

Recombinant poxviruses as mucosal vaccine vectors

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The majority of infections initiate their departure from a mucosal surface, such as *Human immunodeficiency virus* (HIV), a sexually transmitted virus. Therefore, the induction of mucosal immunity is a high priority in the development of vaccines against mucosal pathogens. The selection of an appropriate antigen delivery system is necessary to induce an efficient mucosal immune response. Poxvirus vectors have been the most intensively studied live recombinant vector, and numerous studies have demonstrated their ability to induce mucosal immune responses against foreign expressed antigens. Previous studies have demonstrated that recombinants based on the attenuated modified vaccinia virus Ankara (MVA) vector were effective in inducing protective responses against different respiratory viruses, such as influenza and respiratory syncytial virus, following immunization via mucosal routes. Recent studies performed in the murine and macaque models have shown that recombinant MVA (rMVA) does not only stimulate HIV-specific immunity in the genital and rectal tracts following mucosal delivery, but can also control simian/human immunodeficiency viraemia and disease progression. In addition, a prime-boost vaccination approach against tuberculosis emphasized the importance of the intranasal rMVA antigen delivery to induce protective immunity against *Mycobacterium tuberculosis*. The aim of this review is to summarize the studies employing recombinant poxviruses, specifically rMVA as a mucosal delivery vector. The results demonstrate that rMVAs can activate specific immune responses at mucosal surfaces, and encourage further studies to characterize and improve the MVA mucosal immunogenicity of poxvirus vectors.

Introduction

The majority of human pathogens initiate their infectious process through a mucosal barrier, such as *Human immunodeficiency virus* (HIV), a sexually transmitted virus. Moreover, the impact of HIV infection on mucosal tissues is emphasized by the rapid and marked depletion of CD4⁺ T cells that reside in the gastrointestinal tract and other mucosal tissues, including the lung and vagina (Brenchley *et al.*, 2004; Mehandru *et al.*, 2004). The mucosal route acquires numerous other viral and bacterial infections with significant epidemiological impact. Examples include other sexually transmitted infections caused by *Chlamydia*, *Neisseria gonorrhoeae* and herpes simplex virus (HSV), gastrointestinal tract infections caused by *Helicobacter pylori*, *Vibrio cholerae*, enterotoxigenic *Escherichia coli*, *Shigella* spp., rotaviruses and caliciviruses, and respiratory infections caused by *Mycoplasma pneumoniae*, influenza virus and respiratory syncytial virus (RSV) (Eriksson & Holmgren, 2002). These pathogens represent a challenge for the induction of mucosal immunity in the development of effective vaccine strategies. Of major importance is the choice of an effective antigen delivery system capable of inducing a significant immune response at a mucosal site. In this review, we discuss the basis in the development of mucosal vaccines, specifically the use of recombinant modified virus Ankara (rMVA) and other attenuated poxvirus vectors. Emphasis is given to current strategies, in particular, (i) prime-boost approaches that may be used in the future, (ii) manipulation of the virus vector, and (iii) the use of mucosal adjuvants.

Key issues in the development of mucosal vaccines

Mucosal surfaces represent a critical component of the mammalian immunological repertoire. Mucosal-associated lymphoid tissue (MALT) is a highly specialized and compartmentalized system composed of organized and dispersed lymphoid tissues, which has been well defined in the gastrointestinal, respiratory and nasal tracts, with the inductive sites of aggregated lymphoid tissues (such as Peyer's patches in the gut) and effector sites of the respective mucosal tissues. MALT is divided into two functionally distinct compartments, the inductive and effector sites. Activation of these sites is of paramount importance in the design of an effective vaccine. Various experiments have revealed that inductive sites present in certain locations, such as Peyer's patches and nasopharyngeal-associated lymphoreticular tissue (NALT), can function as primary sources of precursor cells that migrate and then populate remote mucosal tissues (McGhee *et al.*, 1989; Ogra *et al.*, 2001). These findings have led to the notion of a common mucosal immune system (CMIS). Taking advantage of the characteristics of CMIS, one can prevent invasion of pathogens through a specific mucosal surface, such as the genital mucosa, through the induction of a mucosal immune response by vaccinating at other mucosal inductive sites such as NALT (McDermott & Bienenstock, 1979). Of the various mucosal routes tested in humans and non-human primates, nasal immunization provides a better vaginal response than rectal immunization, and has the capacity to elicit rectal responses (Bergquist *et al.*, 1997; Kozlowski *et al.*, 2002; Mestecky & Fultz, 1999). There are several

advantages in using mucosal routes instead of parenteral routes for the administration of vaccines. The most important is based on the general acceptance of needle-free methods for vaccine delivery, whereupon administration could be performed by oral or aerosol (intranasal, i.n.) routes. Furthermore, their attraction is based on the potential to overcome the known barriers of parenteral vaccination caused by pre-existing immunity as a result of previous vaccination (Belyakov *et al.*, 1999).

