In medical research and development, most ongoing efforts focus on the study of candidate vector vaccines against human diseases that are more "difficult" to prevent, e.g. those caused by newly emerging or chronic virus infections, or by bacterial infections, parasites or cancer (for overview see Tab. 1).

A safe and effective human immunodeficiency virus (HIV) vaccine is urgently needed to control the worldwide HIV epidemic. However, the development of a vaccine against AIDS represents a substantial scientific challenge related to HIV antigenic variability, the lacking understanding of immune correlates for protection, limitations of available animal models, and the enormous constraints associated with the probable need for multiple large-scale clinical trials in different parts of the world (for review see [47]). Moreover, the fragile immune system of HIV-infected individuals sets high standards for candidate vaccine safety. Recently, highly attenuated poxviruses have continued to play a major role in the international search for an AIDS vaccine, which also takes advantage of established technologies for vector vaccine production at an industrial scale. For example, safety-

tested VACV strains MVA and NYVAC, and avirulent avipoxviruses are characterized by severe growth deficiencies in human cells; however, they can efficiently express recombinant genes and represent attractive candidate immunodeficiency virus-specific vaccines [48–50] (for review see [6, 51–55]). The data from clinical research with poxvirus recombinant vaccines so far demonstrate induction of humoral and cellular HIV antigen-specific immune responses in humans. In many preclinical experiments, varying degree of protection against homologous immunodeficiency virus infection has been found, predominantly depending on the challenge virus/animal model used for evaluation. However, HIV has an extraordinary genetic diversity and the "Holy Grail" AIDS vaccine would have to cross-protect against different HIV clades. A major scientific challenge is now to find appropriate antigens or epitopes that elicit a cross-protective immune response. For some time, induction of cellular immunity was the primary focus of HIV vaccine development but the generation of broadly neutralizing antibodies is also believed to be indispensable [56]. Concurrently, data from two studies in the macaque model showed that booster vaccinations with oligomeric or native Env proteins enhance Env-binding and virus-neutralizing antibody responses primed by recombinant MVA vaccines, and suggest that such antibodies are indeed likely to play a role in vaccine-induced protection [57, 58].

Hepatitis C is another global health problem caused by a chronic virus infection that still lacks a preventive vaccine, and substantial efforts are currently dedicated to preclinical research in animal model systems [59]. The immunogenicity of the first poxvirus vector vaccines based on recombinant *Canarypox virus* and recombinant MVA have been tested in HLA-transgenic mouse models [60, 61].

The threatening episode of suddenly emerging coronavirus infections in humans causing severe acute respiratory syndromes impressively demonstrated the suitability of recombinant poxvirus vaccines to quickly evaluate candidate vaccines against a previously unknown pathogen [62, 63]. Thus, in view of the current struggle to tune-up well established but rather too simple vaccine technologies for preparation against the global threat of an influenza pandemic, it is tempting to look into the possible usefulness of poxvirus vectors for development of more potent third generation influenza virus-specific vaccines.

In addition, recombinant poxviruses have proven to be excellent aspirants for vaccine development against other disastrous infectious diseases with global impact such as tuberculosis and malaria (for reviews see [64, 65]). The incidence of disease caused by *Mycobacterium tuberculosis* is steadily increasing often on the basis of poverty-impaired health services, widespread HIV infection, or the emergence of resistant *M. tuberculosis*. In recent efforts to elicit more potent anti-mycobacterial immunity, MVA vector viruses served to identify new promising target antigens and resulted in the development of the first subunit vaccines entering clinical testing

[66–68]. Similarly, an effective vaccine against malaria is urgently required and a variety of antigens from *Plasmodium falciparum* has been expressed and tested with recombinant VACV or avipoxviruses. First clinical trials have been initiated using recombinant MVA and fowlpox virus vectors and suggest the usefulness of prime-boost protocols for eliciting enhanced malaria-specific T cell immunity [69, 70].

Therapeutic application of recombinant poxviruses

In HIV-infected patients therapeutic immunization is considered as a possible means to achieve viral containment without maintenance of antiretroviral treatment. First data from clinical evaluation of recombinant canarypox virus and recombinant MVA vaccines are encouraging, with efficient expansion of vaccine-stimulated HIV antigen-specific $CD8^+$ and/or $CD4^+$ T cell responses and first evidence of improved virus control [71–74].

The identification of tumor-associated antigens (TAA), which are displayed by MHC molecules and recognized by specific T cells, showed that vaccination might serve as an effective therapy for a number of malignancies. The particular potential to activate robust cellular MHC class I- and II-restricted $CD8^+$ and $CD4^+$ T cell responses against recombinant antigens make poxvirus vectors attractive as vaccines for immunotherapeutic approaches against cancer. For experimental cancer therapy, virus antigen-associated malignancies seem to be predestined targets for vaccines because these TAA consist of non-self antigens and do not require breaking of immunotolerance. Taylor and co-workers [75] demonstrated the immunogenicity of an Epstein-Barr virus (EBV)-associated nasopharyngeal carcinoma vaccine by reactivating EBV-specific $CD8^+$ and $CD4^+$ memory T cells *in vitro*. There is evidence for the therapeutic efficacy of poxvirus vaccines delivering human papillomavirus (HPV) E2, E6 or E7 antigens against cervical cancer associated with HPV infection in Phase I/II clinical trials [76–80].

