question as to how a molecule that shows such evolutionary variation can serve an important function. The three aspects of IgD gene structure that are very unusual and are conserved between mouse and human are the proximity to the μ gene, the distal coding of the secreted terminus, and the identical cytoplasmic domain of the membrane terminus. All of these are connected with the unusual way in which the expression of the molecule is regulated by mRNA processing. This suggests perhaps that the maintenance of a system of dual antigen receptors on the B-cell surface is more important in evolution than the specific structure of the receptor itself.

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Vaccinia Virus Recombinant Expressing Herpes Simplex Virus Type 1 Glycoprotein D Prevents Latent Herpes in Mice

Abstract. *In humans, herpes simplex virus causes a primary infection and then often a latent ganglionic infection that persists for life. Because these latent infections can recur periodically, vaccines are needed that can protect against both primary and latent herpes simplex infections. Infectious vaccinia virus recombinants that contain the herpes simplex virus type 1 (HSV-1) glycoprotein D gene under control of defined early or late vaccinia virus promoters were constructed. Tissue culture cells infected with these recombinant viruses synthesized a glycosylated protein that had the same mass (60,000 daltons) as the glycoprotein D produced by HSV-1. Immunization of mice with one of these recombinant viruses by intradermal, subcutaneous, or intraperitoneal routes resulted in the production of antibodies that neutralized HSV-1 and protected the mice against subsequent lethal challenge with HSV-1 or HSV-2. Immunization with the recombinant virus also protected the majority of the mice against the development of a latent HSV-1 infection of the trigeminal ganglia. This is the first demonstration that a genetically engineered vaccine can prevent the development of latency.*

In humans, a primary infection of the lips, cornea, or genitalia with herpes simplex virus (HSV) type 1 or type 2 is often followed by the establishment of a latent ganglionic infection that persists for the life of the individual (*1*). These latent infections can reactivate intermittently and give rise to recurrent herpetic lesions even in the presence of high titers of neutralizing antibody (*2*). Our understanding of this process in man is based largely on research carried out on experi-

Table 1. Effect of immunization with vaccinia HSV-1 gD recombinant virus on the lethality of mice challenged with HSV-1 or HSV-2. BALB/c mice, 6 to 8 weeks old, were vaccinated with either 1×10^8 plaque-forming units (pfu) of vaccinia HSV-1 gD (vgD52) or vaccinia HBsAg (vHBs4) or with 1×10^8 pfu of wild-type vaccinia. Sera for antibody determinations were collected from the retro-orbital plexus 4 weeks after immunization. HSV neutralizing antibody titers were determined in a complement-dependent microneutralization assay (*21*). End points were expressed as the reciprocal of the highest twofold serum dilution that prevented a cytopathic effect by 100 tissue culture infectious doses of HSV. The geometric means of the antibody titers were based on data from groups of 14 to 19 mice. In lethality experiments, mice were challenged intraperitoneally with 1×10^8 pfu of HSV-1 (strain F) or 2×10^6 pfu of HSV-2 (strain G).

Immunizing agent	Route of immunization	Antibody titer (geometric mean)	Challenge virus: HSV-1 Dead/inoculated	HSV-1 Mortality (%)	HSV-2 Dead/inoculated	HSV-2 Mortality (%)
None		<4	58/72	81	39/40	98
Vaccinia (wild type)	Footpad	<4	9/14	64	20/21	96
	Tail	<4			19/21	90
Vac HBsAG	Intraperitoneal	<4			18/19	95
	Footpad	<4	18/21	86		
	Tail	<4			20/21	96
Vac HSV-1 gD	Intraperitoneal	54	1/19	5	1/19	5
	Footpad	75	0/28	0	0/20	0
	Tail	60	1/45	2	1/20	5
	Total	62	2/92	2	2/59	3

mental animals. A mouse model has been used to study the establishment, maintenance, and reactivation of HSV (*3–5*). Upon infection of an epithelial surface, such as the lips, HSV is taken up by the nerve terminals at the site of inoculation and spread by axoplasmic transport to nerve cell bodies in the trigeminal ganglia (*3*). A productive infection persists for about 2 weeks, during which infectious HSV can be detected in cell-free homogenates of trigeminal ganglia. Latency then ensues and is defined as the period during which infectious virus can no longer be recovered from cell-free ganglionic homogenates but can be detected by explanting and cultivating trigeminal ganglia on indicator cells such as primary rabbit kidney (*3–5*).

