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Biochemistry

Construction and characterization of an infectious vaccinia virus recombinant that expresses the influenza hemagglutinin gene and induces resistance to influenza virus infection in hamsters

(hybrid vaccinia virus/chimeric gene/live virus vaccine/recombinant DNA)

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ABSTRACT **A DNA copy of the influenza virus hemagglutinin gene, derived from influenza virus A/Jap/305/57 (H2N2) was inserted into the genome of vaccinia virus under the control of an early vaccinia virus promoter. Tissue culture cells infected with the purified recombinant virus synthesized influenza hemagglutinin, which was glycosylated and transported to the cell surface where it could be cleaved with trypsin into HA1 and HA2 subunits. Rabbits and hamsters inoculated intradermally with recombinant virus produced circulating antibodies that inhibited hemagglutination by influenza virus. Furthermore, vaccinated hamsters achieved levels of antibody similar to those obtained upon primary infection with influenza virus and were protected against respiratory infection with the A/Jap/305/57 influenza virus.**

The construction of infectious hybrid viruses by genetic engineering provides a method of producing live vaccines. A candidate vaccine of this type was constructed by inserting the hepatitis B virus surface antigen gene into vaccinia virus (1, 2). Vaccinia virus was selected as a vector because the large genome size and absence of stringent packaging constraints allow the introduction of foreign DNA without loss of infectivity (3–5). In addition, vaccinia virus has a wide host range, permitting veterinary and medical applications, and has been used successfully worldwide for mass immunization against smallpox.

The strategy we use to insert and express foreign DNA in vaccinia virus has been described elsewhere in detail (6, 7). Essentially, the construction of vaccinia virus recombinants is carried out in two stages. First, recombinant DNA techniques are used to assemble a plasmid containing a chimeric gene flanked by vaccinia virus DNA. The chimeric gene has the transcriptional regulatory signals and RNA start site of a vaccinia virus gene (8–10) adjacent to the translational start site and foreign protein coding sequence of a foreign gene. The next stage is the insertion of the chimeric gene into vaccinia virus. This occurs by homologous recombination in cells that have been infected with vaccinia virus and transfected with the plasmid containing the chimeric gene by using protocols similar to those described for marker rescue (11–13). The site of insertion is determined by the flanking DNA sequences present in the plasmid. Although any nonessential region of the vaccinia genome could be used to flank the chimeric gene, we routinely use segments of the vaccinia virus gene for thymidine kinase (TK) (10) because recombinants will then have a TK-negative phenotype and can be selected by plaque formation in the presence of BrdUrd. This process has been simplified by the construction of plasmid insertion vectors that have properly positioned restriction sites for the insertion of the foreign gene segment (6, 7).

In this communication, we describe the formation and properties of a vaccinia virus recombinant that contains the influenza virus gene for hemagglutinin (HA). The HA genes from several influenza subtypes have been cloned and their sequences determined (14–18), and some of these have been expressed in simian virus 40 (SV40) virus vectors (19–22). The product of this gene is probably the most thoroughly studied integral membrane protein: its three-dimensional structure has been determined (23), antigenic sites have been mapped (24), and mutations affecting antigenicity, post-translational processing, and transport have been described (19, 22, 25). Of central importance is the fact that antibodies against this protein neutralize the infectivity of influenza virus (26). The purpose of the present study was to determine whether a cDNA copy of the HA gene could be expressed in a vaccinia virus vector. We found that the HA polypeptide was glycosylated and transported to the surface of the cell and that intradermal inoculation of experimental animals with the vaccinia virus recombinant led to the production of circulating antibodies to HA and protection against respiratory infection with influenza virus.

MATERIALS AND METHODS

Viruses. Vaccinia virus (strain WR) was obtained from the American Type Culture Collection, grown in HeLa cells, and purified from cytoplasmic extracts by sucrose gradient centrifugation (27). Influenza virus A/Jap/305/57 (H2N2) was obtained from the Research Resources Branch of the National Institute of Allergy and Infectious Diseases and grown in 10-day-old chicken embryos.

Cells. HeLa cells were grown in Eagle's medium supplemented with 5% horse serum. Human 143 TK^- cells (obtained from K. Huebner, Wistar Institute) were grown in Eagle's medium with 10% fetal bovine serum and 25 μg of BrdUrd per ml. CV-1 cells were grown in Dulbecco's modified medium containing 10% fetal bovine serum.

