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Genetics

Construction of live vaccines by using genetically engineered poxviruses: Biological activity of recombinant vaccinia virus expressing influenza virus hemagglutinin

(recombinant DNA/eukaryotic virus vector/hemagglutination inhibition/immunogenic response/gene expression)

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ABSTRACT **Recombinant vaccinia viruses containing the cloned hemagglutinin (*HA*) gene from influenza virus were constructed. The biological activity of these poxvirus vectors was demonstrated both *in vitro* and *in vivo*. Expression of HA in cells infected with recombinant vaccinia was detected by using specific anti-HA antiserum and ^{125}I-labeled protein A, showing that HA synthesized under the regulation of vaccinia virus was antigenic. Immunization of rabbits with these recombinant poxviruses resulted in the production of antibodies reactive with authentic influenza HA as detected by radioimmunoassay, by inhibition of HA erythrocyte agglutination, and by neutralization of influenza virus infectivity. The production of antibodies directed against influenza HA suggested that the *HA* gene expressed in vaccinia is immunogenic. These data indicate the potential of genetically engineered poxviruses for use as generic live vaccine vehicles that have both human and veterinary applications.**

The rescue of inactive endogenous DNA sequences by infectious vaccinia virus has been demonstrated in our laboratory by marker-rescue experiments using deletion mutants (1) and by Sam and Dumbell (2) with thermosensitive viral mutants. The vaccinia virus thymidine kinase (TK) gene was physically defined by translational studies (3) and by exploiting the marker-rescue technique (4). The ability to rescue endogenous viral DNA suggested to us a mechanism for the introduction of exogenous genes into poxviruses. Thus, a foreign DNA flanked with contiguous vaccinia DNA sequences could be introduced by transfection procedures into virus-infected cells. Site-specific *in vivo* recombination between the vaccinia sequences flanking the foreign DNA and homologous sequences on replicating viral DNA would allow insertion of the foreign DNA into infectious viral progeny. Pure populations of infectious recombinant vaccinia virus could be obtained by a variety of approaches. The feasibility of this approach has been demonstrated by the expression of the *TK* gene from herpes simplex virus (HSV) in vaccinia vectors (5) and confirmed by Mackett *et al.* (6), who have also inserted the HSV *TK* gene into vaccinia virus.

The utility of poxviruses as eukaryotic expression vectors can be categorized as follows: (*i*) definition of the regulatory events in the viral replication cycle by following the expression of a foreign gene from a variety of early or late promoters or by analysis of foreign genetic elements in a cytoplasmic location, (*ii*) as a eukaryotic expression vector for the synthesis of biological products, and (*iii*) in the construction of live recombinant vaccines directed against both human and veterinary infectious diseases.

The biological activity of vaccinia recombinants expressing a cloned hemagglutinin (*HA*) gene from influenza virus as an example of the production of live vaccines by the use of appropriately modified poxviruses is the subject of this communication.