There are various factors that must be taken into account to achieve successful mucosal immunization, recently summarized in several reviews (Cripps *et al.*, 2001; Eriksson & Holmgren, 2002; Holmgren & Czerkinsky, 2005). Key factors to be considered are: (i) effective delivery of antigen to the mucosal immune induction site; (ii) enhancement of mucosal immune responses by the use of safe mucosal adjuvants; (iii) choosing a regime and route of immunization that will induce protective responses at the desired mucosal site and preferably, systemically; and (iv) choosing an adequate formulation for the vaccine during optimization of the mucosal immunization regime.

Different mucosal delivery systems consisting of microparticles, lipid-based structures, and various live-attenuated bacteria and viruses have been described in detail elsewhere (Eriksson & Holmgren, 2002; Holmgren *et al.*, 2003; Kersten & Hirschberg, 2004).

Live viral vectors as delivery systems to induce mucosal immune responses

Among the different antigen delivery systems, live recombinant viral vectors have the capacity of inducing strong cellular immune responses and can also prime antibody responses against expressed foreign antigens. Further advantages of these vectors include their ability to naturally infect target cells and tissues of interest, their ease of delivery via mucosal routes and their provision of a natural adjuvant effect due to induction of cytokines and chemokines attributable to the vector itself. Among the many live viral vectors described, a number have been used to induce immune responses following mucosal delivery. Adenovirus (Ad) vectors that are natural mucosal immunogens, can be administered orally or intranasally. These vectors have been exploited extensively as gene therapy vectors, and a great deal is known about their molecular biology and host interactions (Voltan & Robert-Guroff, 2003). Principally, these vectors have been manipulated to express foreign antigens and are then delivered via i.n. routes to induce protective immune responses (Prevec *et al.*, 1989). In recent years numerous vaccine studies using these vectors in the HIV field have been described and are summarized in a current review (Gomez-Roman & Robert-Guroff, 2003). Attenuated influenza virus-vectors, including cold-adapted and genetically engineered viruses containing specific attenuating mutations, also have the capacity to induce mucosal immune responses when delivered via the natural route of infection, the nasal mucosa. A number of studies have documented the use of influenza viruses as mucosal delivery vectors (Ferko *et al.*, 1998, 2001; Gherardi *et al.*, 2003; Palese *et al.*, 1997). Vectors based on the *Venezuelan equine encephalitis virus* (VEEV) are also suitable as mucosal vaccines because VEEV targets and replicates in mucosal inductive sites, such as the intestinal Peyer's patches (Voltan & Robert-Guroff, 2003). VEEV recombinant

vectors have been constructed as replicon competent viruses and as replicon particles, with both strategies highlighting their ability to induce mucosal, as well as systemic immune responses (Caley *et al.*, 1997; Harrington *et al.*, 2002).

Poxvirus vectors are the most intensively studied live virus recombinant vectors and have been used with different antigens in numerous animal models. In the different immunization strategies where poxvirus vectors were employed, systemic inoculation was the most frequent route of virus infection. Nonetheless, few studies have evaluated the efficacy of recombinant poxviruses delivered by mucosal routes, a subject discussed in the following sections of this review.

Induction of mucosal immune responses by recombinant poxviruses

Vaccinia virus (VACV), a prototype member of the family *Poxviridae*, was used as a live vaccine to eradicate smallpox (Esposito & Fenner, 2001; Moss, 2001). This virus has proven to be a useful vector for potential vaccination purposes due to its broad host range and ability to generate recombinant viruses that express a variety of foreign antigens, and which confer protection to immunized animals (Giavedoni *et al.*, 1991; Moss, 1984, 1996; Smith *et al.*, 1983). Poxvirus genomes are double-stranded DNA molecules of 130–300 kb in length. VACV is a complex virus with more than 190 open reading frames (ORFs) that allows for the insertion of large foreign DNA fragments (over 25 kb), (Smith & Moss, 1983) and facilitates the expression of recombinant foreign proteins at high levels. Another advantage is its ability to infect a wide host range. Although virus replication occurs in the cytoplasm of infected cells, poxviruses have their own transcriptional system (Broyles, 2003) and consequently viral promoters are necessary in the expression cassette for a foreign gene. The natural promoters P7.5 and H5 are relatively weak, therefore the use of a synthetic early-late promoter (Chakrabarti *et al.*, 1997) is currently the most common choice for achieving high expression of biologically active proteins.

