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Genetics

Construction of live vaccines using genetically engineered poxviruses: Biological activity of vaccinia virus recombinants expressing the hepatitis B virus surface antigen and the herpes simplex virus glycoprotein D

(eukaryotic expression vector/herpes simplex virus neutralization/herpes simplex virus protection/hepatitis B virus surface antigen antibodies)

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ABSTRACT **Potential live vaccines using recombinant vaccinia viruses have been constructed for both hepatitis B and herpes simplex. These recombinant vaccinia viruses express cloned genes of the hepatitis B virus surface antigen (HBsAg) or the glycoprotein D from herpes simplex virus (HSV-gD). The HBsAg synthesized *in vitro* under the regulation of vaccinia virus is secreted from infected cells as a particle of ≈22 nm diameter with a density of 1.2 g/ml as determined on CsCl gradients. Inoculation of rabbits with the recombinant vaccinia virus that expresses the HBsAg elicits the production of high-titered antibodies. Synthesis of the HSV-gD was detected in tissue culture by radioimmunoassay on unfixed cells, suggesting that the HSV-gD synthesized by the recombinant vaccinia virus is membrane associated. Inoculation of rabbits with the recombinant vaccinia virus expressing HSV-gD resulted in the production of antibodies that reacted with authentic HSV-gD as detected by radioimmunoassay. Furthermore, the antiserum was shown by plaque-reduction assay to neutralize the infectivity of herpes simplex virus. Immunization of mice with the vaccinia recombinant expressing HSV-gD gave complete protection on subsequent challenge with lethal doses of live herpes simplex virus.**

The ability to rescue endogenous subgenomic DNA fragments using infectious vaccinia virus (1–3) was extended in our laboratory in the construction of vaccinia virus as a eukaryotic expression vector. This was shown by the expression of the cloned herpes simplex virus (HSV) thymidine kinase gene in infectious recombinant vaccinia virus by our group (4) and by Mackett *et al.* (5). The use of poxviruses as a eukaryotic expression vector in the construction of live recombinant vaccines has been discussed (4) and has been demonstrated in our laboratory by expressing the influenza virus hemagglutinin gene in vaccinia virus recombinants (6). Inoculation of laboratory animals with these recombinant vaccinia viruses elicited the production of antibodies directed against the hemagglutinin gene as detected by neutralization of influenza virus infectivity, hemagglutination inhibition, and other serological tests (6). We have extended this approach of genetically engineered poxviruses for the construction of live recombinant vaccines in the studies reported here. Thus, potential live vaccines directed against an enteric virus, hepatitis B, and a neuro/dermotropic dermotropic virus, herpes simplex, have been constructed by genetically engineering vaccinia virus, and the biological activity of these recombinants was tested in laboratory animals.

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MATERIALS AND METHODS

Construction of Chimeric Donor Plasmids for *in Vivo* Recombination and Isolation of Pure Populations of Infectious Recombinant Vaccinia Viruses. The strategies involved in the construction of chimeric donor plasmids, *in vivo* recombination and the isolation of pure populations of infectious recombinant vaccinia viruses, have been described (1, 4, 6). Construction, analysis, and purification of chimeric donor plasmids were carried out using standard molecular-cloning techniques (4, 6, 7). Cloned hepatitis B virus (HBV) (subtype ayw) genome as a tandem repeat in pBR322 was a generous gift from G. Acs and P. Price (Mt. Sinai, New York). A *Bgl* II fragment containing the surface antigen (sAg) as well as the entire pre-sAg region (8, 9) was inserted into the *Bam*HI site of the internal vaccinia *Hin*dIII F fragment in the plasmid pDP120 next to a vaccinia promoter sequence previously used for HSV thymidine kinase and influenza hemagglutinin expression (4, 6). The plasmid pDP120 contains the *Pst* I subfragment of the internal vaccinia *Hin*dIII F. This chimeric donor plasmid, pDP250B, was used to generate the recombinant vaccinia virus, vP11, by *in vivo* recombination. An *Hha* I fragment derived from the cloned HBV genome and coding for the sAg was modified by the addition of *Bgl* II linkers after filling in the recessed ends with T4 DNA polymerase and inserted into the *Bam*HI site of the vaccinia *Hin*dIII F fragment. This chimeric donor plasmid, pDP232B, was then used to construct the recombinant vaccinia virus, vP59, by *in vivo* recombination.