Preparation of DNA. DNA was extracted from purified vaccinia virus as described (28). Plasmid recombinants were constructed and used to transform bacteria by standard methods (29). After chloramphenicol amplification (30), bacteria were lysed by the alkaline $NaDodSO_4$ method (31), and plasmid DNA was purified by CsCl/ethidium bromide equilibrium density gradient centrifugation. DNA fragments were isolated from agarose gels by electroblotting onto DEAE paper (32).

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Abbreviations: bp, base pair; HA, hemagglutinin; kb, kilobase; $TCID_{50}$, tissue culture infectious dose that infects 50%; TK, thymidine kinase.

Transfection and Isolation of Recombinant Virus. CV-1 cells infected with wild-type vaccinia virus were transfected with calcium phosphate-precipitated plasmid DNA as described (13). TK^- recombinants were isolated by plaque assay in TK^- cells in the presence of BrdUrd and were distinguished from spontaneous mutants by dot-blot DNA:DNA hybridization (4). After two successive plaque purifications, virus was amplified by infecting TK^- cell monolayers in the presence of BrdUrd and then grown in HeLa cells without selection.

Antibody Binding to Viral Plaques. Monolayers of CV-1 cells (25 cm^2), containing plaques formed by virus addition 2 days earlier, were rinsed with phosphate-buffered saline and then fixed to the plastic Petri dishes by addition of 4 ml of cold methanol. After 10 min, the monolayers were washed three times with buffered saline and incubated successively for 1-hr intervals at room temperature on a rocking platform with (*i*) 4 ml of 4% bovine serum albumin/0.02% sodium azide; (*ii*) the same, supplemented with either goat anti-influenza anti-serum or rabbit anti-vaccinia antiserum; (*iii*) the same, supplemented with 0.5 μCi (1 Ci = 37 GBq) of ^{125}I-labeled staphylococcal A protein. The plates were washed extensively with buffered saline before and after addition of staphylococcal A protein.

Polypeptide Analysis. CV-1 monolayers were infected with 30 plaque-forming units per cell of vaccinia virus or similar amounts of influenza virus. From 2 to 6 hr after infection, approximately 3×10^6 cells were labeled with 80 μCi of [^{35}S]methionine (1,000 Ci/mmol, Amersham) in medium otherwise lacking this amino acid. As described (1), cells were lysed with 0.5% Nonidet P-40 and incubated with preimmunization goat serum followed by formalin-treated staphylococcal cells (ref. 33; Calbiochem–Behring). After centrifugation, the supernatant was incubated with goat anti-influenza virus A/Jap/305/57 antiserum followed by staphylococcal cells. Immunoprecipitated polypeptides were resolved by electrophoresis through a 15% polyacrylamide gel and detected by autoradiography.

Animal Studies. Twenty-week-old Golden Syrian hamsters were anesthetized with ether, and their lateral abdominal walls were shaved. One side received an intradermal injection of 10^8 plaque-forming units of either wild-type vaccinia or vaccinia recombinant vInf1 in 0.1 ml, and the other side received the same amount of virus by scarification. A separate group of animals received $10^{5.0}$ $TCID_{50}$ units ($TCID_{50}$ = tissue culture infectious dose that infects 50%) of influenza virus A/Jap/305/57 intranasally in 0.1 ml. The three groups of animals (10 per group) were bled through the retro-orbital plexus on days 0, 12, 22, and 40, and sera from each animal were tested individually for antibodies to influenza HA by the hemagglutination inhibition test (34) with influenza virus A/Jap/305/57 as antigen. On day 40, each hamster was anesthetized by intraperitoneal injection of pentabarbital and inoculated intranasally with $10^{5.0}$ $TCID_{50}$ units of influenza virus A/Jap/305/57 in 0.1 ml. One or 2 days later, the lungs and nasal turbinates were removed (five animals per day per group) and 10% (wt/vol) tissue homogenates were prepared and assayed for influenza infectivity on MDCK cell monolayers as described (35).

Pairs of female white rabbits also were inoculated by intradermal injection of 10^8 plaque-forming units of either wild-type vaccinia or recombinant vInf1 in 0.1 ml. Rabbits were bled from their ears on days 0, 14, 33, and 62, and sera were tested for antibodies to influenza HA as above.