The vaccine used against smallpox was administered intradermally because other routes were thought to be less immunogenic (Moss, 1996; Esposito & Fenner, 2001). Nonetheless, different studies have demonstrated the ability of recombinant poxviruses to induce mucosal immune responses. Studies performed by Hochstein-Mintzel *et al.* (1976) revealed that oral immunization with attenuated VACV was effective in conferring protection against smallpox. A significant example where a poxvirus vaccine was administered by the mucosal route with proven efficacy was in the field immunization programmes, where oral immunization with a recombinant VACV (rVACV) expressing the rabies glycoprotein (VRG) conferred protection against rabies. Consequently, animal species fed with bait containing VRG were protected against rabies infection (Rupprecht *et al.*, 1986; Tolson *et al.*, 1987; Koprowski, 1989). Other studies have suggested that a gut mucosal infection with VACV induced strong VACV-specific T-helper cell and cytotoxic T-lymphocyte (CTL) responses (Issekutz, 1984). Our laboratory group has further characterized the mucosal immune response induced after oral delivery of rVACV. Following oral administration, VACV is able to replicate in the gut-associated lymphoid tissues (GALT), as well as in the spleen, and has the capacity to induce mucosal and systemic

humoral and cellular immune responses against the vector and VACV-expressed recombinant antigens (Gherardi & Esteban, 1999). Further studies performed principally with rVACV based on the WR (Western Reserve) strain have demonstrated mucosal immune responses induced against different recombinant antigens, employing various mucosal routes (Table 1). Thus, after intrajejunal immunization, rVACV conferred significant protection against an influenza virus challenge (Meitin *et al.*, 1994). In another investigation, i.n. immunization of cotton rats with rVACV induced complete protection of the upper respiratory tract following challenge with RSV (Kanesaki *et al.*, 1991). Furthermore, the i.n. delivery of rVACV expressing a CTL epitope of the HSV glycoprotein B was shown to induce protective immunity against i.n. challenge with HSV, inducing both a primary CTL response in the draining lymph nodes and a splenic memory CTL response (Blaney *et al.*, 1998). All of the above studies highlight the efficacy of poxvirus vectors, based on replication competent viruses, as inducers of specific mucosal immune responses against a variety of antigens in different animal models, including mouse, rat, cotton rat, juvenile ferret and macaque (see in Table 1).

Use of rMVA and other attenuated VACV vectors as mucosal vaccines

For safety considerations, the use of replication competent rVACV is not desirable, particularly in young children and immune compromised individuals where disseminated or progressive vaccinia may occur (Lane *et al.*, 1969; Redfield *et al.*, 1987). As a result, several approaches have been taken to eliminate the side effects of VACV through the development of highly attenuated VACV strains (Moss *et al.*, 1996).

One of these strains is MVA, developed after more than 570 passages in chicken-embryo fibroblast (CEF) cells, losing about 30 kb of the genome, and with the ability to have productive replication in most mammalian cells. Viral protein synthesis is, however, unimpaired compared with replication competent VACV (Gallego-Gomez *et al.*, 2003; Sutter & Moss, 1992; Sutter & Staib, 2003). An important feature of this virus is that it was administered as a smallpox vaccine, without complications, to more than 120000 individuals, including those considered at risk from the conventional smallpox vaccine (Hochstein-Mintzel *et al.*, 1975). The novel advances made in the preclinical and clinical evaluation of MVA as a second-generation poxvirus vaccine are described in a recent review (Drexler *et al.*, 2004). Another advantage of this vector system has been its flexibility, demonstrated by the rapid production of an experimental MVA vaccine expressing the coronavirus spike protein for immunization against severe acute respiratory syndrome (SARS) (Bisht *et al.*, 2004).

MVA administration by a mucosal route was first described in 1972 (Hochstein-Mintzel *et al.*, 1972), whereupon following i.n. inoculation, MVA conferred protection against a poxvirus challenge in monkeys and rodents. A recent study using an rMVA-expressing luciferase, defined the fate and biosafety of MVA when inoculated by different routes in female mice (Ramirez *et al.*, 2003). Following i.n. inoculation, expression of luciferase was detected in the nasal-associated lymphoid tissue and the lungs, whilst adverse reactions were not observed in the central nervous system nor the upper and lower airways (Ramirez *et al.*, 2003). Recently, a