A 2.5-kilobase (kb) *Hin*dIII/*Sac* I subfragment encoding HSV glycoprotein D (HSV-gD) was subcloned from an *Eco*RI DNA fragment H derived from HSV type 1 (KOS), the sticky ends were filled in with T4 DNA polymerase, and *Bam*HI linkers were attached. This modified fragment was cloned into pRW120 (6), a pBR325 derivative containing the *Pst* I subfragment of vaccinia *Hin*dIII F. This chimeric donor plasmid, pBL330A, was used to construct the recombinant vaccinia virus vP60.

Chimeric donor plasmids and recombinant vaccinia viruses were constructed so the 5′-to-3′ direction of transcription was from right to left relative to the vaccinia genome.

All recombinant vaccinia viruses described here were isolated by a replica filter technique (4), and purified populations were obtained by two or more cycles of plaque isolation.

Abbreviations: HSV, herpes simplex virus; HSV-gD, herpes simplex virus glycoprotein D; HBV, hepatitis B virus; sAg, surface antigen; HBsAg, hepatitis B virus surface antigen; kb, kilobase pair(s); bp, base pair(s); pfu, plaque-forming units; RIA, radioimmunoassay; vP, poxvirus vector; IU, international units.

Restriction endonuclease analysis was carried out on all the recombinants described. The foreign inserts were localized as expected and no rearrangements of the inserted sequences were detected.

Synthesis and Quantitation of the Hepatitis B Virus Surface Antigen (HBsAg). Synthesis of HBsAg was detected using the qualitative radioimmunoassay (RIA), Ausria II, from Abbott and quantified using a parallel line assay with a HBsAg (subtype ayw) standard (12.3 ng/ml) generously provided by C. Troisi (Baylor College of Medicine, Houston, TX).

Inoculation of Rabbits. Nys:(FG) rabbits were inoculated with highly purified recombinant virus or wild-type virus either intravenously or at two or three sites intradermally. Antiserum was collected at weekly intervals.

Quantitation of Antibody Response in Rabbits Inoculated with the Recombinant Vaccinia Virus Expressing the HBsAg. Levels of antibodies directed against the HBsAg were quantitated in terms of RIA units using the commercially available RIA kit (Ausab) from Abbott. Antibody levels were also quantitated in international units (IU) in parallel-line assays using anti-HBsAg (subtype ayw) serum with a concentration of 45 mIU/ml. The control serum was generously provided by C. Troisi.

Neutralization of HSV. Antiserum from rabbits inoculated with recombinant vaccinia virus expressing the HSV-gD was heat-inactivated, mixed with an equal volume of virus, and plated on CV-1 monolayers after holding at 4°C overnight. Virus plaques were visualized by staining with neutral red and were counted after 48 hr.

Immunization of Mice with the Vaccinia Virus Recombinant Expressing the HSV-gD and Challenge with Infectious HSV. Nya:NYLAR mice, an outbred albino strain maintained at the New York State Department of Health laboratories, were inoculated intraperitoneally with either phosphate-buffered saline, wild-type vaccinia virus, or a vaccinia virus recombinant, vP60, expressing the HSV-gD (0.2 ml per mouse). Three weeks after inoculation the mice were challenged with an intraperitoneal injection of infectious HSV type 1 (strain AA) generously provided by J. McSharry (Sterling Winthrop Research Institute, Rensselaer, NY). The fate of the mice was observed for a 2-month period.

