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Vaccinia Virus: A Tool for Research and Vaccine Development

BERNARD MOSS

Vaccinia virus is no longer needed for smallpox immunization, but now serves as a useful vector for expressing genes within the cytoplasm of eukaryotic cells. As a research tool, recombinant vaccinia viruses are used to synthesize biologically active proteins and analyze structure-function relations, determine the targets of humoral- and cell-mediated immunity, and investigate the immune responses needed for protection against specific infectious diseases. When more data on safety and efficacy are available, recombinant vaccinia and related poxviruses may be candidates for live vaccines and for cancer immunotherapy.

VACCINIA VIRUS WAS SUCCESSFULLY USED FOR THE PREvention of smallpox and the eradication of variola virus, the causative agent of that disease. The protective effect of vaccination was reported by Edward Jenner in 1798 (*1*). Vaccinia virus was initially isolated directly or indirectly from infected cows (Fig. 1, top). Subsequently, some preparations of vaccine came from pox lesions of horses and other lots were purportedly mixed with smallpox virus to increase their "potency"; after a nearly 200-year history, in man and animals, the derivation of present-day vaccinia virus is obscure (*2*). DNA analyses indicate that vaccinia virus is closely related to, but distinct from, variola virus, cowpox virus, and other members of the poxvirus family that occur naturally (*3*).

The novelty of the vaccination procedure led some contemporaries of Jenner to view the idea of using cowpox material with alarm (Fig. 1, bottom), a response similar to the greeting recombinant DNA methodology received in our own time. Nevertheless, the safety of vaccination compared to the practice of variolation (inoculation with small quantities of live variola virus) and its prophylactic value were soon evident. In 1801, Jenner predicted "that the annihilation of the Small Pox, the most dreadful scourge of the human species, must be the final result of this practice [vaccination]" (*4*). Implementation of the Intensified Smallpox Eradication Programme by the World Health Organization (WHO) began in 1967, a year in which there were 131,776 reported cases of smallpox in 31 different

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countries or territories (and probably 100 times that number of actual cases) (*4*). The last case of endemic smallpox occurred in Somalia in 1977; an authoritative history of this singular achievement is now available (*4*). Destruction of the remaining frozen samples of variola virus, after the completion of genomic sequencing, is planned in 1993 to ensure that the disease cannot recur.

WHO recommended in 1980 that smallpox vaccination be universally discontinued except for investigators at special risk of poxvirus infection. Continued studies on vaccinia virus and other members of the Orthopoxvirus genus were encouraged by WHO. Subsequent investigations have established new uses for them as tools for research in molecular biology, cell biology, and immunology. Most exciting, but controversial, are efforts to develop recombinant poxviruses as live vaccines to prevent unrelated diseases and for cancer immunotherapy.

Molecular Biology of Vaccinia Virus

The most unusual features of the poxvirus family of DNA viruses relate to their ability to replicate within the cytoplasm, rather than the nucleus, of the infected cell (*5*). Infectious virions are brick-shaped and 300 to 400 nm in diameter. A lipoprotein envelope surrounds the complex core structure that contains a linear 200,000 base pair (bp) duplex DNA molecule with hairpin loops connecting the two strands at each end. Also present within the core are virus-encoded enzymes, including a multisubunit DNA-dependent RNA polymerase, a transcription factor, capping and methylating enzymes, and a poly(A) polymerase, all of which form a complete apparatus for the synthesis of translatable mRNA. After viral attachment to the cell and membrane fusion, the core enters the cytoplasm and approximately 100 early genes are transcribed by the viral RNA polymerase (Fig. 2). During poxvirus DNA replication, concatemeric intermediates that are resolved into unit-length genomes are formed. The replicated DNA molecules provide the templates for the successive expression of intermediate and late genes. Each temporal class of genes has its own characteristic promoter sequences that are recognized by specific viral proteins. After synthesis of the late structural proteins, progeny virus particles are assembled and some are released with an additional Golgi-derived membrane, thereby completing the infectious cycle (Fig. 2). Nuclear factors, possibly RNA polymerase II, may be involved in the formation of mature, infectious virions (*6*).