The results of laboratory studies indicate that to prevent the establishment of a latent infection, vaccination must take place before the primary infection occurs (*4*). Both HSV subunit (*6*) and recombinant DNA–derived HSV proteins (*7, 8*) have been proposed as candidate vaccines but have not yet been shown to prevent latent HSV infections. The envelope of HSV consists of several glycoproteins designated gB, gC, gD, and gE (*9*), of which gD has been shown to protect mice against lethal HSV infection (*6, 10*). Recently, the HSV-1 gD gene was sequenced (*11*) and expressed in bacteria (*7*), eukaryotic cells (*8*), and a pox-virus recombinant (*12*). We describe here the construction of two live vaccinia virus recombinants that express HSV-1 gD and the use of one of these recombinants to protectively immunize mice against lethal and latent HSV infection.

For these experiments, two vaccinia recombinants, vgD28 and vgD52, were constructed (*13–16*); these recombinants contain the entire coding sequence of the gD gene of HSV-1 (strain KOS) fused to a vaccinia virus promoter and inserted into the thymidine kinase locus of vaccinia virus. Constructs vgD28 and vgD52 contained promoters $P_{7.5}$ and P_{28}, respectively, which were derived from vaccinia virus transcription units encoding proteins of 7,500 (*17*) and 28,000 daltons (*18*). Promoter $P_{7.5}$ contains both early and late regulatory sequences (*16, 19*), whereas promoter P_{28} contains only late regulatory signals (*18*). In each case, the first translation initiation signal following the vaccinia virus RNA start site was derived from the gD gene. This procedure ensured the synthesis of the authentic HSV protein.

To determine whether the vaccinia-HSV gD recombinant viruses expressed an HSV polypeptide, we reacted tissue culture cell monolayers containing plaques of vaccinia-HSV-1 gD recombinant or wild-type vaccinia with antisera to vaccinia or HSV. Antisera to vaccinia reacted with plaques formed by cells infected with recombinant (Fig. 1A) or wild-type vaccinia (Fig. 1C). In contrast, antisera to HSV reacted with plaques formed by recombinant virus (Fig. 1B), but not with the wild-type virus (Fig. 1D). To show that the HSV polypeptide was gD, we grew tissue culture cells infected with wild-type or recombinant vaccinia viruses in the presence of [^{35}S]methionine or [^{3}H]glucosamine. HSV-specific polypeptides were precipitated with antisera to HSV, dissociated with sodium dodecyl sulfate, and resolved by polyacrylamide gel electrophoresis. Autoradiographs of the gels (Fig. 1E) show that recombinant viruses vgD52 and vgD28 (lanes 3 and 4) synthesized HSV polypeptides that migrated with the 60,000-dalton gD of native HSV-1 (lane 1). Other immunoprecipitated protein bands are thought to represent aggregates or processing intermediates of HSV gD. The 60,000-dalton polypeptide also incorporated [^{3}H]glucosamine (not shown). Recombinant vgD52 was used in all subsequent experiments (*20*).

To assess the ability of the vaccinia HSV-1 gD to induce neutralizing antibodies and protective immunity, we inoculated BALB/c mice with the vgD52 recombinant virus intraperitoneally, subcutaneously (rear footpad injection), or intradermally (at the base of the tail) (Table 1). Mice vaccinated intradermally developed a typical "pox" lesion, which healed in about 4 weeks. Sera for the determination of anti-HSV neutralizing antibody titers were obtained at various times after vaccination and assayed in a complement-dependent microneutralization test (*21*). Neutralizing antibody was detected within 7 days after vaccination and reached a titer as high as 1:128 within 3 to 4 weeks. Approximately the same titers were obtained by the three routes of immunization. In comparison, mice vaccinated with wild-type vaccinia or vaccinia expressing the surface antigen of hepatitis B (vHBs4) (*15*) demonstrated no neutralizing antibody to HSV (titer < 1:4). In other experiments, two rabbits were inoculated intradermally with the vgD52 recombinant virus. HSV-neutralizing antibody was detected in sera 10 days after immunization and reached maximum titers of 1:64 and