MATERIALS AND METHODS

Construction of Chimeric Donor Plasmids for *in Vivo* Recombination. Plasmids were constructed, analyzed, and purified using standard techniques. Plasmids were constructed for insertion of plasmid pBR322 DNA into vaccinia as follows. Isolated vaccinia *Hin*dIII fragment F was circularized by ligation with T4 DNA ligase. This circularized fragment F was then cut with *Bam*HI. The linear inverted fragment F was ligated to *Bam*HI-cleaved pBR322 that had been treated with alkaline phosphatase (7) and used to transform (8) competent *Escherichia coli* RR1 (9). Recombinant plasmids were screened by restriction analysis of minilysates (10). Two plasmids that contained the inverted *Hin*dIII fragment F in opposite orientation within pBR322 were designated pDP301A and pDP301B. A plasmid (pJZ102) containing the cDNA sequence of the *HA* gene of influenza strain A/PR/8/34 (HINI) inserted into the *Hin*dIII site of pBR322 was obtained from P. Palese (Mt. Sinai, New York). The *HA* sequence in pJZ102 was reversed at the *Hin*dIII site by cutting with *Hin*dIII and religating. Plasmids with the *HA* sequence in opposite orientation were then designated pJZ102A and pJZ102B. For insertion of the *HA* sequence directly into the *Bam*HI site within the *Hin*dIII fragment F of vaccinia, a plasmid containing a *Pst* I subclone of the *Hin*dIII fragment F was constructed using plasmid pBR325 (11). To have a plasmid that contained only a single *Bam*HI site, the *Bam*HI site of pBR325 was removed by cleaving pBR325 with *Bam*HI, and this was followed by filling in the sticky ends using T4 DNA polymerase (12) and religating the blunt ends. This modified pBR325 plasmid was cleaved with *Pst* I and ligated with the isolated 3.7-megadalton *Pst* I fragment derived from the *Hin*dIII fragment F. This recombinant plasmid was designated pRW120. The *Hin*dIII HA fragment was isolated from pJZ102. The sticky ends were filled in by using T4 DNA polymerase followed by the addition of *Bam*HI linkers (Collaborative Research, Waltham, MA). This fragment was inserted into the *Bam*HI site of pRW120 by using T4 DNA ligase. A recombinant plasmid was isolated that contained the *HA* sequence inserted into the vaccinia fragment such that the 5′ to 3′ direction of transcription of the *HA* gene was right to left with respect to the vaccinia

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Abbreviations: TK, thymidine kinase; HSV, herpes simplex virus; HA, hemagglutinin; P_i/NaCl, phosphate-buffered saline; P_i/NaCl/albumin, P_i/NaCl containing bovine serum albumin at 1 mg/ml; bp, base pair(s); kbp, kilobase pair(s).

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genome. This plasmid was designated pDP122B.

Construction and Isolation of Recombinant Vaccinia Viruses. A vaccinia recombinant, vP53, containing the *HA* gene inserted directly into the unique *Bam*HI site of the internal *Hin*dIII fragment F, proximal to an endogenous vaccinia promoter region was obtained as a pure isolate after *in vivo* recombination using S-variant vaccinia (13) VTK$^-$79 (5) as rescuing virus and donor plasmid pDP122B. Two vaccinia recombinants, vP7 and vP8, constructed by using donor plasmids pDP301A and pDP301B, respectively, and VTK$^-$79 rescuing virus were isolated by using a replica filter technique as described (5). Vaccinia recombinants vP9 and vP10, containing the *HA* gene inserted at the *Hin*dIII site of pBR322, were constructed by using donor plasmids pJZ102A or pJZ102B, respectively, and vP7 or vP8 as rescuing virus. Pure isolates were obtained by replica filter plating and plaque purification. Orientations and copy number of the expected insertions in recombinant progeny virus were verified by restriction analysis of purified DNA. No rearrangements of the inserted sequences were detected.

Radioimmune Assays. African green monkey kidney cells (CV-1) or baby hamster kidney cells (BHK-21) were infected as monolayers. After 48 hr, plaques were visualized by neutral red staining and probed with specific antiserum in phosphate-buffered saline (P_i/NaCl) containing bovine serum albumin at 1 mg/ml for 1 hr at room temperature. The monolayers were washed repeatedly with P_i/NaCl/albumin to remove unreacted immunoglobulin. ^{125}I-Labeled protein A (approximately 0.2 μCi per sample; 1 Ci = 37 GBq) was added in P_i/NaCl/albumin for 1 hr at room temperature. Unreacted ^{125}I-labeled protein A was removed by a series of P_i/NaCl/albumin washes. The monolayers were imprinted onto nitrocellulose filters and the filters were then radioautographed. Rabbit antisera directed against the H1-HA serotype of influenza strain A/PR/8/34 (HINI) or specific for the H3–HA serotype of influenza strain A/AICHI/2/68 (H3N2) (14) were provided by P. Palese (Mt. Sinai, New York).