Poxviruses provide a system for combining biochemical and genetic approaches to the study of transcription (*7*) and replication (*8*) because (i) most or all of the needed enzymes and factors are encoded within the 200,000-bp genome; (ii) RNA and DNA synthesis occur in the cytoplasm and hence are physically separated from the corresponding host events in the cell nucleus; (iii) early transcription components are present in a partially purified state within the virus core; and (iv) virus mutants are readily isolated. Thus, many viral genes involved in the life cycle of poxviruses have been identified. The availability of the sequence of the vaccinia virus genome (*9*) will facilitate the identification of additional genes.

Poxviruses are also valuable for investigating virus-host interactions, since a number of genes are devoted to such activities (*10*). Several of the vaccinia virus–encoded proteins involved in host interactions are secreted from infected cells; these include a functional homolog of epidermal growth factor, a complement control protein, and a 13.8-kD protein that is associated with virulence. Three genes with homology to serine protease inhibitors are present in vaccinia or cowpox virus, and an involvement of one in modulation of the host inflammatory response has been demonstrated. Several host range genes have been discovered, but other genes that confer resistance to interferon and inhibit host macromolecular synthesis are not yet identified. Poxviruses that cause tumors will probably be a source of additional genes involved in cell proliferation and suppression of immune responses.

Construction of Expression Vectors

The study of vaccinia virus has provided many insights into biological mechanisms. Nevertheless, the expanded interest in the virus is primarily because of its development as a vector for the expression of foreign genes. Homologous DNA recombination occurs naturally during the replication of poxviruses (*11*); thus, vaccinia virus genes can be mapped by marker rescue (*12*) and foreign DNA can be inserted into the viral genome (*13*). As a first step in the insertion process, a foreign gene is flanked with vaccinia virus DNA sequences and then the product, usually a plasmid, is introduced into a cell that has been infected with whole vaccinia virus (Fig. 3). The latter procedure, except for the use of whole virus instead of purified viral DNA, is similar to that used for nuclear DNA viruses (*14*); whole virus must be used for vaccinia virus because the DNA of poxviruses requires the enzymes and factors of the core for infection. Recombination leads to the insertion of the foreign DNA into about 0.1% of the progeny virus genomes. At

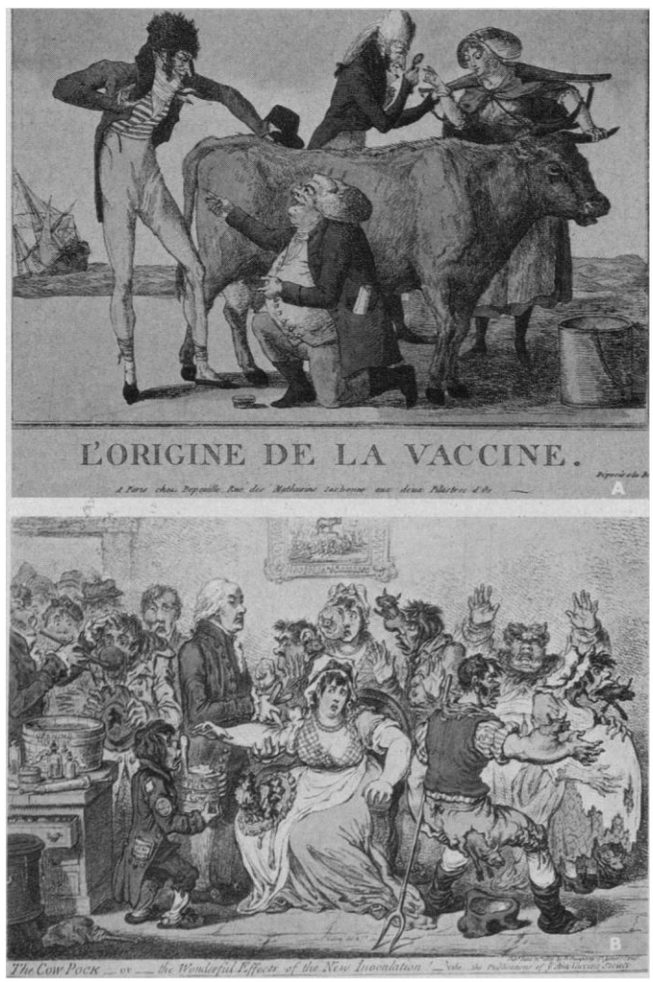

Fig. 1. Smallpox vaccination. (**Top**) French engraving from about 1800, depicting the origin of vaccinia virus. (**Bottom**) English engraving by James Gilray (1802) suggesting the alarm that greeted the introduction of the smallpox vaccine. [Photo courtesy of the National Library of Medicine]

