Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 11349–11353, October 1996
Colloquium Paper

This paper was presented at a colloquium entitled "Genetic Engineering of Viruses and Virus Vectors," organized by Bernard Roizman and Peter Palese (Co-chairs), held June 9–11, 1996, at the National Academy of Sciences in Irvine, CA.

Applications of pox virus vectors to vaccination: An update

(vaccinia/fowlpox/canarypox/NYVAC vaccinia)

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ABSTRACT **Recombinant pox viruses have been generated for vaccination against heterologous pathogens. Amongst these, the following are notable examples. (*i*) The engineering of the Copenhagen strain of vaccinia virus to express the rabies virus glycoprotein. When applied in baits, this recombinant has been shown to vaccinate the red fox in Europe and raccoons in the United States, stemming the spread of rabies virus infection in the wild. (*ii*) A fowlpox-based recombinant expressing the Newcastle disease virus fusion and hemagglutinin glycoproteins has been shown to protect commercial broiler chickens for their lifetime when the vaccine was administered at 1 day of age, even in the presence of maternal immunity against either the Newcastle disease virus or the pox vector. (*iii*) Recombinants of canarypox virus, which is restricted for replication to avian species, have provided protection against rabies virus challenge in cats and dogs, against canine distemper virus, feline leukemia virus, and equine influenza virus disease. In humans, canarypox virus-based recombinants expressing antigens from rabies virus, Japanese encephalitis virus, and HIV have been shown to be safe and immunogenic. (*iv*) A highly attenuated vaccinia derivative, NYVAC, has been engineered to express antigens from both animal and human pathogens. Safety and immunogenicity of NYVAC-based recombinants expressing the rabies virus glycoprotein, a polyprotein from Japanese encephalitis virus, and seven antigens from *Plasmodium falciparum* have been demonstrated to be safe and immunogenic in early human vaccine studies.**

The notion that the work of Edward Jenner could be carried on after the successful global eradication of smallpox as a human infectious disease was provided by early descriptions of the engineering of vaccinia virus to express foreign genes (1, 2). Thus, by splicing genes from heterologous pathogens into the vaccinia virus vector one could immunize against that cognate pathogen. The 14 years since those publications were an exciting period where numerous strains of vaccinia were engineered to express a variety of antigens from a myriad of bacterial, viral, and parasitic pathogens with subsequent evaluation of the recombinants in both animal models as well as target species. Initial safety concerns of vaccinia virus vectors have been addressed by the use of highly attenuated replication-deficient strains of the virus as well as the engineering of host range-restricted pox viruses such as canarypox virus that, while restricted for productive replication to avian species, have been shown to effectively vaccinate nonavian targets. The initial studies on vaccinia virus were extended to other members of the pox virus family so as to provide species specific vectors. An example of this is the engineering of fowlpox-based vectors for use as recombinant vaccines in the poultry industry. Much information has been gained through this period and today some commercial success has been evidenced by the licensing of several products in the veterinary field. Today, in addition to continued work in this area for vaccines, pox virus-based vectors remain as eminent tools for studying the parameters of immune induction and new fields of endeavor are being investigated such as in cancer immunotherapy.

This paper will provide an update, albeit incomplete, of ongoing research with pox virus-based vectors.

Vaccinia-Rabies Glycoprotein G Recombinant

A vaccinia recombinant expressing the rabies virus glycoprotein was an early example of a successful pox virus vector useful in immunization (3). The vector was constructed by the insertion of the encoding cDNA for the rabies virus glycoprotein in the thymidine kinase locus of the Copenhagen strain of vaccinia virus. Disruption of the thymidine kinase locus allowed a biochemical selection of the recombinant as well as an attenuated phenotype to the vector. This recombinant has received a conditional commercial license in both Europe and in the United States.

The recombinant is administered as a live vaccine in baits for oral uptake by foxes in Europe and by raccoons in the United States. Extensive seeding of large geographic regions has provided field safety and efficacy. More recently, vaccine baits for controlling an epizootic of rabies in coyotes and grey fox in Texas has involved the seeding by air of more than 40,000 square miles with this recombinant vaccine.