RESULTS

Construction of Recombinant Vaccinia Viruses Containing the Gene Coding for the HBsAg. The nucleotide sequence of the 3,182-base pair (bp) HBV (subtype ayw) genome has been elucidated (8). Within this sequence, there is an open reading frame that contains the region coding for the sAg. This open reading frame extends ≈618 bp upstream from the ATG codon for the NH_2-terminal methionine of the sAg and contains two additional in-phase initiation codons. In cells transformed with HBV DNA, it has been shown that this upstream pre-sAg sequence must be intact to allow synthesis of sAg (9–11). Deletion of any part of the pre-sAg region results in abortive transcription with no detectable expression of sAg. We therefore chose the 2.3-kb *Bgl* II fragment for insertion into vaccinia virus. This fragment contains both the pre-sAg and sAg sequences (Fig. 1) in which a 2.3-kb sAg mRNA has been localized (9). Fig. 1 shows the *Hin*dIII restriction map of the VTK⁻79, S variant of the WR strain (4, 12) of vaccinia virus used in these studies as well as the genomes of two vaccinia recombinants containing sequences from HBV. The vaccinia recombinant vP11 contains the *Bgl* II fragment from HBV with the first ATG localized 10 bp from the *Bam*HI site of the vaccinia *Hin*dIII F fragment. Also shown in Fig. 1 is the vaccinia recombinant vP59, in which the modified 1.1-kb HBV *Hha* I fragment encoding the sAg is inserted at the *Bam*HI site of vaccinia virus. In this construct, the single ATG codon is located 20 bp from the *Bam*HI site.

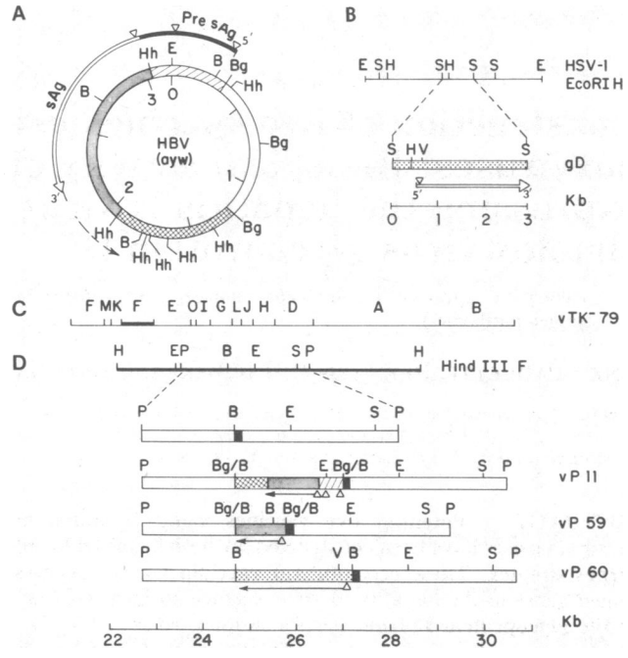

FIG. 1. Schematic representation of recombinant vaccinia viruses containing sequences coding for the HBsAg or HSV-gD. (*A*) A brief map of the HBV (subtype ayw) (8) genome is given showing the sequences coding for the sAg (shaded region) as well as the pre-sAg sequences (hatched region). ▽ denotes ATG codons, and direction of transcription is shown by the arrow. The *Hin*dIII physical map of the S-variant vaccinia virus VTK⁻79 is shown in *C*. The internal *Hin*dIII fragment F is amplified in *D* with additional restriction sites shown. A *Bgl* II fragment from the cloned HBV sequences was isolated and cloned into the *Bam*HI site of pDP120, a plasmid containing the *Pst* I subfragment of *Hin*dIII F in pBR322. This chimeric plasmid was used as donor DNA for *in vivo* recombination with vaccinia virus. A recombinant vaccinia virus, vP11, containing the HBV sequences inserted at the *Bam*HI site of *Hin*dIII F was isolated. An *Hha* I fragment encoding the HBsAg containing a single ATG codon was cloned into pRW120 (6), *Pst* I subfragment of *Hin*dIII F cloned into pBR325, and used for *in vivo* recombination with VTK⁻79 to generate vP59. The *Eco*RI fragment H derived from HSV type 1 (KOS) is shown in *B*. A *Hin*dIII to *Sac* I subfragment of the *Sac* I fragment, known to contain the HSV-gD coding sequence, was also modified and used for *in vivo* recombination. A novel vaccinia recombinant, vP60, was isolated containing the HSV-gD coding sequences inserted into the *Bam*HI site of the *Hin*dIII F fragment. Restriction sites noted are Hh, B, Bg, E, S, H, V, and P for *Hha* I, *Bam*HI, *Bgl* II, *Eco*RI, *Sac* I, *Hin*dIII, *Pvu* II, and *Pst* I, respectively.