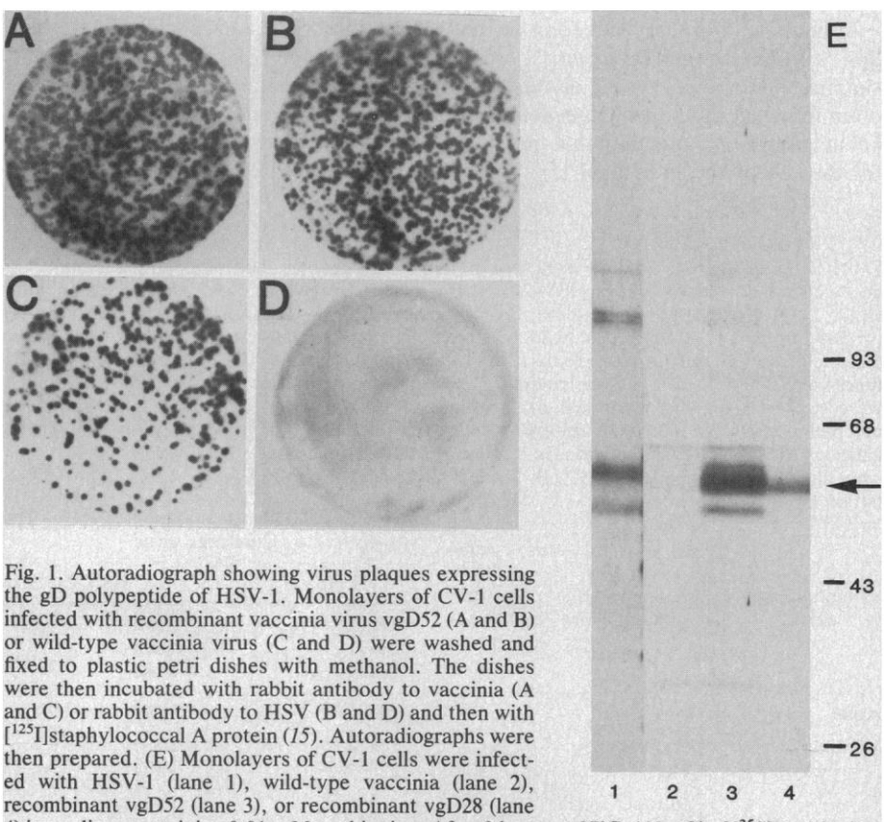

Fig. 1. Autoradiograph showing virus plaques expressing the gD polypeptide of HSV-1. Monolayers of CV-1 cells infected with recombinant vaccinia virus vgD52 (A and B) or wild-type vaccinia virus (C and D) were washed and fixed to plastic petri dishes with methanol. The dishes were then incubated with rabbit antibody to vaccinia (A and C) or rabbit antibody to HSV (B and D) and then with [^{125}I]staphylococcal A protein (*15*). Autoradiographs were then prepared. (E) Monolayers of CV-1 cells were infected with HSV-1 (lane 1), wild-type vaccinia (lane 2), recombinant vgD52 (lane 3), or recombinant vgD28 (lane 4) in medium containing 0.01 m*M* methionine. After 2 hours at 37°C, 100 μCi of [^{35}S]methionine (1000 Ci/mmol) was added and the incubation was continued for approximately 10 hours. Cytoplasmic extracts were prepared and precipitated with a monoclonal antibody to HSV-1 gD (obtained from M. Zweig) (lane 1) and rabbit antiserum to HSV (lanes 2 to 4). Immunoprecipitated polypeptides were dissociated with sodium dodecyl sulfate and resolved by polyacrylamide gel electrophoresis (*15*). The positions of marker polypeptides are at the right (molecular mass × 10^{-3}). The arrow designates the position of the 60,000-dalton HSV protein.

1:128 in 3 weeks. No decrease in titer was observed over the next 9 months.