Fowlpox Virus-Based Recombinants

The engineering of fowlpox virus-based vectors has direct application for recombinant vaccines in the poultry industry. Fowlpox virus is a pathogen in poultry. Attenuated fowlpox virus vaccines have been used for decades in the poultry industry to prevent wild-type virus infection. These attenuated fowlpox vaccine strains provide starting material for further construction of recombinant vaccines. The vector approach in poultry is confronted by issues similar to the general vaccine discipline and specifically to the vector approach. One such issue is how will preexisting maternal immunity influence the outcome of vaccination with a recombinant vector approach. In the poultry industry, this problem is generally twofold since the mother is immune to both the pathogen whose genes are to be expressed in the vector and to the fowlpox vector itself. The results of such a situation are detailed in ref. 5, where a fowlpox virus recombinant expressing the hemagglutinin neuraminidase and the fusion glycoproteins of Newcastle disease virus (NDV) are studied. A single inoculation in specific

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Abbreviations: NDV, Newcastle disease virus; JEV, Japanese encephalitis virus; PRV, Pseudorabies virus; FeLV, feline leukemia virus; CTL, cytotoxic T lymphocyte.

pathogen-free birds at 1 day of age provided hemagglutinin-inhibiting antibodies that were maintained for the 8-week test period, which is the lifespan of a commercial broiler. Protective immunity was demonstrated against a combined intramuscular velogenic NDV challenge and a respiratory NDV challenge. Significantly, vaccination of commercial broiler chickens that retained a level of maternal immunity against both NDV and the vector resisted a subsequent challenge against both a lethal intramuscular NDV challenge, as well as a virulent fowlpox virus challenge. However, the NDV-specific immune response was at a reduced level. A fowlpox virus recombinant expressing NDV glycoproteins has received commercial licensure in the United States.

Avipox Virus Vectors in Nonavian Species

Members of the *Avipox* genus such as fowlpox and canarypox are distinguished by their host restriction for replication to avian species. It was discovered that inoculation of avipox-based recombinants into mammalian cells resulted in expression of the foreign gene and that inoculation into mammals resulted in the induction of protective immunity (6, 7). This surprising finding provided a significant safety profile to these vectors. Immunization could be affected in the absence of productive replication while eliminating the potential for dissemination of the vector within the vaccinate and, therefore, the spread of the vector to nonvaccinated contacts or to the general environment.

For reasons still not understood, it was demonstrated that a recombinant canarypox vector was a 100 times more efficient than a comparable fowlpox vector in inducing protective immunity and similar to a thymidine kinase-disrupted replication competent vaccinia virus vector (8).

Numerous examples have now been provided demonstrating the safety, immunogenicity, and protective efficacy of canarypox-based recombinants in both experimental animal models and target species. A prime example has used canarypox-based recombinants expressing the rabies virus glycoprotein G. Rabies virus infection and immunization are issues for both veterinary and human medicine. A great deal of information is available in rabies virus immunization, experimental animals and target species are readily available for study, and the parameters of successful immunization are understood. The safety and immunogenicity of a canarypox-based rabies glycoprotein recombinant was demonstrated in a number of nonavian species (9). Protection of vaccinated experimental animals or target species cats and dogs was demonstrated.

To appreciate the duration of immunity that could be engendered by vaccination with a canarypox-based recombinant, naive beagles were vaccinated by a single subcutaneous dose of the vaccine followed by rabies challenge with rabies virus. All vaccinated dogs seroconverted with maximal titers at 1 month. At various times after vaccination, a subset of dogs was challenged. At 6 and 12 months postvaccination, all dogs vaccinated with a single dose of the vaccine resisted challenge that was lethal to all the control animals. At 24 months after vaccination, 11 of 12 vaccinated dogs survived challenge with similar protection observed at 36 months postvaccination (10). These studies demonstrated that a single vaccination was immunogenic and that a protective immune response was primed such that recall as long as 3 years later was protective against a rabies virus challenge in the target species.

Successful vaccination in the presence of rabies-specific maternal antibodies was demonstrated in the following experiment using beagles. A worst scenario situation was established wherein pregnant bitches with immunity to rabies were revaccinated 2 weeks before whelping to maximize the antirabies antibody titers transferred from the bitch to the offspring. At 2 weeks after birth, the pups were vaccinated with a single dose of a canarypox-based rabies vaccine recombinant. Serological responses were followed to monitor either the decay of maternal antibodies in the nonvaccinated control pups or the effect on antibody titers on the pups vaccinated in the presence of maternal antibodies. At 3 months, immunity was challenged by inoculation of live rabies virus in the temporal muscle. The maternal antibody titer in the unvaccinated pups decayed with the expected kinetics. Pups vaccinated with the recombinant virus showed a slight increase in rabies virus neutralizing titer at 2 weeks postvaccination that fell to undetectable levels at the time of challenge. In a vaccine dose-dependent fashion, pups immunized in the presence of maternal immunity survived the rabies virus challenge that was lethal to all the nonvaccinated pups (10). This study demonstrated that young animals could be successfully vaccinated in the presence of maternal immunity.