RESULTS

Construction of Plasmid and Virus Recombinants. The construction of a chimeric gene containing the transcriptional regulatory signals and RNA start site of an early vaccinia virus gene and the translational start site and coding sequences of the influenza HA gene are diagrammed in Fig. 1. The starting plasmids were pJHB16, which contains a 1.7-kilobase (kb) segment of the HA gene of influenza virus A/Jap/305/57 (20), and pGS20, which contains a 265-base-pair (bp) segment including the transcriptional regulatory signals and RNA start site of an early vaccinia virus gene translocated within the body of the TK gene (4, 6, 7). In pJHB16, a synthetic *Hin*dIII linker precedes the first nucleotide of the HA translation initiation codon, and a *Bam*HI site occurs at the distal end of the gene. The *Hin*dIII site of pJHB16 was changed to a *Bam*HI site so that the HA segment could be cloned into the unique *Bam*HI site of pGS20. The resulting plasmids, pGS36 and pGS37, contain the HA gene correctly and incorrectly oriented with respect to the vaccinia promoter and were distinguished by agarose gel electrophoresis of *Sal* I restriction endonuclease digests. The two plasmids were then used to transfect CV-1 cells infected with wild-type vaccinia virus. Homologous recombination between vaccinia TK sequences in the plasmid and virus genome resulted in insertion of the HA gene into vaccinia virus. The virus progenies were then plaque-assayed on TK^- cells in the presence of BrdUrd to select TK^- recombinants. The latter were distinguished from spontaneous TK^- mutants by dot-blot hybridization to ^{32}P-labeled influenza virus HA DNA. After two plaque purifications, recombinant virus stocks derived from pGS36 and pGS37 were called vInf1 and vInf2, respectively.

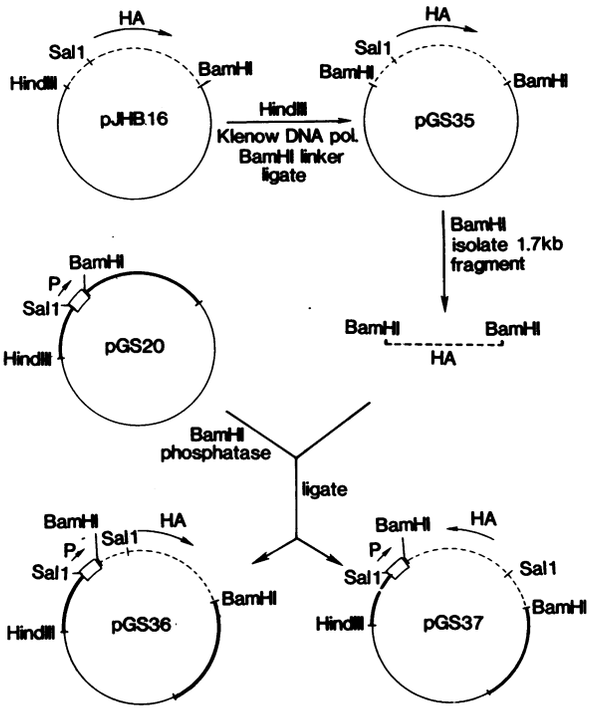

FIG. 1. Construction of recombinant plasmids. ---, Influenza HA DNA; □, vaccinia transcriptional regulatory sequence (P) including RNA start site; ——, flanking vaccinia virus DNA including interrupted TK gene; ——, plasmid DNA.

Analysis of Recombinant Virus DNA. DNA was extracted from purified virus particles, digested with appropriate restriction endonucleases, and separated by agarose gel electrophoresis. DNA fragments were transferred to duplicate nitrocellulose sheets by bidirectional blotting and hybridized to ^{32}P-labeled DNA from the TK-containing *Hin*dIII J fragment of vaccinia virus or the HA gene of influenza virus. Autoradiographs of *Hin*dIII digests demonstrated that the 5-kb *Hin*dIII-J fragment of wild-type vaccinia virus was replaced by a 7-kb fragment in both vInf1 and vInf2 (Fig. 2 *Left*). As predicted,