Immunization of Rabbits. New York State (NYLAR) rabbits were administered 4 A_{260} units of purified virus intravenously and immune sera were obtained at various intervals.

Hemagglutination Inhibition Assay. Immune serum was monitored for its ability to inhibit the agglutination of either guinea pig or chicken erythrocytes by authentic influenza HA by standard protocols (15).

Neutralization of Viral Infectivity. Sera were monitored for the ability to neutralize vaccinia or influenza viral infectivity by using plaque reduction assays. Vaccinia virus was mixed in equal parts with serum dilutions and kept at 4°C overnight until plated out on CV-1 monolayers. Influenza virus was mixed with equal volumes of diluted sera and after 2 hr at 4°C plated on Madin–Darby canine kidney cell monolayers. Plaque formation conditions were identical except that trypsin (2 μg/ml) was included in the agar overlay for influenza virus. In addition to plaque reduction, neutralization of influenza virus infectivity was also monitored by reduction in HA production in infected CV-1 monolayers.

RESULTS

Construction of Recombinant Vaccinia Viruses Containing pBR322 and Influenza HA Sequences. Construction of cloned chimeric DNA that would serve as a donor DNA for insertion into vaccinia virus requires the following protocol. The foreign DNA must be inserted into vaccinia DNA sequences at a site that does not disrupt any essential vaccinia gene function. The foreign gene, to be expressed, must be adjacent to functional regulatory sequences appropriate for the vaccinia transcriptive machinery. Furthermore, the constructs must be made in a plasmid or appropriate cloning vehicle so that sufficient quantities of the donor DNA can be obtained for transfection. To facilitate our early work with regard to investigating a variety of foreign DNAs or the analysis of exogenous or endogenous regulatory sequences, vaccinia vectors were constructed containing the entire pBR322 plasmid sequence at a site in the vaccinia genome previously shown to be nonessential and proximal to a functional vaccinia promoter (5). Additional genetic elements can be readily inserted into these viral vectors by using pBR322 flanking sequences as the homologous DNA sequences for *in vivo* recombination. Thus, the construction of donor plasmids does not require flanking additional endogenous vaccinia sequences because they can be cloned directly into pBR322.

Two vaccinia recombinants, vP7 and vP8, that contain pBR322 sequences inserted into the unique *Bam*HI site of the internal *Hin*dIII fragment F of the S-variant, VTK$^-$79, vaccinia genome are shown in Fig. 1. The orientation of the plasmid sequence in vP7 is opposite to that in vP8. The utility of these vectors for inserting additional foreign DNA into vaccinia has been shown by the construction of two additional vaccinia recombinants, vP9 and vP10, containing the cloned *HA* sequence inserted into the *Hin*dIII site of pBR322 (Fig. 1). The *HA* sequences in vP9 and vP10 have identical polarity. Fig. 1 also shows a vaccinia recombinant, vP53, in which the *HA* gene is inserted directly