safety study performed in simian immunodeficiency virus (SIV)-infected macaques and severe combined immunodeficient (SCID) mice demonstrated that MVA can be considered safe for application in phase I clinical trials in HIV-1-infected human subjects (Hanke *et al.*, 2005). Although a majority of studies have analysed MVA immunogenicity after systemic inoculation, a number of groups have demonstrated the efficiency of MVA as a mucosal vaccine vector. Principal studies using rMVA to induce mucosal immunity are summarized in Table 1. The administration of rMVA via mucosal routes proved to be efficient in generating protective immune responses to airborne viruses such as influenza and RSV in mice (Bender *et al.*, 1996; Wyatt *et al.*, 1999), as well as *Human parainfluenza virus* type 3 in cotton rats and monkeys (Durbin *et al.*, 1998; Wyatt *et al.*, 1996). Another mucosal path, by which rMVA can activate mucosal immune responses, is by the intrarectal (i.r.) route. When this route was used for immunization, MVA expressing gp160 of HIV-1 89.6 induced both a mucosal (Peyer's patch and lamina propia) and systemic CTL response (Belyakov *et al.*, 1998a). Recently, our laboratory group compared the differences in mucosal immunogenicity between replication competent and non-competent rVACVs-expressing HIV-1 Env IIIB (WRenv and MVAenv) after inoculation of mice by i.n., intravaginal (i.vag.) and subcutaneous routes (Gherardi *et al.*, 2004). After i.vag. inoculation, rMVA induced a poor immunogenic response in mice compared with the WR strain, since there was not any significant expression of the luciferase reporter gene in the urogenital tract nor in the remaining target organs following inoculation (Ramirez *et al.*, 2003). Nonetheless, by the i.n. route, rMVA was able to induce a significant splenic immune response against HIV-1 Env antigen following a single inoculation. Additionally, the immunogenicity achieved by this route was greatly enhanced by its subsequent co-delivery with the mucosal adjuvant cholera toxin (Gherardi *et al.*, 2004).

Other attenuated poxviruses that have been applied by mucosal routes with promising results are NYVAC and ALVAC. NYVAC was derived from the Copenhagen strain of VACV by the deletion of 18 ORFs responsible for virulence and host range regulation (Tartaglia *et al.*, 1992). ALVAC, however, is a canary poxvirus whose replication is restricted to avian species and does not cause disseminated infection in mammalian hosts (Taylor *et al.*, 1992, 1995). Mucosal vaccination with recombinant NYVAC or canarypox virus (ALVAC) constructs containing the canine distemper virus (CDV) haemagglutinin and fusion genes, protected juvenile ferrets against i.n. challenge with virulent CDV (Welter *et al.*, 1999). In another study, NYVAC-expressing SIV Gag, Pol and Env products (NYVAC/SIVgpe), inoculated by various routes and assayed for its immunogenicity against an SIV mac 251 peptide Gag, revealed that both mucosal and systemic immunization routes were effective in inducing mucosal immune responses (Stevceva *et al.*, 2002).

Recently, a human trial was performed with the aim to compare systemic and mucosal delivery of two canary poxvirus vaccines, either expressing HIV-1 genes or the gene for rabies virus G protein (Wright *et al.*, 2004). The results revealed a mucosal CTL response in four of the eight individuals after administration via the i.r. route, and a limited immunoglobulin (Ig) A response at the same site. The final conclusion of this study was that the canary poxvirus vector was not an effective mucosal immunogen. While a low mucosal immunogenicity of canarypox-

based vaccines was reported, many studies described above strongly suggest that attenuated VACV vectors such as MVA can induce mucosal immune responses, which can be enhanced further through the optimization of immunization protocols. The main studies described are depicted in Table 1.

Strategies to improve mucosal immune responses employing poxvirus vectors

The prime-boost approach. An effective method of increasing the cellular immunity against a specific pathogen is through repeated vaccination. The idea of boosting immune responses has been around since the appearance of vaccines, and repeated administration of the same vaccine (homologous boosting) has been shown to enhance humoral immune responses. Nonetheless, this approach is relatively inefficient for boosting cellular immune responses, since prior immunity to the vector impairs good presentation and the generation of appropriate signals. To overcome this problem, the sequential administration of vaccines based on different vectors or antigen delivery systems was developed and is highly efficient in inducing potent cellular immune responses. This concept, known as 'prime-boost', was described initially for CD8⁺ T-cell responses with influenza and VACV vectors in the murine malaria model (Li *et al.*, 1993), and has been used in a variety of models, defining that the order of the inoculating vector in the immunization protocol is critical, since the VACV vector is most effective when administered as a booster (Zavala *et al.*, 2001; McShane, 2002; Newman, 2002; Ramshaw & Ramsay, 2000; Woodland, 2004). The vectors most commonly employed as the booster dose are based on poxvirus and adenovirus, both of which exert a synergistic effect on the magnitude of the cellular immune response and induce a selective enrichment of high avidity T cells as well as an increased efficacy against subsequent pathogen challenge (Estcourt *et al.*, 2002; McShane, 2002).