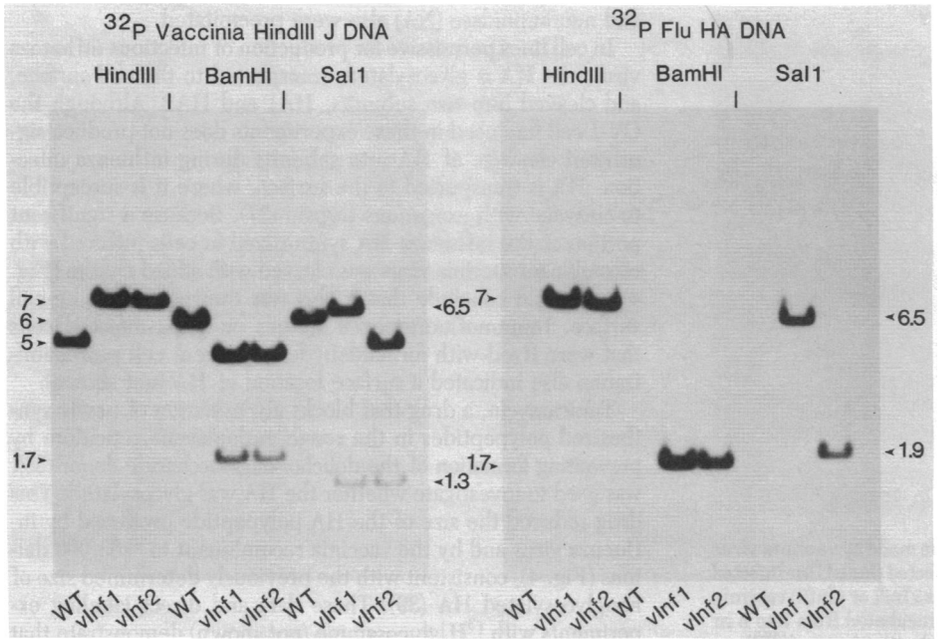

FIG. 2. Analysis of recombinant virus DNA. DNA extracted from purified virus was digested with restriction endonucleases *Hin*dIII, *Bam*HI, or *Sal* I. DNA fragments were resolved by electrophoresis through a 0.6% agarose gel and transferred by blotting to sheets of nitrocellulose placed above and below the gel. The sheets of nitrocellulose were hybridized to either the purified *Hin*dIII J DNA fragment of vaccinia virus or the HA gene of influenza virus labeled with ^{32}P by nick-translation (36). WT, wild-type vaccinia virus; vInf1 and vInf2, recombinant vaccinia viruses derived from plasmids pGS36 and pGS37, respectively. The sizes of DNA fragments are indicated in kb.

the 7-kb fragments hybridized to influenza virus HA DNA (Fig. 2 *Right*). Upon *Bam*HI digestion, two bands of 4.6 kb and 1.7 kb that hybridized to the vaccinia *Hin*dIII J fragment were produced from each recombinant instead of the single 6-kb fragment produced from wild-type virus (Fig. 2 *Left*). The sizes of these fragments are consistent with the presence of a *Bam*HI site upstream of the TK gene (J. Weir, personal communication) and the introduction of new *Bam*HI sites in the recombinants. A single 1.7-kb fragment that hybridized to the influenza HA gene (Fig. 2 *Right*) was released from both vInf1 and vInf2. This represents the entire inserted HA fragment, and its similarity in size with the *Bam*HI fragment that hybridized to vaccinia *Hin*dIII J DNA is coincidental.

The orientation of the inserted HA gene was demonstrated by *Sal* I digestion. Cleavage of vInf1 DNA produced a fragment of 6.5 kb that hybridized to both influenza HA and vaccinia *Hin*dIII J DNA probes (Fig. 2). This band contained the entire HA gene (except for 90 bp at the 5′ end) and nearly 5 kb of vaccinia DNA including sequences downstream of the TK gene. Another 1.3-kb fragment containing sequences upstream of the TK gene hybridized only to the vaccinia probe. A smaller fragment of 390 bp, representing 90 bp of the HA gene joined to the translocated vaccinia promoter, ran off the gel. Cleavage of vInf2 DNA with *Sal* I produced 5-kb and 1.3-kb fragments that hybridized to vaccinia DNA and a 1.9-kb fragment that hybridized to influenza HA DNA (Fig. 2) and represented the translocated vaccinia promoter joined to the distal 1.64 kb of the HA gene. Hybridization of the 5-kb fragment to HA DNA was not detected because only 90 bp of the latter was present.