The concept of using a nonreplicating avipox virus vector, a canarypox-based rabies recombinant, has been evaluated for safety and immunogenicity in human clinical studies (11, 12). Rabies naive healthy adult volunteers were inoculated with increasing doses of the recombinant in a schedule including a boost at 1 and 6 months. For comparison, the standard inactivated human diploid cell rabies vaccine was used. All inoculations with the recombinant canarypox vaccine were well-tolerated with only mild and short-lived reactions at the inoculation site reported. In these two clinical trials, induction of antirabies immune responses were demonstrated, and it was demonstrated that canarypox recombinants could be used either by themselves or in a protocol wherein the priming vaccination with the vector could be followed by a booster with the inactivated rabies vaccine.

Although the immune responses to the experimental canarypox recombinant were comparable but not demonstrated to be superior to those obtained with the standard inactivated rabies vaccine, it perhaps is not surprising given the relative low doses of the recombinant vaccine used in these studies and the comparison with an optimized and highly immunogenic licensed vaccine.

Other examples demonstrating the utility of canarypox virus-based vectors for veterinary species have been provided. Canarypox virus recombinants expressing the measles virus fusion and hemagglutinin glycoproteins have been used to vaccinate dogs. Comparison of these recombinants with vaccinia virus vectors expressing the same genes were shown to provide similar levels of immune response and protection against a challenge with the related Morbilli virus, canine distemper (13).

Construction of specific canine distemper virus recombinants expressing the fusion and hemagglutinin have been evaluated in the highly susceptible ferret model and dog host and were demonstrated to provide protection against challenge (unpublished data).

Canarypox-based recombinants expressing the hemagglutinin from equine influenza virus were shown to be immunogenic when inoculated in horses and provided protection against a naturally occurring equine influenza virus infection (14).

Two canarypox virus-based recombinants were constructed, each expressing the entire *gag* gene and either the intact subgroup A *envelope* of feline leukemia virus (FeLV) or a modified version of the *envelope* from which the putative immunosuppressive region was deleted (15). These recombinants were evaluated for protective efficacy in kittens of 8–9 weeks of age. Two inoculations of the recombinants at 5 and 2 weeks before challenge failed to induce measurable FeLV neutralizing antibodies. Nevertheless, 50% of the cats receiving the mutated *envelope* recombinant and 100% of the cats receiving the intact *envelope* recombinant were protected against an oronasal challenge with the FeLV-A/Glasgow-1 isolate. Protection was assessed by evaluating p27 antigenimea, detecting FeLV antigen in blood smears, and the attempted recovery of infectious FeLV. This was the first description of

a successful immunization against a retrovirus provided by pox virus-based recombinants.

The above observations provided an impetus to further investigate the potential of canarypox-based vectors for immunization against other retrovirus with particular attention on the lentiviruses with focus on HIV, the causative infectious agent of AIDS. The entire *envelope* protein of the human T-cell leukemia/lymphoma virus type I was expressed in a canarypox virus vector. Two inoculations of the recombinant vaccine candidate were administered to rabbits. Five months after the last inoculation, the animals were exposed to a human T-cell leukemia type-I cell associated challenge from a primary culture of the *bou* isolate. The animals were protected. The protected animals were again challenged 5 months after the initial challenge exposure with 5 ml of blood from an infected rabbit. Immunity failed this relatively large challenge exposure. Of interest in these studies (16) was the observation that if a subunit *envelope* booster was administered in alum after the priming vaccination with the canarypox recombinant protection was not obtained. Interpretation of this observation can lead to interesting speculation.

Other interesting observations using canarypox-based recombinants expressing antigens from either HIV-I or II, as well as simian immunodeficiency virus, have been reported. In laboratory rodents, induction of both humoral immunity as well as cytotoxic T lymphocyte (CTL) can readily be demonstrated (17).

Recombinants expressing HIV-II *gag*, *pol*, or *envelope* genes have been evaluated in macaques in several studies with some level of protection described (18, 19). Significant and raising concerns for those involved in vaccine development correlates of protective immunity are not revealed in these studies. Multiple immunization allowing for the maturation of the immune response is suggested by some studies (20). An intriguing observation was the cross protection against HIV-II challenge in monkeys vaccinated with HIV-1 recombinant pox viruses (21). A likely interpretation of this data is the induction of and protection by cross-reactive CTL. However, the basis of this cross protection is currently unknown.