least 25,000 bp of DNA can be added to the vaccinia virus genome without any deletions being required (*15*); this capacity exceeds that of other vectors used in mammalian cells and is sufficient for more than a dozen genes of average size. A nonessential region of the genome is usually targeted for insertion. Thus, the recombinant virus will be capable of independent replication.

The time of onset and amount of foreign gene expression obtained with vaccinia virus vectors is determined by the poxvirus promoter sequences. Therefore, expression directed by early promoters begins during the first few hours after infection but then usually diminishes, whereas expression directed by intermediate or late promoters is delayed until DNA replication occurs. Whether due to intrinsic promoter strength, number of templates, or prolonged duration of expression, the highest amounts of protein are obtained with late promoters (*16–18*).

Foreign genes must not contain introns because splicing does not occur in the cytoplasm (*19*). Early poxvirus genes signal transcriptional termination 20 to 50 nucleotides downstream of TTTTTNT sequences in the noncoding strand (*20*). Thus, cryptic termination sequences in foreign genes should be altered when early class promoters are used (*21*).

Special insertion or transfer vectors simplify the construction and isolation of recombinant viruses and allow consistently high expression of foreign genes (*16, 18, 22, 23*). These vectors consist of a plasmid with a segment of vaccinia virus DNA that is interrupted by an expression cassette consisting of a vaccinia virus promoter and unique restriction endonuclease sites for cleavage and ligation to the foreign gene. Because recombination is frequent, virus plaques can be screened by DNA hybridization or for expression of the foreign gene product (*24*). Nevertheless, selection or general screening procedures are very helpful (*16, 22, 25–28*). Frequently, procedures such as thymidine kinase (TK) negative selection and β-galactosidase expression are combined. Detailed laboratory protocols for making recombinant viruses are available (*29*).

Bacteriophage-Vaccinia Hybrid Systems

A powerful alternative approach to the construction of vaccinia virus expression vectors makes use of the efficient and specific single subunit DNA-dependent RNA polymerase of bacteriophage T7 and its close relatives. The bacteriophage T7 (*30–32*) or related bacteriophage T3 (*33*) RNA polymerase genes, under control of a vaccinia virus promoter, have been integrated into recombinant vaccinia viruses. Cells are infected, express functional bacteriophage RNA polymerase, and transcribe genes adjacent to the bacteriophage promoter. For analytical purposes, such as screening for mutations, the target genes can be delivered in plasmids by transfection. This avoids the additional steps required for production of recombinant viruses. The same or similar plasmids can be used for expression in *Escherichia coli* and in cell-free translation systems. For larger-scale expression, the gene regulated by the phage promoter has been integrated into the genome of a second vaccinia virus so that expression occurs when cells are doubly infected. An advantage of using a recombinant virus instead of a plasmid is that infection is much more efficient than transfection and can be done with many liters of cells.

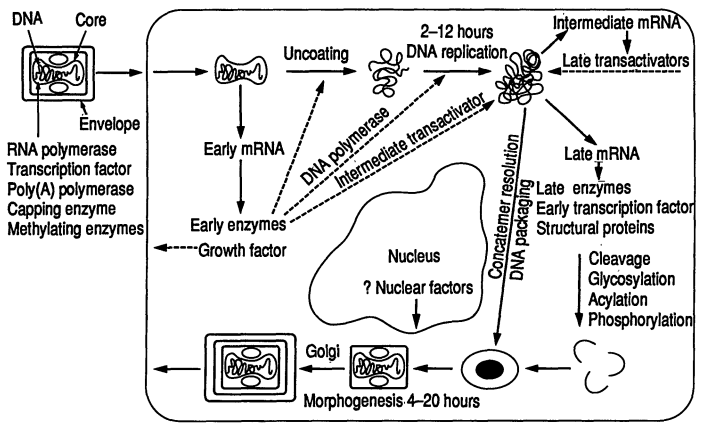

Fig. 2. The infectious cycle of vaccinia virus.