Expression of the HBsAg in the Vaccinia Recombinant vP59. Table 1 documents the levels of expression and cellular distribution of the HBsAg synthesized under vaccinia virus regulation. No serologically crossreactive protein was detected in wild-type vaccinia virus-infected cells. Significant levels of HBsAg expression were detected in cells infected with the vaccinia recombinant vP59. In repeated experiments, 150–200 ng of HBsAg was synthesized in a 24-hr infection per 10^6 cells, using vP59 recombinant vaccinia at a multiplicity of infection of ≈2 plaque-forming units (pfu) per cell. Not surprisingly, the majority of the HBsAg was secreted from the infected cells and was localized in the medium. This is known to occur in hepatoma cells (13) and in other tissue culture cells transformed with HBV and expressing the HBsAg (9–11). The presence of HBsAg in the medium is not due to lysis of the infected cells because under these conditions >90% of the viral infectivity remained cell-associated. Significant levels of HBsAg in vP59-infected cells, however, were not detectable by RIA (6) using unfixed infected cells, suggesting that sAg does not accumulate in the cell

Table 1. Synthesis and cellular distribution of the HBsAg

Inoculum	Fraction	Total pfu	% pfu distribution	HBsAg, total ng synthesized	% HBsAg distribution
VTK⁻79	Supernate	7.2×10^5	7	0	0
	Cellular	90.0×10^5	93	0	0
vP59	Supernate	10.9×10^5	7	131	76
	Cellular	146.0×10^5	93	42	24

Monolayers of CV-1 cells were infected at 2 pfu per cell with either wild-type vaccinia, VTK⁻79, or vaccinia recombinant expressing the HBsAg vP59. The supernate was collected 24 hr after infection; the cells were washed with saline and the wash combined with the supernate. The washed monolayer of cells was collected in saline. The fractions were titered for pfu to determine the distribution of progeny virus or assayed for HBsAg using the Ausria test kit. The mean of three determinations is shown.

membrane. The level of expression of HBsAg by the vaccinia recombinant vP59 is several thousand times greater than that detected in vP11 (data not shown). It is not clear at this time whether the multiple in-frame translation initiation codons affect efficient translation or whether the pre-sAg region is expressed and not subsequently processed. Nevertheless, it is clear that the pre-sAg sequences are not required for faithful synthesis of HBsAg under vaccinia virus regulation.

Biophysical Characterization of the HBsAg Synthesized by the Vaccinia Recombinant vP59. The HBsAg secreted from cells infected by the vaccinia recombinant vP59 showed bands at the characteristic density of 1.2 g/ml on CsCl gradients (14, 15) as detected by RIA using the commercial Ausria II test from Abbott (Fig. 2). An electron micrograph (Fig. 3) shows spherical particles present in the peak CsCl gradient fraction. The average diameter of these particles is ≈22 nm, which is similar to the size of those found in carriers or released from transformed cells (16–18).

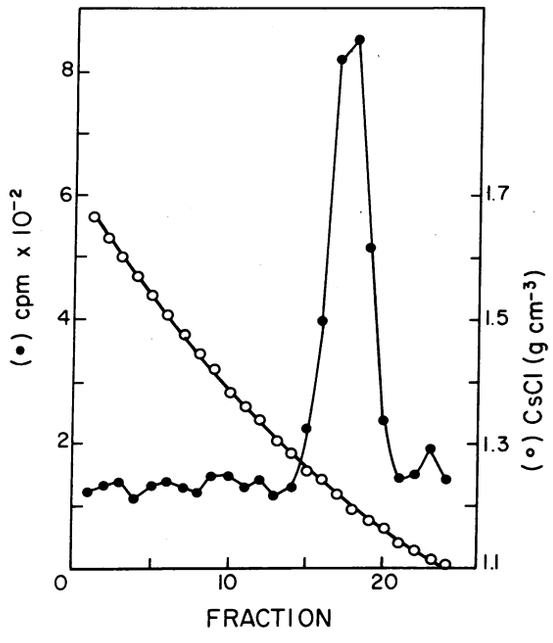

FIG. 2. Isopycnic centrifugation of HBsAg synthesized by the vaccinia recombinant vP59. Supernatant fraction from CV-1 cells infected with the recombinant vaccinia virus vP59 expressing the HBsAg was concentrated by centrifugation at 73,000 × *g* for 24 hr at 4°C in an SW 41 rotor. The pellet was suspended in 0.5 ml of 10 mM Tris·HCl (pH 7.4). Two hundred microliters was layered onto a 1.1–1.6 g/ml CsCl gradient and centrifuged at 35,000 rpm for 36 hr at 18°C in an SW 41 rotor. Fractions (0.5 ml) were collected from the bottom, diluted 1:10 with phosphate-buffered saline and assayed for HBsAg using the Ausria II test kit. Densities were calculated from refractive indices.