A series of recombinant canarypox virus-based recombinants expressing an increasing complexity of HIV-I strain MN antigens have been constructed and evaluated in human clinical trials for both safety and immunogenicity. The earliest of these studies in HIV seronegative healthy adult volunteers have been reported (22). A vaccine regimen providing the best results to date involve one or two doses of the recombinant canarypox virus vector followed by one or two doses of an adjuvanted recombinant *envelope* subunit. The induction of binding, HIV neutralizing, and both CD4 and CD8 CTL have been reported (22–24).

More recent data using the more complex recombinants and higher doses of vaccine in a vector prime/subunit antigen boost protocol have demonstrated better levels of neutralizing antibody induction and a more complex reactivity of CTL to multiple HIV antigens. Further comparison of separate phase I trial data a prime/boost protocol using the canarypox vector fares favorably when compared with a prime boost protocol using a replication competent vaccine vector as a primer (unpublished data). In this light, the failure of the canarypox vector to replicate in the mammalian host provides advantage over the replication competent vaccinia virus vector. The general safety profile of the HIV-I canarypox recombinants in human volunteers is similar to that observed with the canarypox recombinants expressing the rabies virus glycoprotein discussed above.

Attenuated Vaccinia-Based Vector: NYVAC

The global smallpox eradication program was made possible by several biological features of the pathogen and the vaccine. The pathogen had only a single host for infection and propagation—man. There were no animal reservoirs from which the pathogen could recrudesce. Defined outbreaks of the infection could be circumscribed and contained by vaccination. Vaccinia, the vaccine, could be produced efficiently and at low cost in regional centers. The ability to retain potency of the vaccine as a freeze-dried preparation allowed storage and transport to remote regions of the globe. The successful smallpox eradication program, however, was not without vaccine-associated risk. Vaccine reactogenicity with some severe or lethal outcomes was associated with the vaccine in general and specifically higher rates of adverse events were evidenced in certain populations or with certain vaccine strains or preparations. Early attempts to manufacture the vaccine under more defined and regulated laboratory conditions were abandoned with the success of the eradication effort. The known reactogenicity of the vaccinia vaccine was therefore a concern to be addressed when the virus was proposed as a vector for new engineered vaccines. This concern has been addressed in several ways such as the provision of naturally host-restricted vectors described above or by the targeted attenuation of existing vaccine strains. This approach is demonstrated by the engineering of the NYVAC strain of vaccinia virus. The Copenhagen strain of vaccinia was chosen as a vaccine substrate. The entire DNA sequence of the genome was established (25). With this information and the extant knowledge of virulence-related and other genetic functions related to host range replication competency unwanted genetic information was precisely deleted from the vaccinia virus genome. The resultant vector, NYVAC, was highly attenuated as demonstrated in a series of studies in animal surrogates (26). Intracranial inoculation of newborn or young adult mice demonstrated a very favorable dose range compared with either the parent or other vaccine strains, and significantly no disseminated viral infection was observed in immunocompromised hosts. In numerous tissue culture cells of human origin, the vector was shown to be highly debilitated for replication consistent with the deletion of host range genes. The modified NYVAC vector, while highly attenuated, retained the ability to induce protective immune responses to foreign antigens in a fashion similar to the thymidine kinase mutant of the parent strain.

A number of examples using the NYVAC vector as a recombinant vaccine delivery system have been provided in animal model systems and in target species including humans. A series of NYVAC recombinants were generated to express glycoproteins from Pseudorabies virus (PRV) and the immunity afforded by these recombinants was evaluated in the target species of PRV infection, the pig. PRV neutralizing antibodies were induced following two intramuscular inoculations 28 days apart. The NYVAC recombinant expressing the PRV glycoprotein gp50 induced levels of PRV neutralizing antibodies and afforded protection against a virulent oronasal PRV challenge that was comparable to vaccination with inactivated PRV vaccine (27). The advantage of a recombinant vaccine is that one is allowed to discriminate between a naturally infected versus vaccinated animal since the recombinant vaccine displays a defined subset of the antigens of the pathogens. This discrimination allows the agricultural industry to properly track infections and cull infected herds.

A NYVAC-based recombinant expressing two hemagglutinin glycoproteins of the A1 and A2 equine influenza serotypes induced hemagglutinin inhibiting antibodies when inoculated into horses and afforded significant protection when the vaccinated horses became exposed to a natural equine influenza virus infection (14).

The polyprotein of Japanese encephalitis virus (JEV) encoding prM/M, E, and NS1 was expressed in NYVAC recombinants and the vector used to vaccinate swine, a major natural host of JEV infection and a reservoir for mosquito transmis-

sion of the virus to man. Hemagglutinin-inhibiting and JEV-neutralizing antibodies were induced on vaccination. The nonvaccinated challenged animals succumbed to JEV infection, whereas the vaccinated group had levels of JEV challenge viremia insufficient to be transmitted by mosquitoes (28) Both a NYVAC- and a canarypox-based Japanese encephalitis recombinant are currently being evaluated in human clinical trials.