Several poxvirus vaccine candidates directed against auto-TAA are also in preclinical and clinical development, using carcinoembryonic antigen (CEA) [81, 82] and prostate-specific antigen (PSA) [83], and a number of melanoma-associated antigens [84, 85], like gp100, tyrosinase or Melan-A are the furthest developed vaccination strategies and are summarized by Kwak et al. in [86]. Often these strategies are combined with either cytokines like IL-2 [87, 88], costimulatory molecules such as B7-1 [89–91], CTLA-4 blockade [92] or cellular adjuvants like dendritic cells, to enhance immune responses against antigens that are likely tolerogenic self proteins [93, 94].

One approach in experimental cancer therapy is based on oncolytic viruses (OV) that were selected or engineered to replicate, propagate and spread exclusively in tumor cells, leading to their destruction, while not

affecting normal cells. This targeting is possible because OV exploit the cellular defects that permit tumor cell growth. To date several types of OV have been developed and have entered clinical trials. These trials demonstrated an acceptable safety profile of OV, but limited therapeutic efficacy when used as monotherapy. However, improved performance was noted when OV were used in combination with traditional therapies (chemotherapy or radiation) (reviewed in [95]).

Replicating VACV is being developed as an oncolytic agent (for review see [96, 97]) because it is able to infect and spread in a large variety of cells and confers an anti-tumor effect by virus-mediated cell death. The first Phase I clinical trial with a VACV recombinant expressing granulocyte-macrophage colony-stimulating factor (GM-CSF) applied intratumorally showed that the vector is well tolerated and efficient to a limited extent in the treatment of cutaneous melanoma [98]. VACV variants have been engineered to improve safety by causing inefficient replication in normal cells but retaining high propagation efficiencies in tumor cells [99]. Deletions of the TK and vaccinia growth factor (VGF) genes were shown to decrease VACV virulence [100, 101]. TK/VGF-negative VACV double mutants are further attenuated and showed an enhanced growth capacity in tumor cells [102]. Preferential replication in tumor cells is attributed to the requirement of TTP for DNA synthesis from the nucleotide pool present in highly dividing cells and the activation of the epidermal growth factor receptor (EGFR) signaling pathway, a frequent abnormality in cancer cells. Another attenuation strategy makes use of the ability of cancer cells to evade the induction of apoptosis. The additional deletion of the viral anti-apoptotic genes SPI-1 and SPI-2 from the VACV genome resulted in a recombinant VACV that preferentially replicated in transformed or p53-negative cells and displayed a significant anti-tumor effect in mouse models [103]. A rabbit poxvirus, *Myxoma virus* (MV), which causes myxomatosis in European rabbits but is nonpathogenic in man, has also been developed as an oncolytic virus candidate. MV encodes proteins that counteract rabbit interferons but are unable to antagonize interferons of other species, including humans. In normal interferon-responsive human cells, MV replication is blocked [104]. However, MV productively infects a variety of human tumor cells, which are non-responsive to interferon [105]. Since the virus does not infect man there is no pre-existing immunity in the human population. This, together with the apparent inherent tropism for human tumor cells suggests the potential for exploiting MV as novel OV platform.

Outlook

Live poxviral vectors are particularly attractive because they mimic natural infections, while allowing for *de novo* synthesis of heterologous vaccine antigens. Hereby, poxviral vector vaccination is expected to elicit appro-

priate "danger" signals to the immune system resulting in a preferential recognition and presentation of target antigens. Concerns about the safety of poxviruses, including VACV as the former vaccine successfully used to eradicate human smallpox, have been addressed by the application of viruses that are replication defective and avirulent when tested *in vivo*. To date multiple types of poxvirus vectors have been developed and have entered clinical trials, particularly in the areas of HIV/AIDS or cancer vaccine development. Many results suggest satisfying safety and efficacy of poxvirus vector vaccines with regard to eliciting specific immune responses to selected target antigens in humans. Nevertheless, the complexity involved in inducing protective immunity against infections with immunodeficiency viruses or in eliciting potent immune responses against tumour-associated self-antigens suggests that the immunogenicity of candidate vaccines may still need a booster to achieve protective vaccination against AIDS, malaria or cancer. Hereby, exciting recent results from basic poxvirus research help to reveal an astonishing versatility of poxviral strategies to counteract the innate host immune response and will lead to the generation of optimized vectors and even better poxvirus vaccines [106–108].

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