Immunogenicity of the HBsAg Synthesized by the Vaccinia Recombinant vP59. To determine whether the HBsAg synthesized under the regulation of vaccinia virus was immunogenic, rabbits were inoculated either intradermally or intravenously with highly purified virus preparations of the recombinant vaccinia vP59. As noted in Fig. 4, significant levels of antibody were detected for the first 6 weeks after inoculation with the recombinant virus. Between 10^5 and 10^6 RIA units per ml of serum were detected regardless of whether the inoculation was intravenous or intradermal. Similar levels of antibody production were obtained over the 20-fold range of concentrations of inoculum tested, well within the variation of responses expected from individual rabbits. It should be noted from Fig. 4 that rabbits inoculated with a mixture of recombinant viruses (vP59, expressing the HBsAg, and vP60, expressing the HSV-gD) gave similar responses in antibody production directed against the HBsAg. These rabbits were also shown to have responded to the HSV-gD antigen by other immunological tests (see below), thus indicating the feasibility of an individual animal responding immunologically to two foreign antigens simultaneously expressed under vaccinia virus regulation. Seven weeks after inoculation, the antibody response in some rabbits was $>3.2 \times 10^5$ mIU/ml, levels significantly greater than required to confer immunity to hepatitis B in humans were one to extrapolate from these laboratory experiments.

Construction of Recombinant Vaccinia Virus Expressing the Glycoprotein D (gD) Gene from HSV. A number of glycopro-

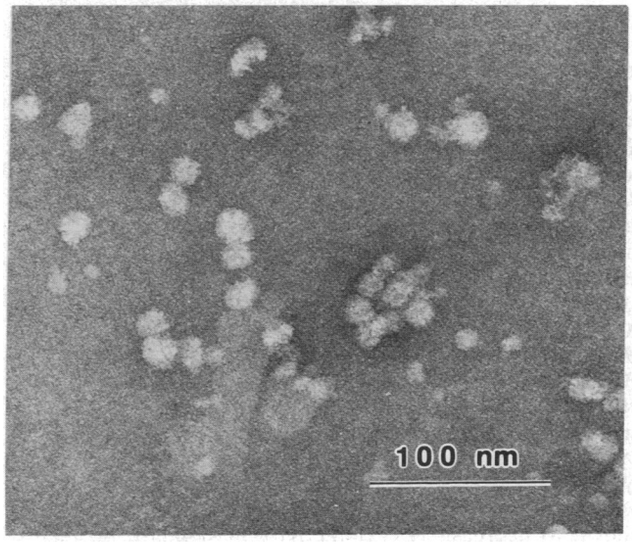

FIG. 3. Electron micrograph of spherical 22-nm-diameter HBsAg particles. Negatively stained (1% uranyl acetate) preparation of immunoreactive material banding at 1.2 g/ml on CsCl density gradients is shown. The electron micrograph was generously provided by W. Samsonoff (Center for Laboratories and Research).