These data indicate that both vInf1 and vInf2 contain the entire influenza HA gene inserted into the TK gene of vaccinia virus. However, in vInf1 the HA gene is correctly oriented with respect to the translocated vaccinia promoter, whereas in vInf2 it is incorrectly oriented. Additionally, the absence of the 5-kb *Hin*dIII J fragment in either of the recombinants indicates that there is no detectable wild-type virus. The latter conclusion was reached independently by the finding of identical virus titers upon plaque assay in TK^- cells in the presence and absence of BrdUrd (not shown), and by plaque–antibody binding experiments described below.

Expression of the Influenza HA Gene. Evidence for the expression of influenza HA in cells infected with vInf1 was obtained by the binding of antibody prepared against influenza A/Jap/305/57 to virus plaques. Antibody binding was detected by incubation with ^{125}I-labeled staphylococcal A protein followed by autoradiography (Fig. 3). A direct comparison of the stained cell monolayer with the autoradiograph indicated that all vInf1 plaques bound antibody. In contrast, binding of antibody was not detected with plaques formed by wild-type

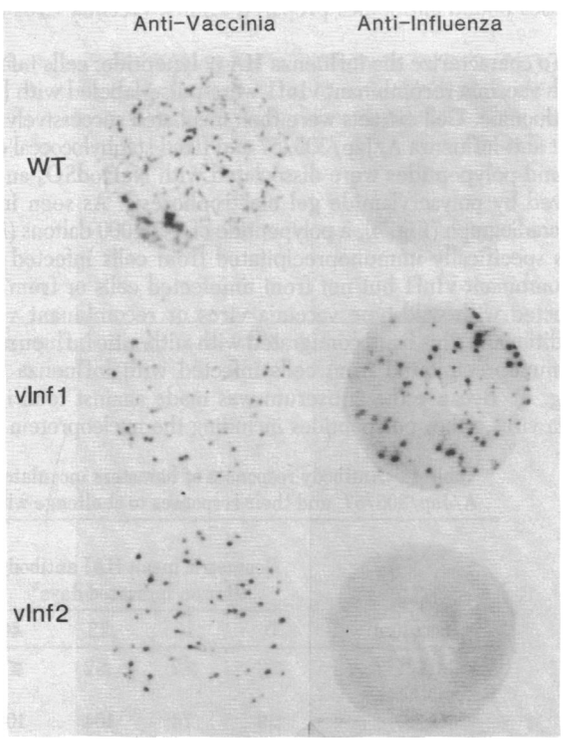

FIG. 3. Detection of HA expression by individual virus plaques. Duplicate monolayers of CV-1 cells containing plaques produced by wild-type vaccinia virus (WT) or recombinant viruses (vInf1 and vInf2) were washed and fixed to the plastic Petri dishes. Binding of anti-vaccinia (*Left*) or anti-influenza (*Right*) antibodies followed by incubation of ^{125}I-labeled staphylococcal A protein was carried out as described. Autoradiographs are shown.

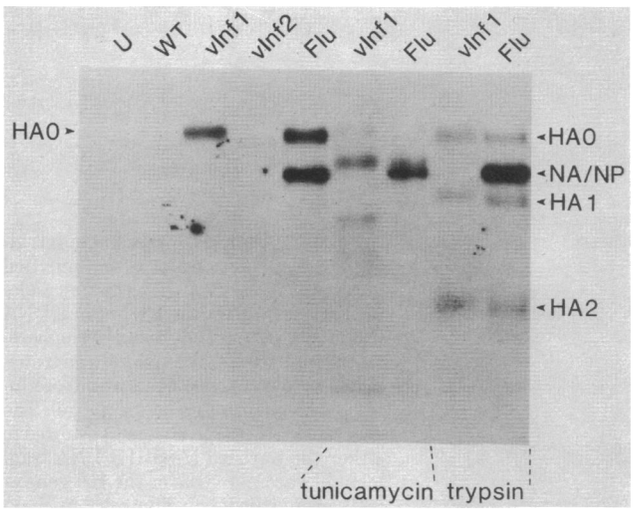

FIG. 4. Characterization of HA polypeptide made by vaccinia virus recombinant. CV-1 monolayers were mock-infected (lane U) or infected with wild-type (lane WT) or recombinant (lanes vInf1 or vInf2) vaccinia virus or influenza virus (lane Flu) and then incubated from 2 to 6 hr later in methionine-free medium supplemented with 80 μCi of [^{35}S]methionine. Additional monolayers were incubated in parallel with tunicamycin (2 μg/ml) or trypsin (2 μg/ml). HA polypeptides were then immunoprecipitated with goat anti-influenza A/Jap/305/57 antiserum and fixed staphylococcal cells. Bound polypeptides were resolved by electrophoresis on a 15% polyacrylamide gel. An autoradiograph is shown. HA0, polypeptide of ≈75,000 daltons; NA/NP, neuraminidase/nucleoprotein.