Although large amounts of RNA were made in this hybrid expression system, formation of the cap structure, which is naturally present at the 5′ ends of eukaryotic mRNAs and enhances ribosome binding, occurred inefficiently and restricted the amount of protein synthesized (*32*). To overcome this problem, the cardiovirus cap-independent mechanism of translation was used. Translation was considerably enhanced by incorporating a DNA copy of a cardiovirus untranslated leader sequence downstream of the T7 promoter (*34*). Under favorable conditions more than 10% of the total cell protein was the recombinant protein within 24 hours.

The use of two separate recombinant viruses to produce polymerase and template presents no problem for laboratory studies and may avoid expression of potentially toxic proteins during preparation of recombinant virus stocks. Nevertheless, the double infection protocol requires accurate determination of virus titers and adds to the expense of large-scale production. Vaccinia virus with both the T7 RNA polymerase and a T7 promoter–regulated gene in the same viral genome seems to have poor viability (*31*). Two independent solutions to the problem were developed. Cell lines with integrated copies of the bacteriophage RNA polymerase gene have been constructed (*35, 36*), and these can be infected with a single recombinant vaccinia virus that contains the bacteriophage promoter-regulated foreign gene. The other approach was to limit expression of the T7 RNA polymerase gene with the *E. coli lac* operator-repressor system until inducer was added (*37*).

Experimental Applications

In general, proteins synthesized by vaccinia virus vectors are biologically active and are processed and transported in accord with the primary structure of the protein and the inherent capabilities of the host cell. The wide host range of vaccinia virus provides considerable latitude in choosing cells. As vaccinia virus proteins normally undergo a variety of post-translational modifications (*38*), it is not surprising that *N*- and *O*-glycosylation, phosphorylation, myristylation, proteolytic cleavage, polarized membrane and nuclear transport, and secretion also occur with recombinant proteins (*39*).

Simple transfection-infection protocols can be used for analytical studies and may have advantages over transient expression systems that depend on the transport of plasmids to the nucleus. When cells infected with vaccinia virus are transfected with a plasmid that contains a late vaccinia promoter–regulated gene, it is expressed in the cytoplasm (*40*). Alternatively, the hybrid bacteriophage T7-vaccinia system is particularly convenient for transient expression. Thus, genes cloned into plasmids with bacteriophage T7 promoters can be expressed in cells infected with a recombinant vaccinia virus that contains the T7 RNA polymerase gene. Use of the untranslated leader region of encephalomyocarditis virus, a cardiovirus, is recommended for maximum expression. Either the calcium phosphate (*30*) or the cationic lipid (*41*) methods may be used for plasmid transfection, with greater than 90% of the cells expressing the gene (*42*). Cell lines that stably express bacteriophage RNA polymerase

still require vaccinia virus infection for high-efficiency transient expression (*36, 43*). This transfection system has been applied to analysis of the interactions of CD4 with the envelope glycoprotein from human immunodeficiency virus (HIV) (*44, 45*) and a membrane-associated tyrosine kinase (*46*), the rescue of temperature-sensitive virus mutants (*47*), epitope mapping of monoclonal antibodies (*48*), virus-mediated cell fusion (*49*), virus assembly (*41*), and expression of voltage-gated ion channels (*50*), neurotransmitter receptors (*51*), and the cystic fibrosis transmembrane conductance regulator (*52*). Several plasmids can be transfected simultaneously: all five proteins of vesicular stomatitis virus were adequately expressed from cloned cDNAs, which led to the replication, assembly, and budding of defective interfering particles (*53*). Although vaccinia virus cannot replicate in amphibian oocytes, some viral protein synthesis occurs (*54*); oocytes can be injected with virus and plasmids to obtain transient expression of bacteriophage T7 promoter–regulated genes (*50*).