The use of poxviruses in the boost phase of prime-boost immunization protocols has been shown to be a very efficient vaccination approach in different animal models, particularly in their ability to induce specific cellular immune responses and, more importantly, to trigger protection against different pathogens (Gherardi *et al.*, 2001; McShane, 2002; Ramshaw & Ramsay, 2000; Schneider *et al.*, 1999; Vuola *et al.*, 2005). In these studies, the immunogens commonly used during priming were DNA vectors. Nonetheless, other immunogens, such as proteins, peptides, virus-like particles and attenuated viral vectors, have also been employed.

Of major importance in prime-boost strategies is the development of appropriate vectors that must be safe, promptly delivered, easily manipulated, poorly immunogenic to itself and possess the ability to induce both systemic and mucosal immunity. Taking advantage of influenza virus' capacity to target the mucosal tissue, recently we described the administration of prime-boost immunizations with a recombinant influenza virus vector expressing the well-characterized CD8⁺ T-cell epitope from the V3 loop of HIV-1 (clade B) and two rVACVs expressing the entire HIV-1 Env protein (WRenv and MVAenv). By comparing how these vectors activate specific cellular immune responses when administered to mice by systemic and mucosal routes, we found that the prime-boost combination of influenza and MVA can activate

specific cellular immune responses in the genito-rectal draining lymph nodes (GRLNs) and in the spleen, when the vectors are delivered by i.n./intraperitoneal (i.p.) or i.n./i.n. routes, respectively (Gherardi *et al.*, 2003). Thus, the combined use of two viral vectors, such as influenza and MVA, can activate immune responses against selected antigens at critical sites such as the GRLNs.

Although DNA vectors have been employed in different models, naked DNA vectors have been more effective at triggering specific immune responses in prime-boost protocols with heterologous vectors, when given during priming rather than at boosting. This may be due to the fact that lower doses of the antigen are delivered by DNA vectors compared with those synthesized by viral vectors. Indeed, the efficacy of these vectors can be augmented by increasing their transcriptional efficiency and persistence of DNA *in vivo*. Although DNA vaccines have most often been administered systemically, they can also be administered mucosally to generate effective responses, as seen by the ability of an i.n. DNA vaccine to protect mice from lethal challenge with influenza (Okuda *et al.*, 2001). The success of DNA vaccination of the mucosal epithelia is linked to the optimal presentation of plasmid DNA-encoded protein by resident antigen presenting cells. Several promising technologies are currently in development for optimizing plasmid DNA delivery and transfection efficiency at the mucosal epithelia (Hobson *et al.*, 2003), including co-delivery of conventional enterotoxins as adjuvants, the combination of immunomodulatory molecules with DNA vaccines (Staats *et al.*, 2001) or DNA adsorption into positively charged poly (DL-lactide-co-glycolide) (PLG) microparticles (Singh *et al.*, 2001).

Systemic vaccination strategies based on prime-boost regimens that employ a DNA prime immunization followed by a booster with recombinant poxvirus have established that this approach elicits potent cellular immune responses against a variety of antigens, including SIV and HIV (Amara *et al.*, 2001; Hanke & McMichael, 2000; Kent *et al.*, 1998). Due to the urgency of optimizing protocols for generating efficient mucosal immunity, recent studies have employed the prime-boost approach by delivering the vectors through mucosal routes (Bertley *et al.*, 2004; Eo *et al.*, 2001; Gherardi *et al.*, 2004; Makitalo *et al.*, 2004; Ramsay *et al.*, 1997; Wang *et al.*, 2004; Wierzbicki *et al.*, 2002). In these studies, different routes were utilized, and in some cases, the inclusion of cytokines or enterotoxin mucosal adjuvants improved the mucosal immune response. In one study (Wang *et al.*, 2004), immunity achieved after rectal DNA/MVA vaccination of rhesus macaques delayed the progression of AIDS following challenge with simian human immunodeficiency virus (SHIV) 89.6P. In another study, using the macaque model, a combined parenteral and mucosal administration of a DNA prime/MVA boost vaccine regimen induced an enhanced cellular immunity and systemic control of SHIV infection after homologous intravenous (i.v.) SHIV-4 challenge (Makitalo *et al.*, 2004). Animals immunized intramuscularly (i.m.) and mucosally showed a stronger cellular immune response and reduction of viral load following subsequent challenge when compared with animals immunized only by the i.m. route. Interestingly, this study found a correlation between the breadth and magnitude of the immune response induced and the protective efficacy of the vaccination, since the animals with the lowest virus loads demonstrated broad (CD8⁺, CD4⁺ T cells and antibody) and

strong immune responses. Data from another study suggested that SIV/HIV-specific mucosal immunity can be boosted by peripheral MVA immunization after oral priming with a salmonella vector vaccine (Evans *et al.*, 2003). The importance of i.n. mucosal delivery of antigen from rMVA was emphasized by the results of a study in which a prime-boost approach induced a complete protective immunity against the aerosol challenge with *M. tuberculosis* (Goonetilleke *et al.*, 2003).