vaccinia virus or vInf2. However, plaques formed with all three viruses bound antibodies prepared against vaccinia virus (Fig. 3).

To characterize the influenza HA polypeptide, cells infected with vaccinia recombinant vInf1 were pulse-labeled with [^{35}S]-methionine. Cell extracts were then incubated successively with goat anti-influenza A/Jap/305/57 and fixed staphylococcal cells. Bound polypeptides were dissociated with $NaDodSO_4$ and resolved by polyacrylamide gel electrophoresis. As seen in the autoradiograph (Fig. 4), a polypeptide of ≈75,000 daltons (HA0) was specifically immunoprecipitated from cells infected with recombinant vInf1 but not from uninfected cells or from cells infected with wild-type vaccinia virus or recombinant vInf2. Additionally, this band comigrated with authentic influenza HA immunoprecipitated from cells infected with influenza virus (Fig. 4). Because the antiserum was made against total influenza virus, other polypeptides including the nucleoprotein (NP) and neuraminidase (NA) also were precipitated.

In cell lines permissive for production of infectious influenza virus, the HA is glycosylated, transported to the cell surface, and cleaved into two subunits, HA1 and HA2. Although the CV-1 cell line used in these experiments does not produce significant cleavage of HA into subunits during influenza infection, HA is transported to the surface, where it is susceptible to cleavage with exogenous trypsin (37). Because a significant portion of the influenza HA synthesized in cells infected with recombinant vaccinia virus was cleaved with added trypsin (Fig. 4), we could conclude that it also was transported to the cell surface. Immunofluorescence studies on vInf1-infected cells that were fixed with formaldehyde to prevent cell permeabilization also indicated a surface location of HA (not shown).

Tunicamycin, a drug that blocks glycosylation of newly synthesized polypeptides in the rough endoplasmic reticulum by preventing formation of the dolichol-oligosaccharide donor (38), was used to investigate whether the HA was glycosylated. This drug reduced the size of the HA polypeptide produced by influenza virus and by the vaccinia recombinant to ≈63,000 daltons (Fig. 4), consistent with the previously determined size of nonglycosylated HA (39). These data and direct labeling experiments with [^{3}H]glucosamine (not shown) demonstrate that the HA produced by vInf1 is glycosylated in a manner similar to authentic influenza HA.

Vaccination of Animals. The ability of vaccinia virus recombinants to elicit an antibody response to influenza HA was tested initially in rabbits. The recombinant virus used was purified by sucrose gradient centrifugation and, at concentrations 50-fold higher than that used for immunization, had no detectable influenza HA as judged by the inability to agglutinate chicken erythrocytes. Accordingly, synthesis of influenza HA by the recombinant vaccinia virus in inoculated animals would be required to stimulate antibody production. Two pairs of rabbits were inoculated intradermally with either wild-type vaccinia virus or vInf1, and sera were assayed for antibodies to influenza HA by hemagglutination inhibition test on 0, 14, 33, and 62 days after vaccination. In both rabbits vaccinated with vInf1, significant antibody levels were detected by day 14, and the titers increased to 1:128 and 1:64 on day 62. No antibodies to HA were detected in the sera of animals vaccinated with wild-type virus.