Recently, Paoletti *et al.* (*12*) reported that intraperitoneal injection of a vaccinia virus recombinant that expresses HSV-1 gD antigen protected mice against a subsequent intraperitoneal challenge with HSV-1. When mice inoculated with our vaccinia–HSV-1 gD recombinant, by any of the three routes, were challenged with a lethal dose of HSV-1 (strain F), only 2 percent of the animals died (Table 1). In contrast, 81 percent of the nonvaccinated mice, and 64 and 86 percent, respectively, of the mice vaccinated either with wild-type vaccinia or the vaccinia recombinant expressing hepatitis B surface antigen (HBsAg) (*15*) died from a lethal challenge of HSV-1 (Table 1). In other experiments, 100 percent of the mice vaccinated with the vaccinia–HSV-1 gD recombinant and challenged with a lethal dose of HSV-1 (MacIntyre strain) were protected (20 of 20 survived), whereas none of 20 unvaccinated mice and none of 20 mice vaccinated with the recombinant expressing HBsAg were protected.

To see whether vaccinia HSV-1 gD would protect mice against HSV-2, we challenged immunized animals with a lethal dose of HSV-2 (strain G). As seen in Table 1, 98, 93, and 95 percent, respectively, of controls (unvaccinated), mice vaccinated with wild-type vaccinia, and mice vaccinated with vaccinia expressing HBsAg (*15*) succumbed to the lethal challenge with HSV. In contrast, only 3 percent of mice vaccinated by any of three routes with the vaccinia HSV-1 gD recombinant virus died after lethal challenge with HSV-2. These data demonstrate that a single inoculation with the vaccinia HSV-1 gD recombinant elicits an immune response against an antigenic determinant common to gD of both HSV-1 and HSV-2 (*6, 22*) and protects mice against a lethal infection by either virus.

To determine whether vaccination would prevent the development of a latent HSV infection, we immunized mice intradermally (tail route) with the vaccinia HSV-1 gD recombinant and 4 weeks later challenged them with the KOS strain of HSV-1 by the lip route. Unimmunized mice and mice immunized with wild-type vaccinia served as controls. After 24 days, the trigeminal ganglia were removed and assayed for latent virus. In two separate experiments (Table 2), approximately two-thirds of the ganglia from mice vaccinated with the recombinant virus were protected from the development of a latent infection.

The protection afforded against the establishment of a latent infection with the vaccinia recombinant compares favorably with earlier results in which live HSV was used as a vaccine (*4*). Nonetheless, approximately one-third of the vaccinated animals were not protected. To see whether this was due to differences in antibody titer among vaccinated mice, we collected serum from individual animals immediately before challenge with HSV and assayed it for neutralizing antibody. Several weeks later, trigeminal ganglia were removed and examined for the presence or absence of latent virus. Analysis of the data revealed that the prechallenge antibody titer was the same (geometric mean titer, 1:16) in those animals that did and in those animals that did not develop a latent infection.

The potential advantages and disadvantages of using vaccinia virus recombinants as vaccines have been discussed (*12, 15*). In the present study, the vaccinia HSV-1 gD recombinant that we constructed proved to be highly effective in raising antibody and protecting mice against lethal challenge by either HSV-1 or HSV-2. Results from past (*4*) and current experiments, however, indicate that the protection against the development of a latent infection may not be solely dependent on the titer of neutralizing antibody. In one hypothesis, protection may be related to whether antibody reaches and neutralizes the infecting virus before the virus enters the sensory neuron where it becomes inaccessible to antibody (*4, 5*). Whether antibody reaches the virus would depend on the route of infection and the state of the epithelial surface at the site of infection (for example, the presence or absence of serum exudate containing antiviral antibody). In humans there is still relatively little precise information about the natural route of HSV infection. Thus, the most effective vaccine in raising neutralizing antibody, whether a live attenuated virus (*23*), a subunit preparation (*24*), a synthetic polypeptide (*25*), or a recombinant virus, may not be totally effective in preventing latency. The answer to this question will only be known after this or other HSV vaccines are tested in controlled clinical trials in humans.