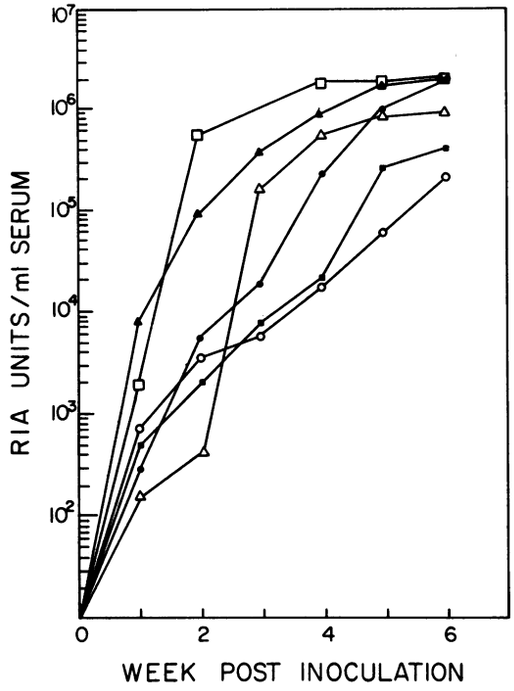

FIG. 4. Antibody response in rabbits inoculated with recombinant vaccinia virus vP59 expressing the HBsAg. Antiserum was collected at weekly intervals and screened for antibodies reactive with HBsAg using a commercially available RIA kit (Ausab) from Abbott. Rabbits were inoculated intradermally at two or three sites with recombinant vaccinia virus vP59 using 1.8×10^7 (●), 1.8×10^8 (○), or 3.6×10^8 (■) pfu of virus. One rabbit (□) was inoculated with 3.6×10^8 pfu intravenously. Mixtures of recombinant vaccinia virus vP59 and the vaccinia recombinant expressing the HSV-gD vP60 were injected intravenously (▲) or intradermally (△) using 1.8×10^8 pfu of each recombinant. Antibody levels are noted in RIA units per ml of serum as defined by the supplier of the RIA kit.

teins are associated with HSV (19). The gD is particularly important because it contains type-common antigenic sites such that antiserum produced against gD from one serotype will also neutralize the other. The coding sequences for the gD have been localized to genome map units 0.9–0.945 by analysis of intertypic recombinants (20) and, more rigorously, to a *Sac* I DNA fragment (21). Recently, the nucleotide sequence of the coding region of the HSV type 1 *gD* gene has been reported (22). The *Eco*RI DNA fragment H (Fig. 1) was purified from HSV type 1 (KOS) and cloned into pBR322. A *Hin*dIII/*Sac* I, 2.5-kb subfragment was derived, the recessed ends were filled in with T4 DNA polymerase, and *Bam*HI linkers were attached. This fragment, lacking the endogenous HSV-gD promoter, was subcloned into pRW120 and designated pBL330A. This chimeric donor plasmid was used to construct the recombinant vaccinia virus vP60 (Fig. 1). The HSV-gD coding sequences are located such that the ATG codon is ≈75 bp from the vaccinia *Bam*HI site in *Hin*dIII F. Preliminary evidence for the expression of the HSV-gD was detected by RIA (6) in unfixed monolayers of vP60-infected cells. To determine that the HSV-gD synthesized by the vaccinia recombinant vP60 was immunogenic, rabbits were inoculated either intradermally or intravenously. Antisera from these rabbits were shown to be reactive with authentic HSV-gD by serological tests and were also shown to decrease HSV infectivity in standard plaque-reduction assays. Thus, using 250 HSV plaques as a test dose, rabbit antiserum obtained 3–5 weeks after inoculation decreased HSV infectivity, as measured by plaque reduction, by >80% at a final serum dilution of 1:160 and by 50% at a final serum dilution of 1:320. It is interesting to note that

Table 2. Protection of mice against challenge with HSV type 1 by immunization with recombinant vaccinia vP60 virus expressing the HSV-gD

Immunizing agent	No. of mice	Survivors	% survival
Phosphate-buffered saline	40	18	45
Vaccinia	40	12	30
Recombinant vaccinia vP60	40	40	100

Mice were inoculated intraperitoneally with saline, 4.5×10^7 pfu of wild-type virus, or 4.5×10^7 pfu of the recombinant vaccinia virus vP60 expressing the HSV-gD. After 3 weeks, the mice were challenged with an intraperitoneal inoculation of infectious HSV type 1 (AA strain), 2.4×10^4 pfu per mouse.

virus-neutralizing antiserum was also obtained from rabbits simultaneously inoculated with vP60 and vP59, the HBsAg expressing vaccinia recombinant (see above).