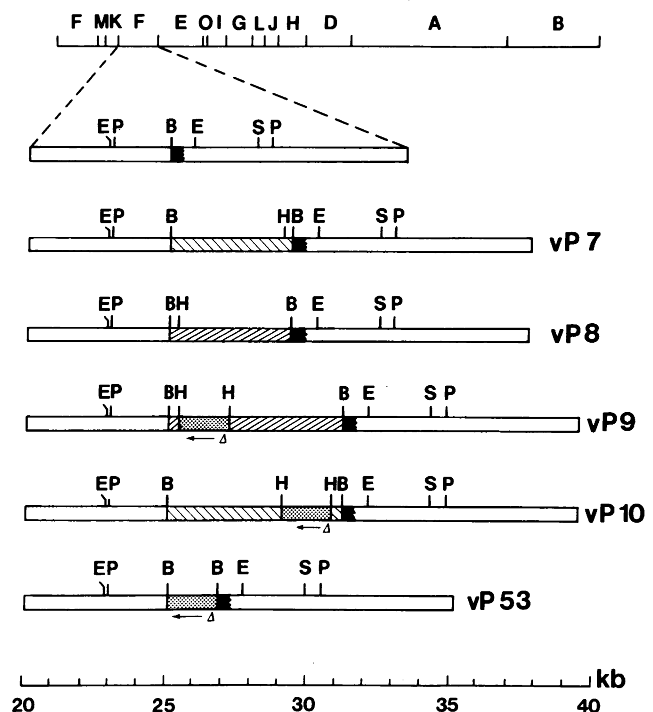

FIG. 1. Vaccinia recombinants containing the influenza *HA* and pBR322 sequences. Physical map of the S-variant (13) vaccinia genome with the internal *Hin*dIII fragment F magnified to show the location of foreign inserts at the unique *Bam*HI site of this fragment. vP7 and vP8 are vaccinia recombinants in which pBR322 sequences are inserted in opposite polarity. vP9 and vP10 are recombinants in which the influenza HA sequence is inserted with identical polarity at the *Hin*dIII site of the pBR322 sequence of vP7 and vP8. vP53 is a vaccinia recombinant in which the influenza *HA* is inserted directly into the *Bam*HI site proximal to an endogenous vaccinia promoter region. Restriction sites noted are P, *Pst* I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sst* I. Vaccinia, pBR322, and influenza *HA* sequences are denoted by clear, slashed, and speckled bars, respectively, while the dark bar denotes the endogenous vaccinia promoter region. The direction of transcription of the *HA* gene is noted by the arrow and Δ indicates the *HA* translation start site.

at the unique *Bam*HI site of the *Hin*dIII fragment F.

Expression of the Influenza *HA* Gene in Vaccinia Vectors. We have previously shown that insertion of a foreign gene into the unique *Bam*HI site of the internal *Hin*dIII fragment F does not disrupt essential gene function and that this locus is sufficiently proximal to a vaccinia promoter to allow detectable levels of expression of the HSV *TK* gene contained within a 3.4-kilobase-pair (kbp) insert (5). In those recombinants, the AUG codon of the *TK* gene was approximately 850 base pairs (bp) from the *Bam*HI site. When the HSV *TK* endogenous promoter was removed and the AUG codon was located approximately 60 bp from the *Bam*HI site, increased (8- to 10-fold) levels of HSV *TK* expression were obtained (unpublished data). Additional unpublished results indicated that the HSV *TK* transcriptional start site was at or very near the *Bam*HI site. These data suggested that this endogenous vaccinia promoter can be used to regulate the expression of additional foreign genes. As expected, insertion of cloned sequences coding for the influenza virus HA in vaccinia virus recombinant vP53 resulted in expression of the *HA* gene as detected by radioimmunoassay (Fig. 2). Since the radioimmunoassay was carried out on unfixed cells, the data suggest that the influenza HA is integrated in the cellular membrane.

Having established the utility of vaccinia recombinants vP7 and vP8, containing pBR322 sequences, by generating the additional vaccinia recombinants vP9 and vP10, containing the *HA* sequences within the plasmid sequence carried by vaccinia vectors vP7 and vP8, we next investigated whether HA was expressed by these recombinants. The *HA* gene as inserted in vP10 placed the AUG codon approximately 380 bp from the *Bam*HI site while more than 4 kbp separated the AUG in the *HA* from the endogenous vaccinia promoter region in vaccinia recombinant vP9. Surprisingly, radioimmunoassay of these recombinants detected expression of the *HA* gene by vP9, although at lower levels than vP53 (Fig. 2) while no antigenic HA expression was detected with the vP10 recombinant.