To investigate whether immunization with vaccinia virus recombinants would protect animals against influenza virus infection, a hamster model system was used (35). Groups of 10 animals were inoculated with either wild-type vaccinia, recombinant vInf1, or influenza A/Jap/305/57, and serum taken from each animal on days 0, 12, 22, and 40 was tested for antibodies

Table 1. Antibody responses of hamsters inoculated with wild-type vaccinia, vaccinia recombinant vInf1, and influenza A/Jap/305/57, and their responses to challenge with influenza A/Jap/305/57

Inoculum	Geometric mean HAI antibody titer on indicated days*				*N* with >4-fold increase in HAI titer	Response to challenge with influenza A/Jap/305/57	
	0	12	22	40		*N* yielding virus	Mean $\log_{10}$ titer ($TCID_{50}$/g lung day 1)
Vaccinia	≦2	≦2	≦2	≦2	0	8	4.4 ± 0.6
Recombinant vInf1	≦2	74	104	104	10	2†‡	2.6 ± 0.1§‖
Influenza A/Jap/305/57	≦2	49	97	49	10	1†‡	2.6 ± 0.1§‖

Ten animals were tested with each inoculum. HAI, hemagglutination inhibition; *N*, number of animals.
* Antibody titers are reciprocals.
† Significantly different from animals inoculated with wild-type vaccinia virus; $P < 0.05$ by Fisher's exact test.
‡ Virus recovered from these animals was at the lowest level detectable ($10^{3.0}$ $TCID_{50}$/g of lung).
§ Significantly different from animals inoculated with wild-type vaccinia virus; $P < 0.01$ by Student's *t* test.
‖ For calculation of mean titers, animals from which virus was not recovered were assigned maximum possible values of $10^{2.5}$ $TCID_{50}$/g of lung.

to influenza HA. Antibodies to HA were not found in animals vaccinated with wild-type vaccinia virus; however, in all animals inoculated with recombinant vInf1 or influenza A/Jap/305/57, antibodies to influenza HA were detected (Table 1). Moreover, the mean levels of antibodies in these two groups of animals were only significantly different on day 40, when the level of antibodies in animals vaccinated with recombinant vInf1 significantly exceeded the levels in animals inoculated with influenza virus (Table 1).

The results of challenging all hamsters with influenza virus 40 days after initial inoculation are also shown in Table 1. These data show that hamsters that initially received either the vaccinia recombinant vInf1 or influenza virus were resistant to challenge as indicated by a reduction in the number of animals from which virus was recovered and by a decrease in the quantity of virus recovered. Additionally, the resistance of these two groups of animals to influenza infection did not significantly differ.

DISCUSSION

In this communication, we describe the construction and properties of a vaccinia virus recombinant that contains the influenza HA gene. The recombinant is infectious and is stable upon repeated passage in tissue culture cells. The influenza HA coding sequences are under control of an early vaccinia virus promoter, and the chimeric gene is expressed in tissue culture cells. The product appears to be authentic because it comigrates with HA made by cells infected with influenza virus and is glycosylated and transported to the cell surface, where it can be cleaved to HA1 and HA2 subunits.

This and other influenza HA genes previously have been expressed in SV40 vectors (19–22). The novel aspect of the vaccinia virus vector is that the infectivity and wide host range of the original virus is retained. This feature can be important in a variety of experimental situations particularly those in which animals are involved. Animals inoculated intradermally with vaccinia virus recombinant vInf1 develop antibodies to influenza HA. Moreover, the antibody levels were equivalent to or higher than those obtained after infection with live influenza virus. By inserting mutated HA genes into vaccinia virus, it should be possible to analyze their effects on both humoral and cell-mediated immunity. This would seem to be a unique method of determining which regions of the HA molecule are important for an immune response during a live infection.

The high level of antibody produced by hamsters vaccinated with the recombinant virus was correlated with resistance of those animals to challenge 6 weeks later with infectious influenza virus. Perhaps most significant was the fact that the animals were vaccinated intradermally on their backs and were challenged by the intranasal route. The acquisition of sufficient local immunity to prevent respiratory infection suggests that vaccinia virus recombinants could be used as a vaccine to prevent infection with influenza or other respiratory viruses in man or other animals. Because at least 25 kb of foreign DNA can be inserted into vaccinia virus vectors (5), different genes or multiple serotypes of the same gene can be used simultaneously. In the case of influenza virus infections of man, the rapid antigenic variation still poses serious problems. Although new vaccinia virus recombinants could rapidly be constructed (7), frequent vaccinations would undoubtedly limit the intradermal growth of the recombinant and the production of antigen. Attempts are currently being made to produce high expression vectors that might produce sufficient antigen after relatively few rounds of virus replication during secondary vaccinations. It also should be possible to produce more attenuated forms of vaccinia virus that produce milder primary vaccination reactions.

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