All of the above studies demonstrate that the use of prime-boost immunization protocols by mucosal routes induced immune responses at mucosal sites and in some cases can confer protection against subsequent pathogen challenge.

Co-delivery with mucosal adjuvants. The effectiveness of mucosal vaccines using poxvirus vectors can be enhanced by the addition of adjuvants. Toxin-based adjuvants, CpG motif DNA, and cytokine-based adjuvants activate cells of the innate and acquired immune system (Yuki & Kiyono, 2003). Toxins, such as the cholera toxin (CT) and a mutant of labile toxin, can enhance mucosal responses and induce systemic immune responses to mucosal vaccines (Pizza *et al.*, 2001; Yuki & Kiyono, 2003). Recently, we have characterized different protocols of immunization in order to enhance the immunogenicity of rMVA expressing the HIV-1 Env IIIB antigen delivered intranasally (Gherardi *et al.*, 2004). It was found that rMVA immunogenicity was enhanced significantly by its co-delivery with the mucosal adjuvant CT. Moreover, a DNA prime/rMVA boost i.n. immunization scheme in the presence of CT, induced a potent specific cellular immune response in the spleen and, more importantly, at mucosal effector sites of the urogenital tract and genital draining lymph nodes. An important finding of these studies was that these immunization schemes induced specific CD8⁺ T-cell populations that secreted interferon (IFN)- γ and different β -chemokines in response to the antigen. Moreover, mucosal-specific antibodies to Env in vaginal washings were detected (Gherardi *et al.*, 2004). Thus, a broad specific immune response was induced by combining DNA/rMVA with CT, and this broad response is necessary to obtain protection against SHIV infection as demonstrated recently in the macaque model (Makitalo *et al.*, 2004).

Another way to potentiate DNA vaccination is by mucosal co-delivery with genetic vaccines encoding adjuvant molecules. In most instances, the adjuvant and antigen system have been administered through the nasal mucosa and responses measured at distal mucosal sites, such as the gut or genital mucosa. Encouraging studies involving the cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL) 2, IL12, IL18 and many others have been reported (Toka *et al.*, 2004). The enhancing effect of cytokines on mucosal prime-boost approaches was demonstrated in some studies. Accordingly, in a mucosal DNA prime/rVACV boost vaccination protocol involving oral administration of PLG-encapsulated plasmid DNA and rVACVs, the optimal responses were obtained when a DNA plasmid encoding IL2 was co-administered. IL2/Ig-mediated activities were associated with higher levels of antigen-specific CTL and antibody responses in mucosal tissues, demonstrating the effectiveness of mucosal DNA vaccines in conjunction with IL2 (Wierzbicki *et al.*, 2002). A more recent study performed in the macaque model described that application of an i.n. DNA

prime/MVA boost immunization scheme administering IL2 during the priming, can induce a specific immune response that is able to control SHIV viraemia and disease progression after mucosal challenge with the pathogenic SHIV 89.6P (Bertley *et al.*, 2004). This report demonstrated that the i.n. route of vaccination induced systemic immune responses sufficient to control viraemia to a degree similar to that induced after i.m. immunization in the same SHIV 89.6P model, and also demonstrated that IL2 produced an adjuvant effect after i.n. administration.

The studies discussed in this section demonstrate that by the aid of mucosal adjuvants, mucosal prime-boost immunization strategies with poxvirus vectors could be improved significantly. The main studies are summarized in Table 2.

Incorporating or deleting immunomodulatory molecules in the virus vector backbone

The poxvirus family members encode proteins that interfere with the immune system to evade host-immune responses (Smith *et al.*, 1997; Smith, 1999; McFadden, 2005). Vectors such as MVA lack several of the immunomodulatory proteins but still encode other proteins capable of counteracting immune responses. For optimal use of poxvirus vectors as vaccines, it will be important to determine if deleting selective viral genes and/or adding genes into the virus genome will improve the immunogenicity of the pox vector (Blanchard *et al.*, 1998). One strategy to modulate the immunogenicity of the poxvirus vector involves the incorporation of genes encoding cytokines into the poxvirus genome (Ramshaw & Ramsay, 2000). Thus, expression of IL12 from rVACV enhanced the immune response to HIV Env in a dose-dependent manner (Gherardi *et al.*, 1999, 2000) and expression of GM-CSF that was fused to HIV Env, also enhanced the immune response to Env (Rodriguez *et al.*, 1999). Recent studies demonstrated that an rVACV lacking the virus serpin genes (B13R and B22R) and co-expressing IFN- γ , induced potent immune responses to the recombinant product conferring protection against subsequent vesicular stomatitis virus infection (Legrand *et al.*, 2005). These observations highlight how the deletion or addition of genes with immunomodulatory roles may be used to improve the poxvirus vector efficacy. This approach might be used as another way to increase the desired immune response against the recombinant antigen in systemic and mucosal sites.