If the same protein is to be expressed on repeated occasions or in large amounts, a recombinant virus containing the foreign gene can be used. Similar amounts of protein can be made with the use of a strong vaccinia promoter or a bacteriophage promoter for gene expression. If the expressed protein exhibits viral or cellular toxicity, then the bacteriophage T7–vaccinia hybrid system is preferred. Although vaccinia vectors are most commonly used for small-scale production, the unenhanced T7-vaccinia hybrid system synthesized the full-length membrane-associated envelope protein of HIV-1 with yields of about 11.2 mg per liter in Vero-cell microcarrier cultures of 40 liters (*55*).

Vaccine development usually requires prior identification of the type and target of protective immune responses. Information of this nature can be obtained by immunizing animals with recombinant vaccinia viruses that express genes of an infectious agent. This approach is simple, because protein isolation is unnecessary, and is valuable for proteins that may lose conformational epitopes when extracted or purified. The antibody response may be manipulated by altering the presentation of a protein. For example, the addition of signal and anchor sequences moves certain proteins to the plasma membrane and results in an increased antibody response (*56*). Immunization with recombinant vaccinia viruses is also a convenient way to produce monoclonal antibodies to native gene products (*57*).

The principal difficulty in determining the targets of CD8-bearing cytotoxic T lymphocytes (CTLs) is that antigens must be processed intracellularly and presented in association with matched major histocompatability complex (MHC) class I molecules for recognition. Recombinant vaccinia virus vectors can be used to infect autologous or MHC-matched target cells, which then express the test antigen. The targets are labeled with chromium and then incubated with lymphocytes from a virus-infected human (*58*) or experimental animal (*59*). The advantage of this method over making stable transfectants is that a single recombinant vaccinia virus can be used to infect cells from a variety of individuals. Human B cells can be immortalized with Epstein-Barr virus to provide a stable source of autologous cells. Recombinant vaccinia viruses can also be used to prime CTLs in vivo and provide an experimental system for studying their protective effects. Antigen presentation by class I molecules may be diminished at late times in infection (*60*), and therefore the use of early (or tandem early-late) promoters is recommended. The methods for using recombinant vaccinia viruses for CTL studies, possible pitfalls, and a list of references are provided in a recent review (*61*).

Influenza CTL targets were comprehensively analyzed with a panel of recombinant vaccinia viruses that express each of the 10 known influenza virus genes (*62*). At least eight were recognized by CTLs from one or more of seven different mouse strains (*63*). Neither the amount of the protein nor its intracellular location was the determining factor in its ability to serve as a target. The same panel of recombinant vaccinia viruses was used for human CTL studies, and similar conclusions were reached (*64*). Peptides of 15 amino acids could be presented to class I–restricted CTLs, a result that suggests that no additional sequences are needed for peptide transport to the endoplasmic reticulum, where binding to MHC is believed to occur (*65*).

Recombinant vaccinia viruses are used to study the CTL response during various stages of acquired immunodeficiency disease syndrome (AIDS). CTLs typically exist only transiently after infection with most viruses, but individuals infected with HIV-1 have persistent circulating CTLs (*66*). Both structural, enzymatic, and regulatory HIV-1 proteins serve as CTL targets (*66–69*). Panels of recombinant vaccinia viruses expressing truncated HIV-1 proteins provided a means of localizing epitopes (*68, 70*). Although the use of vaccinia virus to determine the targets of MHC class I–restricted CTLs has been emphasized here, vaccinia vectors are also used to analyze the presentation of endogenous antigens with MHC class II molecules (*71*).

Experimental animals inoculated with recombinant vaccinia viruses that express genes from many DNA and RNA virus families are usually partially or completely protected against disease caused by subsequent infection. For influenza, parainfluenza, respiratory syncytial, measles, Japanese encephalitis, rabies, rinderpest, Venezuelan equine encephalitis, vesicular stomatitis, herpes simplex, pseudorabies, and Epstein-Barr viruses, protection induced by the recombinant vaccinia vector correlates with neutralizing antibody to viral envelope proteins (*39, 72*). Sometimes, as in the case of chimpanzees inoculated with a recombinant vaccinia virus that expresses hepatitis B surface antigen, vaccination provided only a priming effect so that protection was associated with an anamnestic antibody response (*73*). In a few instances, such as with murine cytomegalovirus, lymphocytic choriomeningitis virus, and possibly Friend leukemia virus, protection was correlated with CTL rather than neutralizing antibody (*74, 75*).