The serologic specificity of the HA expressed by vaccinia recombinants is demonstrated in Fig. 3. Neutral red-stained monolayers of CV-1 cells infected with recombinant vP9 are shown in Fig. 3 (*A* and *B*). As expected, radioimmunoassay using anti-H1-specific serum resulted in binding of radiolabeled protein A (Fig. 3*D*). No immunological crossreactivity of the HA synthesized by vaccinia was detected when anti-H3-specific serum was used (Fig. 3*C*). Radioimmunoassays with vP7, vP8, and the VTK$^-$79 parent were negative.

Induction of Antibodies Directed Against Influenza HA by Rabbits Immunized with Vaccinia Recombinant vP9. To show that the HA expressed in vaccinia recombinants is immunogenic, rabbits were immunized with vP9 and the following experiments were carried out. Monolayers of BHK cells were infected with the A/PR/8/34 strain of influenza virus and allowed to react with either homologous anti-influenza, anti-vaccinia (VTK$^-$79), or anti-vP9 rabbit sera. No reactivity was detected with anti-vaccinia (VTK$^-$79) serum, but immunological reactivity was observed with both the control homologous anti-influenza serum and the antiserum obtained after immunization of rabbits with vaccinia recombinant vP9 (Fig. 4 *A–C*). Immunological reactivity with either anti-vaccinia (VTK$^-$79) or anti-vP9 sera directed against vaccinia virus-infected monolayers of CV-1 cells and the absence of crossreactivity with anti-influenza serum is shown in Fig. 4 *D–F*.

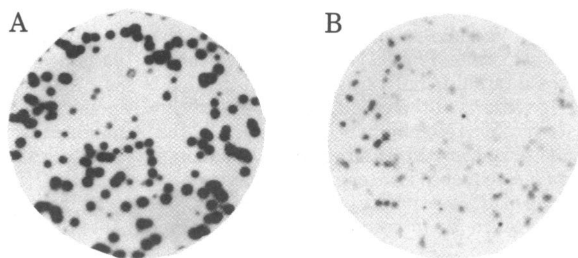

FIG. 2. Radioimmunoassay for influenza *HA* expression in cells infected with vaccinia recombinants vP53 (*A*) and vP9 (*B*). CV-1 monolayers infected with vP53 or vP9 were monitored for *HA* expression 48 hr after infection. Radioautographs of monolayers imprinted onto nitrocellulose indicate levels of ^{125}I-labeled protein A binding.

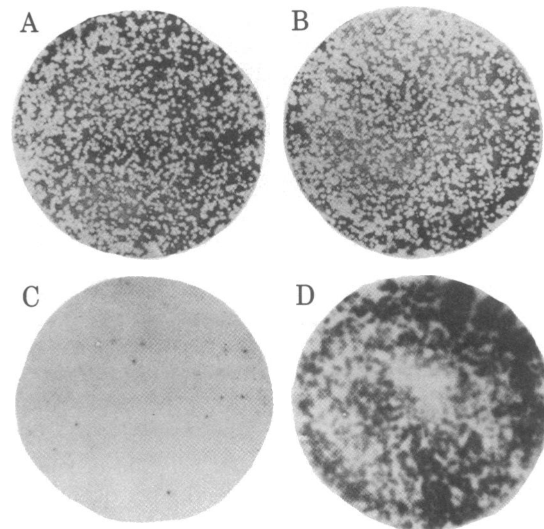

FIG. 3. Specificity of influenza HA produced by vaccinia recombinant vP9. CV-1 monolayers, infected with vP9, were stained with neutral red (*A* and *B*) to visualize plaques 48 hr after infection. The monolayer in *A* was probed with anti-H3 serum (*C*) while the monolayer in *B* was probed with anti-H1 serum (*D*). After exposure to ^{125}I-labeled protein A, the monolayers were imprinted onto nitrocellulose and radioautographed. The spots noted on the filter shown in *C*, probed with anti-H3 serum, are due to incomplete washing of the monolayers.