Conclusions and future applications of mucosal poxvirus-based vaccines

The majority of pathogens are transmitted mucosally, but undoubtedly HIV is the virus with the greatest epidemiological and social impact. HIV infection is a predominantly mucosal acquired disease. Moreover, recent studies have shown that, as in macaques infected with SIV (Veazey *et al.*, 1998), intestinal CD4⁺ T cells are selectively and rapidly depleted in the intestine of HIV-infected patients, at all stages of infection regardless of highly active antiretroviral therapy (Brenchley *et al.*, 2004; Mehandru *et al.*, 2004). These findings highlight the importance of mucosal immunity in HIV infection and the necessity to develop vaccines and/or immunization

strategies to induce local mucosal immune responses against this pathogen. The results summarized in this review indicate that VACV vectors, and especially rMVA, can be employed at local sites to induce mucosal immune responses against the recombinant antigen. Importantly, many studies performed with poxvirus-based vectors described in this review have been accomplished with recombinant vectors expressing HIV or SIV antigens. Some of the studies performed in murine models have shown that rMVA can induce mucosal immune responses (cellular and humoral) when delivered by i.n. or i.r. routes (Belyakov *et al.*, 1998b; Gherardi *et al.*, 2004). On the other hand, studies performed in the macaque model showed protective immunity after an i.n. DNA prime/rMVA boost with IL2 exerting adjuvant effect during priming (Bertley *et al.*, 2004), and i.r. protection after SHIV DNA prime/MVA-SHIV boost (Wang *et al.*, 2004). Protection against other relevant mucosal pathogens further highlights the importance of mucosal targeted prime/MVA boost vaccination approaches, for example the ability of such a protocol to confer complete protection against an aerosol challenge with *M. tuberculosis* (Goonetilleke *et al.*, 2003).

Results from the first clinical trials testing MVA vaccines for prophylaxis or immunotherapy against AIDS, malaria or cancer have been promising overall (Drexler *et al.*, 2004; Vuola *et al.*, 2005). However, recent data from HIV phase I clinical trials performed with MVA and NYVAC vectors (Guimaraes-Walker *et al.*, 2004; Harari *et al.*, 2004) revealed vaccine-specific responses limited to 18 % (MVA) or 50 % (NYVAC) of volunteers, respectively. This indicates that improvement of the vector immunogenicity is necessary through the removal or the incorporation of immunomodulators and by formulations with appropriate adjuvants.

In conclusion, results from the different studies described here indicate that it is necessary to explore further mucosal immunization schemes based on rMVA-expressing antigens of HIV or *Mycobacterium* (as two mucosal pathogens with epidemiological relevance), as well as with other attenuated poxvirus vectors, in order to determine the optimal immunization regimens capable of stimulating high levels of both mucosal and systemic immunity, as these responses might be sufficient to control the infection locally.

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Table 1. Principal studies in which mucosal delivery of poxvirus vectors was applied

Vector	Recombinant antigen	Route*	Immune response induced				Protective immunity	Animal model	Reference
			Cellular		Humoral				
			Mucosal	Systemic	Mucosal	Systemic			
VACV	Vector antigens	Enteric	Strong Th and CTL-response in MLN	ND	ND	ND	ND	Rat	Issekutz (1984)
VACV	HIV-1 Env, β -galactosidase	i.o.	Th and CD8 ⁺ T cells in MLN†	Spleen CD8 ⁺ T cells and Th	IgA in gut and vaginal washes	Serum IgG1 and IgG2a	ND	Mouse	Gherardi & Esteban (1999)
VACV	Epitope from HSV glycoprotein B	i.n.	CTL in local hilar and submaxillary LN	Memory CTL in spleen	ND	ND	Protection from lethal challenge with HSV-2	Mouse	Blaney <i>et al.</i> (1998)
VACV	RSV protein	i.n./enteric	ND	ND	IgA in respiratory tract	Serum IgG	Complete protection after i.n. immunization	Cotton rat	Kanesaki <i>et al.</i> (1991)
MVA	Haemagglutinin and nucleoprotein genes from H1N1 influenza	i.g.	Pulmonary CTL activity	Splenic CTL activity	IgA in vaginal, nasal and gut washes	Serum IgG1 neutralizing antibodies	Complete lung protection	Mouse	Bender <i>et al.</i> (1996)
MVA	HIV-1 89.6P Env	i.r.	CTL in PP† and LP† lymphocytes	Splenic CTL	ND	ND	ND	Mouse	Belyakov <i>et al.</i> (1998a)