The site of inoculation may have a significant effect on the immune response. Nasal inoculations with recombinant vaccinia viruses that express envelope glycoproteins from influenza and respiratory syncytial viruses provide better protection against upper respiratory infections than do intradermal inoculations, but both routes protect against lower respiratory infection (*76*). Mucosal immunity also was achieved by intestinal inoculation with recombinant vaccinia virus (*77*).

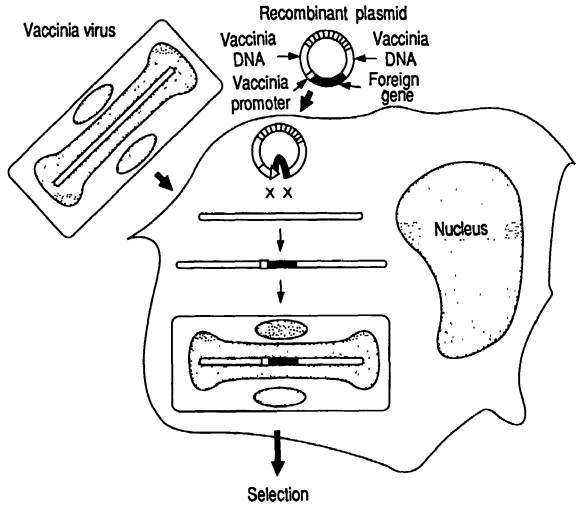

Fig. 3. Formation of a recombinant vaccinia virus by homologous recombination.

Recombinant vaccinia viruses may be used to express genes of nonviral pathogens including bacteria, rickettsia, and protozoa (*39*); in some cases protection of experimental animals was demonstrated (*78*).

Experimental tumor prophylaxis or therapy has made use of vaccinia virus vectors. These include recombinants that express the envelope glycoprotein gene of Friend leukemia virus (*75*), the T antigen genes of polyoma virus (*79*), the rat *neu* oncogene (*80*), and human melanoma-associated antigen gene (*81*). The protection provided in these models was probably due to cell-mediated immunity.

Recombinant Vaccinia Viruses as Live Vaccines

The concept of recombinant vaccinia virus as a live vaccine (*82*) stems from its successful use against smallpox. Those properties of vaccinia virus that contributed to its effectiveness as a smallpox vaccine included stimulation of humoral- and cell-mediated immunity, low cost, heat stability, simple methods of administration, visible proof of successful vaccination, and a long duration of effectiveness after a single dose (*4*). Additionally, the host range of vaccinia viruses (or other poxviruses) would make them suitable for veterinary purposes. Several different genes can be stably integrated into a single recombinant vaccinia virus (*83*) to obtain protection against multiple pathogens (*84*).

The smallpox vaccine approved for use in the United States was made in calf skin; it was not sterile and undoubtedly would not meet modern standards of purity and safety were it introduced today. Tissue culture production and evidence for the absence of adventitious agents would be required. The adverse reaction rates of about 1 in 50,000 that were reported for smallpox vaccination (*4*), would also need improvement. Vaccination was contraindicated for infants with eczema or persons suffering from immune dysfunctions, and disseminated vaccinia occurred recently in one individual with acquired immune deficiency disease (*85*). Clearly, smallpox vaccination was previously acceptable because the disease it prevented was so dangerous. Several attenuated strains of vaccinia were produced by serial virus passage, but the lower potency of some strains and the risk of losing momentum in the drive to eradicate smallpox were factors in the failure to adopt any for general use (*4*). Now, genetic engineering methods can be used to more selectively alter the phenotype of the virus. Deletion of vaccinia virus genes encoding thymidine kinase (*86*), a growth factor (*87*), hemagglutinin (*88, 89*), 13.8-kD secreted protein (*90*), and ribonucleotide reductase (*91*) all decrease the virulence of vaccinia virus in experimental animals. The effects of these genetic alterations on the immunogenicity of the vector have not been adequately studied yet, but some combination of alterations will probably allow both safety and efficacy. As an alternative to mutated vaccinia, other members of the poxvirus family with restricted host range in mammals, such as avipoxviruses, are being tested as vaccine vectors (*92*).