Hemagglutination Inhibition. Influenza virus agglutinates a variety of erythrocytes because of the *HA* gene product. Diagnostic evidence that antibodies directed against influenza virus and its HA are present is routinely shown by hemagglutination inhibition assays. Evidence that antiserum produced in rabbits immunized with vaccinia recombinant vP9 inhibits the agglutination of erythrocytes by authentic influenza HA is presented in Fig. 5. Inhibition of 4 HA units is clearly shown at a reciprocal serum dilution of 320. No inhibition was observed with serum raised against vP10. Antisera produced in rabbits immunized with vaccinia recombinant vP53, the more strongly HA-expressing virus, gave reciprocal hemagglutination inhibition titers of 5,120 (data not presented).

Neutralization of Influenza Virus Infectivity. A fundamental property of antiviral antibodies is the ability to interact with mature virus and neutralize its infectivity. To show that antiserum produced in rabbits immunized with vaccinia recombinant vP9 neutralized influenza virus infectivity, the following experiments were carried out. Influenza virus was incubated at 4°C for 2 hr in the presence of various dilutions of normal or immune serum and either the production of HA or the reduction in plaque number was monitored. Sera from rabbits immunized with vP9 resulted in both decreased levels of HA production (unpublished data) and plaque numbers diagnostic of reduced viral infectivity. A 1:320 dilution of antiserum resulted in a 50% decrease in plaque number of influenza virus. A 50%

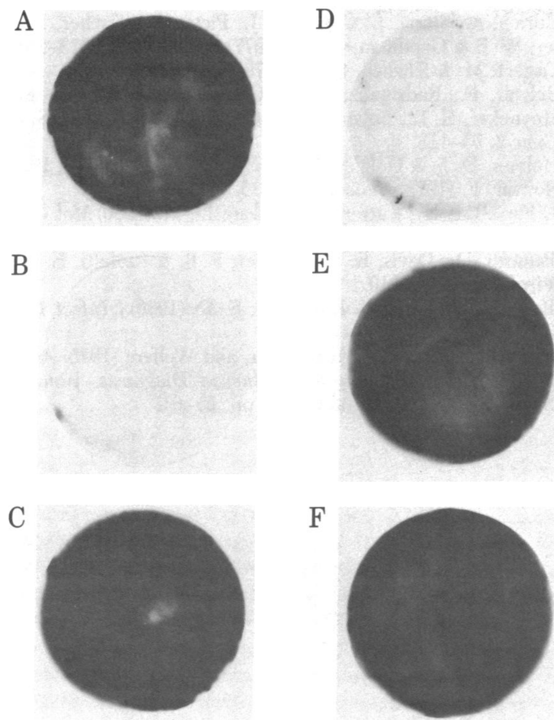

FIG. 4. Radioimmunodetection of influenza HA by antibodies elicited in rabbits by vaccinia recombinant vP9. Monolayers of BHK cells (*A–C*) were infected with influenza virus and monolayers of CV-1 cells (*D–F*) were infected with wild-type S-variant vaccinia virus. Radioautographs of radioimmunoassay using rabbit anti-H1 serum (*A* and *D*), anti-S-variant vaccinia serum (*B* and *E*), and anti-vaccinia recombinant (vP9) serum (*C* and *F*) are shown.

reduction of vaccinia virus plaques was obtained with the same serum at a dilution of 1:64,000.