NYVAC ALVAC	CDV haemagglutinin and fusion genes	i.n./enteric	ND	ND	ND	Serum CDV neutralizing antibodies	Complete protection after i.n. immunization	Juvenile ferrets	Welter <i>et al.</i> (1999)
NYVAC	SIV Gag Pol Env	i.n/i.r.	CD8 ⁺ T cells in rectal and vaginal samples	CD8 ⁺ T cells in PBMC†	ND	ND	ND	Macaque	Stevceva <i>et al.</i> (2002)

*Route employed for immunization: i.o., intraorally; i.n. intranasal; i.g. intragastrically; i.p., intraperitoneal; i.r. intrarectal.

†LN, lymph nodes; LP, lamina propia; MLN, mesenteric lymph nodes; PP, Peyer's patches; PBMC, peripheral blood mononuclear cells.

Table 2. Mucosal immunogenicity of poxvirus vectors during prime-boost immunization schemes

Prime vector/route*	Boost vector/route*	Recombinant antigen	Immune response induced				Protective immunity	Animal model	Reference
			Cellular		Humoral				
			Mucosal	Systemic	Mucosal	Systemic			
Influenza/i.n.	VACV/i.n.or i.p. MVA/i.n or i.p.	HIV-1 Env	CD8 ⁺ and Th cells in GRLN†	CD8 ⁺ and Th cells in spleen	ND	Serum IgG1 and IgG2a	ND	Mouse	Gherardi <i>et al.</i> (2003)
DNA/i.r.	MVA/i.r.	SIV Gag, SIV Pol HIV Env	CD8 ⁺ and CD4 ⁺ T-cells in colon	CD8 ⁺ and CD4 ⁺ T-cells in PBMC	IgA in rectal secretions	IgG	Delayed progression to AIDS, after challenge with SHIV 89.6P	Rhesus macaques	Wang <i>et al.</i> (2004)

DNA/i.m., i.r and i.o.	MVA/i.m., i.r and i.o.	HIV Env, Gag, RT, Rev, tat and nef	ND	CD8 ⁺ and Th- cells in PBMC	No IgG nor IgA in saliva or rectal washes	Weak serum IgG responses	Reduction of SHIV virus burden, after i.v. challenge	Rhesus macaques	Makitalo <i>et al.</i> (2004)
BCG/i.n.	MVA/i.n.	<i>M.</i> <i>tuberculosis</i> antigen 85A	CD8 ⁺ and CD4 ⁺ T-cell responses in LLN†	CD8 ⁺ and CD4 ⁺ T-cell responses in spleen	ND	ND	High levels of protection in both lungs and spleen, after aerosol challenge with <i>M.</i> <i>tuberculosis</i>	Mouse	Goonetilleke <i>et al.</i> (2003)
DNA+CT/i.n.	MVA+CT/i.n.	HIV-1 Env	CD8 ⁺ T-cell responses in GRLN† and GT†	CD8 ⁺ T-cell response in spleen	IgA and IgG in vaginal washes	Serum IgG1 and IgG2a	ND	Mouse	Gherardi <i>et al.</i> (2004)
DNA+IL2/i.o.	VACV	HIV-1 Env	Th-cell responses in LP†	Th and CTL responses in spleen	IgA in stool, saliva and vaginal washes	Serum neutralizing Abs	ND	Mouse	Wierzbicki <i>et al.</i> (2002)
SHIV DNA +IL2/i.n.	MVA	SIV Gag, Pol and HIV Env 89.6P	CD8 ⁺ T-cells in MNC† from rectal mucosa	CD8 ⁺ T-cells in PBMC†	IgA in rectal secretions	Non-systemic IgG	Significant protection from disease progression after rectal challenge with SHIV 89.6P	Macaque	Bertley <i>et al.</i> (2004)

*Route employed for immunization: i.o., intraorally; i.m., intramuscular; i.n. intranasal; i.r. intrarectal.

†GRLN, genito-rectal lymph nodes; GT, genital tract; LLN, lung lymph nodes; LP, lamina propia; MNC, mononuclear cells; PBMC, peripheral blood mononuclear cells.