Protection of Mice by Immunization with the Recombinant Vaccinia Virus vP60 Expressing the HSV-gD Against Challenge with Infectious HSV. Certain strains of mice are highly susceptible to HSV (23, 24). On intraperitoneal injection of live HSV, the mice develop an encephalitis within 5–7 days, with a high mortality rate after another day or two. The mortality rate is dependent on the infectious dose of HSV administered. To determine whether the vaccinia recombinant expressing the HSV-gD would confer immunity to mice challenged with live HSV, the following experiment was carried out. Three sets of mice were inoculated intraperitoneally with saline, wild-type vaccinia virus, or vaccinia recombinant vP60 expressing the HSV-gD. Three weeks later the mice were challenged with intraperitoneal injections of infectious HSV type 1. The results are shown in Table 2. Fifty-five percent of the mice inoculated with a saline solution and 70% of the mice inoculated with wild-type vaccinia virus did not survive the challenge with live HSV. On the other hand, there was a 100% survival rate in the group of mice challenged with HSV but previously immunized with the recombinant vaccinia virus vP60. This experiment convincingly shows protective immunity to HSV conferred by a recombinant vaccinia virus vaccine.

DISCUSSION

Hepatitis B is a worldwide health problem. It is estimated that this infectious disease is a persistent problem for ≈200 million people as either an acute, chronic, or oncogenic process (18, 25). Because of the inability to replicate the viral agent in the laboratory, live or attenuated vaccines directed against hepatitis B have been difficult to obtain. Potential subunit vaccines derived from serum of chronic carriers or from recombinant DNA technology have been tested and shown to be effective. These processes, however, are costly and serious logistic problems exist in terms of worldwide distribution, thus limiting the usefulness of these approaches. The HBsAg, localized on the surface of the infectious Dane particle is considered to be the antigen responsible for conferring immunity to infection with hepatitis B. We have inserted the coding sequences for the HBsAg into recombinant vaccinia virus. Under the control of vaccinia regulatory sequences, levels of expression of the HBsAg sufficient to elicit production of high-titered antibodies in laboratory animals were obtained. If extrapolated to humans, the antibody levels observed in rabbits immunized with the recombinant vaccinia virus expressing the HBsAg would be more than sufficient to confer immunity. Thus, such a live vaccine would be less costly than those currently available, and it could be available for worldwide distribution because of the inherent stability of vaccinia virus. Vaccinia virus has been used for almost 200 years as a live vaccine for smallpox immunization, and the efficacy of vaccinia as a live vaccine has al-

ready been adequately shown in the successful smallpox eradication program. While this manuscript was in preparation, Smith *et al.* (26) reported similar results of HBsAg expression in vaccinia virus recombinants and production of antibody against the HBsAg on inoculation of rabbits.

HSV type 1 and type 2 are known to cause both persistent and latent infections. Recurrent cutaneous manifestations, lethal occurrences in the neonate, encephalitis, and other clinical syndromes have been described (27). In addition, HSV type 2 has been implicated in cervical carcinoma. Genital infections caused primarily by HSV type 2 are thought to affect approximately 9 million people in the United States alone. No vaccine is currently available. The gD is located on the surface of the herpes simplex virion and on the surface of herpes-infected cells. Antiserum directed against the HSV-gD has been shown to neutralize viral infectivity. The gD derived from either HSV type 1 or type 2 contains both type-common antigenic determinants as well as type-specific epitopes. The type-common antigenic determinants are sufficiently crossreactive that antiserum directed against either type 1 or type 2 gD is capable of neutralizing the infectivity of both serotypes of HSV *in vitro* and *in vivo*. We have constructed vaccinia virus recombinants expressing the HSV-gD from HSV type 1. The HSV-gD, as synthesized under the regulation of vaccinia virus, was shown to be antigenic *in vitro*, as defined by reactivity with authentic anti-HSV-gD antiserum. *In vivo*, we have observed immunogenicity in laboratory animals. Rabbits inoculated with the vaccinia recombinant expressing the HSV-gD produced antibodies reactive with authentic HSV-gD, and the antiserum was capable of neutralizing the infectivity of HSV in tissue culture assays. Most important, this recombinant vaccinia virus was shown to elicit protective immunity in mice against a lethal challenge with live HSV. These data suggest an encouraging approach in the control of herpetic infections. Descriptions of vaccinia recombinants expressing the HSV-gD from HSV type 2 and recombinants expressing both type 1 and type 2 HSV-gD will be reported elsewhere.

It is interesting to note that laboratory animals can respond to more than one foreign antigen expressed in vaccinia virus recombinants. This shows the feasibility of using modified vaccinia virus as a polyvalent vaccine vector. In fact, such constructs have been generated and will be described elsewhere.

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