Table 2. Prevention of latent HSV infection by vaccinia HSV-1 gD recombinant virus. Female BALB/c mice were vaccinated with 1×10^8 pfu of vaccinia or vaccinia HSV-1 gD virus by tail scarification. Four weeks later, the mice were challenged bilaterally by the lip route (that is, to infect both trigeminal ganglia) with 6×10^6 pfu of the KOS strain of HSV-1 in experiment 1 or with 2×10^6 pfu in experiment 2. After 24 days, the animals were killed, and the trigeminal ganglia were removed and cultured separately on primary rabbit kidney cells for 3 weeks and then observed for cytopathic effects indicating reactivation of latent HSV. The results are expressed as the ratio of the number of positive ganglia to the total number assayed. We calculated the percentage protection by determining the difference between the fraction (number positive/number tested) of latently infected ganglia in the unimmunized and immunized groups and dividing by the fraction of latently infected ganglia in the unimmunized group (×100).

Vaccination	Number of mice	Trigeminal ganglia (positive/tested)	Protection (%)
Experiment 1			
None	29	55/58	0
Vaccinia (wild type)	30	54/60	5
Vac HSV-1 gD	30	16/60	71
Experiment 2			
None	29	35/58	0
Vaccinia (wild type)	30	40/60	0
Vac HSV-1 gD	30	13/60	63

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13. Construction of vgD28: Nucleotide sequence data indicated the presence of a Hind III site within the untranslated leader segment of the HSV gD gene (*11*). A plasmid pACP13, obtained

from A. Poley, contains an 8.4-kilobase (kb) Hind III–Eco RI fragment of HSV-1 (strain KOS) DNA including the entire coding sequence of the gD gene. Excess DNA beyond the distal end of the gD gene was removed by cleaving the plasmid with restriction endonucleases Sst I and Eco RI, removing the single-stranded ends with T4 DNA polymerase, and recircularizing the plasmid with T4 ligase. The new plasmid, containing a 2.5-kb HSV insert, was designated pMM27. Conveniently, ligation of the two blunt ends regenerated an Eco RI site. The Hind III–Eco RI segment of pMM27 was excised, the staggered ends were filled in with Klenow fragment of DNA polymerase, and then blunt end–ligated into the single Sma I site just downstream from the $P_{7.5}$ vaccinia virus promoter in the plasmid pGS20 (*16*). A resulting plasmid, containing the $P_{7.5}$ promoter and HSV coding segment in the correct orientation, was called pMM28. The HSV gene under control of the vaccinia virus promoter was then inserted into the TK locus of the vaccinia virus genome by homologous recombination as described (*15, 16*) and TK^- recombinants were selected and plaque purified. The predicted structure of the recombinant DNA was confirmed by restriction endonuclease digestion and hybridization to appropriate HSV DNA probes.

14. Construction of vgD52: The staggered ends of the 2.5-kb Hind III–Eco RI segment of pMM27 were filled in with the Klenow fragment of DNA polymerase, and the resulting DNA was blunt end–ligated into the single Hinc II site just downstream from the P_{28} vaccinia virus promoter in plasmid pLTP1 (*19*). A resulting plasmid having the P_{28} promoter and HSV coding segment in correct orientation was called pMM52. Homologous recombination was used to insert the gene into the TK locus of vaccinia virus.
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20. All of the plaques formed by vgD52 reacted with antibody to HSV, whereas only 20 percent of the plaques formed by vgD28 reacted with anti-HSV antibody, even after repeated plaque purification. This difference did not appear to be due to instability of the vaccinia promoter, since promoter $P_{7.5}$ had been used to make stable vaccinia virus recombinants expressing other gene products, including the hepatitis B virus surface antigen (*15*) and the influenza virus hemagglutinin [G. L. Smith, B. R. Murphy, B. Moss, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 7155 (1983)]. Because of this problem, vgD52 was used in all immunization experiments.
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Identification of Human Glucocorticoid Receptor Complementary DNA Clones by Epitope Selection