A NYVAC vector has been engineered to express the rabies glycoprotein gene. In mice, cats, and dogs, the recombinant was shown to be safe and to provide protection against a lethal rabies virus challenge. The recombinant is now being evaluated in phase I human clinical trials for safety and immunogenicity.

Pox virus vectors have been used to determine the immunogenic potential of antigens from *Plasmodium* spp. in an effort to understand the design of an effective vaccine against malarial infections. In this regard a NYVAC vector reconstituted with the K1L host range gene was constructed to express intact or mutated forms of the circumsporozoite protein of *Plasmodium berghei*. Vaccination of the target host, the mouse, induced both binding antibody and CTL. Vaccinated and control mice were challenged either by the intravenous injection of sporozoites or by allowing infected mosquitoes to feed on the subjects. Protection was scored as the absence of blood stage parasetemia as determined by microscopic analysis of blood films from individual mice from 5–15 days after challenge. In a number of challenge experiments, ≈80% protection was obtained. This is to be compared with the consistent 100% level of protection obtained by vaccination with irradiated sporozoites. Protection in the recombinant virus-immunized mice apparently did not correlate with antibodies but a good correlation was established between CTL and protection. *In vivo* antibody depletion of $CD8^+$ T cells before challenge abrogated protection (29).

With this data as an inducement, a complex NYVAC-based recombinant was constructed to express multiple antigens from *P. falciparum*. To address the multiple stages of the parasite life cycle, multiple antigens from the various stages were used. Thus, a recombinant expressing seven parasite antigens was provided. This recombinant was evaluated in rodents and in monkeys where safety and immunogenicity were established (30). This recombinant is now being evaluated in clinical trials where the vaccinated subjects are exposed to the bites of infected mosquitoes. Appearance of parasites in the blood of the infected volunteers will terminate the challenge followed by administration of antimalarial drugs to thwart further replication of the parasite. Since ethical and medical considerations require treatment on appearance of blood-stage parasites, only the antisporozoite and liver-stage immunity engendered by the vaccine can be evaluated. Full evaluation of blood-stage and transmission-blocking immunity cannot be evaluated in this limited clinical setting.

To date, all the above-mentioned abstracted data provided from human clinical trials using NYVAC-based vectors have described a good safety profile and the induction of some level of immunity to the expressed heterologous antigens.

Other Applications of Pox Virus-Based Vectors

The use of pox virus-based vectors as recombinant vaccines for heterologous bacterial, viral, or parasitic pathogens was the first practical application of this technology deriving from the fact that vaccinia virus was an established vaccine. However, the pox virus vectors can be looked at as general delivery systems for genes for other applications. For example, these vectors can be used *in vitro* to stimulate and expand CTL reactivities from the peripheral blood of chronically infected or tumor-bearing individuals (31). The antigen-specific stimulation and expansion of such cultures might provide some therapeutic benefit when reintroduced to the donor patient.

For cancer immunotherapy, numerous pox virus-based recombinants expressing tumor-associated antigens or biological response modifiers have been described (32). Of particular note, recombinants expressing the carcinoembryonic antigen were shown to elicit both antibody and cellular immune responses in mice and monkeys and to protect mice from tumor cell challenge (33, 34). Whether vaccinia or canarypox-based recombinants expressing the carcinoembryonic antigen will have any therapeutic benefit is currently being investigated in the clinic in patients with colorectal carcinomas.

A recent publication (4) reported the protection of mice vaccinated with a p53 expressing recombinant against challenge with an isogenic and highly tumorigenic mouse fibroblast tumor cell line expressing high levels of a mutant human p53 but lacking endogenous murine p53. Expression of the mutant form of p53 in the recombinant virus was not essential since the wild-type p53 afforded similar efficacy. This may be an important observation since p53 is an attractive target for cancer immunotherapy. Mutations of p53 represent the most common genetic changes demonstrated in human tumors.

Discussion

The excitement of the 1982 proposal to use pox virus-based vectors as heterologous vaccines and the ensuing years of extensive pursuit of this idea have provided numerous working examples in laboratory animal model systems as well as in target species. In the veterinary field, products have now been licensed for commercialization and a significant number of clinical studies have been and continue to be pursued for both infectious diseases, *ex vivo* therapies, and cancer immunotherapy. The immediate future looks to be as exciting as the recent past.

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