DISCUSSION

In this communication, we have reported the construction and biological activity of recombinant vaccinia viruses expressing the *HA* gene of influenza virus. Synthesis of functional HSV TK (5) and antigenic and immunogenic expression of the *HA* gene described here show the fidelity of expressing foreign genes in this eukaryotic virus vector system. Insertion of the *HA* gene proximal to an endogenous vaccinia promoter in recombinant vaccinia virus vP53 resulted in the expected expression of HA. It is not clear why vaccinia recombinant vP10 failed to express detectable levels of HA. Significant HA expression in vaccinia recombinant vP9 was unexpected because the *HA* gene was localized more than 4 kbp from the vaccinia promoter region. It should be noted that insertion of the *HA* gene at the *Hin*dIII site of pBR322 interrupts the tetracycline-resistance gene. Apparently, a functional regulatory sequence recognizable by the endogenous vaccinia transcriptive machinery was fortuitously constructed in inserting the *HA* gene into the pBR322 sequence resulting in HA synthesis. Insufficient data are currently available to define precisely what constitutes a vaccinia promoter. We have, however, identified a number of both early and late endogenous vaccinia regulatory sequences that allow expression of foreign genes. In contrast, well-defined eukaryotic or prokaryotic promoters such as the early simian virus 40 promoter, the bacteriophage P_L promoter, the long terminal repeat sequences from retroviruses, and a number of promoters from herpes simplex virus have resulted in nominal expression of foreign genes, suggesting the unique nature of vaccinia promoters. Quantitation of the expression levels of HSV TK, influenza virus HA, the surface antigen of hepatitis virus, glycoproteins from herpes simplex virus, as well as other genes, from a variety of endogenous vaccinia promoters will be presented in other communications.

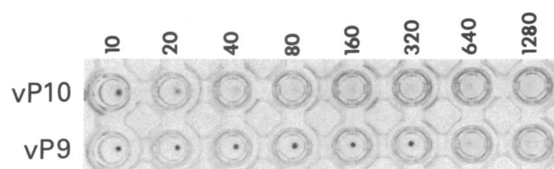

FIG. 5. Hemagglutination inhibition by antiserum from a rabbit immunized with vaccinia recombinants expressing the influenza *HA* gene. Hemagglutination inhibition of sera was tested using 4 HA units and chicken erythrocytes. The antisera used were raised in rabbits in response to immunization with vP10 or vP9. The reciprocal of the antiserum dilution is shown. Similar results were obtained with guinea pig erythrocytes.

It is significant that the level of expression of HA in vaccinia recombinant vP9, although much lower than that found with vP53, can elicit the production of specific antibodies in rabbits. This points out one of the major advantages of live vaccines—i.e., the ability to amplify the antigen during replication in the host, thus presenting the host's immunological system sufficient antigenic mass. It is important to note that the foreign antigen can still elicit antibody production even though competing with the varied and numerous endogenous vaccinia antigens. It is reasonable to expect that increased levels of expression of the foreign antigen from stronger promoters would be reflected in an even more potent immunological response. Nevertheless, the hemagglutination inhibition titers obtained in rabbits in response to immunization with vP9, if extrapolated to humans, would be considered serologic evidence of immunity to influenza.

One can become immune to infectious diseases by a variety of ways. Survival of a natural infection usually results in lifelong immunity. Active immunity can be obtained through an immunization program using live or killed vaccines. With live vaccines, there is always the potential of reversion of the attenuated agent to the pathogenic state. Some etiologic agents may be difficult or impossible to obtain in an attenuated form. Large quantities of material are required for killed vaccines in order to retain sufficient antigenic mass. In addition, killed vaccines are often contaminated with undesirable byproducts retained during their preparation. Heterologous live vaccines using appropriately engineered vaccinia, which is itself a vaccine, seem almost an ideal generic immunogen. Vaccine strains currently available are generally safe and it is reasonable to expect that additional modification of the virus by genetic engineering will result in an even safer virus. The efficacy of the virus, as a vaccine, is attested to by the successful worldwide eradication of smallpox. The genome is sufficiently large to allow the construction of polyvalent vaccines. Indeed, we have already constructed infectious recombinants containing more than 20 kbp of foreign DNA and recombinants expressing several foreign genes (unpublished data). Appropriately engineered, recombinant vaccinia viruses can be used for the production of live vaccines directed against both human diseases and a variety of veterinary diseases. Any infectious agent, whether it be viral, bacterial, or parasitic, may well be amenable to control through such a generic approach. Much work remains to be done but, from this vantage point, this appears to be a promising direction against infectious diseases.

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