The insertion of lymphokine genes into the genome of vaccinia virus can also decrease virulence. Recombinant vaccinia viruses that express interleukin-2 (IL-2) (*88, 93*) or interferon-γ (*94*) are less pathogenic than wild-type virus for immunodeficient athymic nude mice. IL-2–expressing virus is rapidly cleared by natural killer and other cells secreting interferon-γ (*95*). The skin lesions in immunocompetent monkeys produced by a recombinant vaccinia virus that expressed IL-2 were smaller than those made by nonexpressing recombinants (*96*). Apparently, IL-2 expression does not significantly decrease immunogenicity (*88, 96*).

While prevention is far better than cure, an effective treatment for generalized or progressive vaccinia infection could provide a safety net if an immunocompromised individual was inadvertantly vaccinated. Vaccinia immune globulin was probably beneficial in cases of eczema vaccinatum and generalized vaccinia but not for progressive vaccinia in which the immmunodeficiency was usually a cellular one (*97*). Several chemicals inhibit vaccinia replication in cell culture, but none are very effective in clinical circumstances, except perhaps methisazone (*98*). Because vaccinia virus encodes many enzymes, the potential for developing an effective chemotherapeutic agent should be higher than that for less complex viruses. Commercial interest in developing a chemotherapeutic agent that would be reserved for a rare complication, however, is probably low.

Improved safety cannot be overemphasized, but the immune response to the recombinant protein must also be increased. In several cases, antibody titers in higher primates were lower when compared to those in smaller animals. Higher protein expression, retention of a functional thymidine kinase gene, better presentation of the immunogen, and expression of various lymphokines are factors to consider for improving the vaccine.

In adults that have had a smallpox vaccination, existing immunity to vaccinia virus may restrict the replication of the vaccinia vectors and decrease the immune response to the recombinant protein. This is not a concern with young children, however, because smallpox vaccination ceased more than a decade ago. Should more than one vaccinia-based vaccine be developed, then their simultaneous administration as a cocktail or construction of a polyvalent virus might be necessary to avoid the problems associated with immunity to the vector.

Although no recombinant vaccinia virus vaccines have been generally licensed either for veterinary or human use, a rabies virus vaccine has been field-tested in Europe and the United States. An oral bait form of this thymidine kinase–negative vaccinia virus vector that expresses the glycoprotein gene of rabies virus is effective for wildlife including raccoons and foxes (*99*). A native North American raccoon orthopoxvirus also has been engineered as a live recombinant oral rabies vaccine (*100*). Initial testing in cattle of recombinant vaccinia viruses that express the envelope glycoprotein genes of rinderpest, a disease of cattle endemic in Africa, seems promising (*101*).

A phase 1 trial of the safety and immunological response of 36 healthy individuals to a thymidine kinase–negative live vaccinia virus vaccine expressing HIV-1 envelope glycoprotein has been reported (*102*). The vaccine appeared safe, and vaccinia naive persons were primed to HIV as indicated by the development of readily detectable and persistent in vitro T-cell proliferative and serum antibody responses. Individuals that had previously received the smallpox vaccine exhibited a highly variable response, evidently due to their prior immunity to vaccinia. None of the recipients developed neutralizing antibody to HIV, but the recombinant vector used in that study was a "first generation" one and considerably higher expression of HIV envelope protein is now possible. A phase 1 trial was initiated with a subunit vaccine of full-length HIV-1 envelope (gp160) protein purified from cells infected with a bacteriophage T7–vaccinia virus expression vector (*55*), but no results are yet available.

In conclusion, the value of recombinant vaccinia vectors as an experimental tool has been established. Widespread use of vaccinia as a live vaccine, however, depends on improving safety while achieving an even higher immune response to the recombinant protein.

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