Abstract. *Steroid hormones regulate cellular differentiation and physiologic functions predominantly through gene transcription. Regulation is achieved by the interaction of specific steroid receptor proteins and target genes. Expression cloning techniques were used to select human glucocorticoid receptor complementary DNA clones in order to define the mechanism by which the receptor exerts its transcriptional control. Immobilized fusion proteins from individual clones were used to select epitope-specific antibody which was subsequently eluted and identified by binding to protein blots of cellular extracts. Three cross-hybridizing clones containing inserts expressing antigenic determinants of the human glucocorticoid receptor were isolated.*

The regulation of eukaryotic gene expression in response to intercellular signals such as hormones represents a critical strategy for development and homeostatic regulation. Such regulation is modulated by compounds that bind intracellular receptors and those that interact with plasma membrane receptors. Steroid hormones regulate transcription as a consequence of binding specific intracellular receptors (*1*). The interaction between the steroid hormone and receptor initiates a transformation of the complex, after which it is capable of binding high-affinity receptor sites on chromatin and regulating transcription of a limited number of genes (*2*). The rates of transcription of mouse mammary tumor virus (MMTV), mouse metallothionein, and rat growth hormone genes are stimulated by one class of steroid hormones, the glucocorticoids, in cultured cell lines (*3*). Purified rat liver glucocorticoid receptor complexes bind a specific region of cloned MMTV DNA in vitro, suggesting that steroid receptors modulate transcription by binding specific regulatory sequences near promoters (*4*). Furthermore, deletion analysis of the human metallothionein II gene and of the MMTV promoter has defined regions that identify upstream control elements necessary for steroid response (*5*).

One model suggests the existence of distinct steroid-binding and DNA-binding domains in the receptor polypeptide as well as a major immunogenic region (*6*). Although sufficient protein for a direct structural analysis has not been available, polyclonal and monoclonal antibodies have been prepared against partially purified glucocorticoid receptor (*7, 8*). The receptor has been characterized as a 94-kilodalton (kD) polypeptide by biochemical and immunological criteria (*7–9*) as well as by covalent binding studies with labeled steroid analogs (*10*). In the absence of amino acid sequence information, we have attempted to isolate human glucocorticoid receptor complementary DNA (cDNA) clones with a polyclonal antiserum that is reactive with several proteins in addition to the receptor. An epitope selection technique has been used for the identification of these clones.

Rabbit polyclonal antiserum recognizing glucocorticoid receptor (GR 884) (*8*) was initially characterized for its ability to recognize denatured receptor epitopes by immunoblot analysis. Cytoplasmic extracts from human cells producing glucocorticoid receptor were fractionated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) (*11*) and transferred to nitrocellulose filter paper (*12*). This filter was then incubated with GR 884 and specific binding was revealed by subsequent exposure of the filter to ^{125}I-labeled *Staphylococcus* protein A. This antibody identified a number of immunoreactive proteins (Fig. 1). If the antiserum was affinity purified with rat liver glucocorticoid receptor (*13*), only immunoreactivity against the 94- and 79-kD proteins was detected (Fig. 1, lane 3). This agrees with data from human and other mammalian species in which a 94-kD protein frequently copurified with a 79-kD putative cleavage product (*8, 9*).

Receptor from steroid-treated cells has a high affinity for chromatin and remains tightly associated with the nucleus, while receptor from untreated cells has a low affinity for chromatin and thus is found in the cytoplasm during the isolation procedure (*14*). There was a dramatic reduction in the levels of the 94- and 79-kD molecules in cytoplasmic extracts from cells after steroid treatment (Fig. 1, lanes 1 and 2). The relative levels of other nonreceptor proteins, however, were not affected. These data indicate that the antisera recognized an epitope of the glucocorticoid receptor.

To efficiently screen a large number of clones, a λgt11 complementary DNA (cDNA) library was prepared with size-fractionated polyadenylated [poly(A)$^+$] mRNA from the human IM-9 B-cell line (*8*). These cells contain 10^5 receptor molecules per cell, which is approximately tenfold more glucocorticoid receptors than overproducing tissues such as normal liver and lymphoid cells. The procedure used was based upon that of Young and Davis (*15*) and gave a library of approximately 2×10^5 independent members, 75 percent of which contained inserts. Approximately 7.5×10^5 recombinant phage were screened with GR 884