

# Replication-Defective Mutants of Herpes Simplex Virus (HSV) Induce Cellular Immunity and Protect against Lethal HSV Infection

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**Live viruses and live virus vaccines induce cellular immunity more readily than do inactivated viruses or purified proteins, but the mechanism by which this process occurs is unknown. A trivial explanation would relate to the ability of live viruses to spread and infect more cells than can inactivated virus. We have used live but replication-defective mutants to investigate this question. Our studies indicate that the immune responses of mice to live virus differ greatly from the responses to inactivated virus even when the virus does not complete a replicative cycle. Further, these studies indicate that herpes simplex virus-specific T-cell responses can be generated by infection with replication-defective mutant viruses. These data indicate that the magnitude of the cellular immunity to herpes simplex virus may be proportional to the number or quantity of different viral gene products expressed by an immunizing virus.**

Because herpes simplex viruses (HSVs) are ubiquitous human pathogens which cause infections in newborn and immunocompromised patients and considerable morbidity in otherwise healthy people, considerable efforts have gone into producing herpesvirus-specific vaccines (2, 7, 20, 31, 39, 43-46). Although transient protection has been achieved by immunizing mice with purified glycoproteins (2), the search for long-lasting immunity has led investigators to concentrate on the development of live vaccines which are better stimulators of T cells (7, 24, 27-29, 31, 36, 43). The use of live viruses such as vaccinia virus as vectors is controversial because of the toxicity of the parental strain itself (13, 21).

Experiments in mice have defined the requirements for induction of T-cell immunity to HSV. While purified proteins are capable of inducing excellent antibody responses (3), live virus was much more effective at inducing cell-mediated responses (9, 11, 27, 28, 37). Inactivation of virus by heat or UV light markedly reduces the ability of virus to induce HSV-specific cytotoxic T lymphocytes (CTL) (22). Schmid and Rouse demonstrated that the failure of inactivated HSV-1 (as opposed to live virus) to stimulate CTL activity (or increase precursor numbers) was due to an inability of these preparations to stimulate helper T cells (38).

Infection of cells by HSV leads to expression of a sequence of different virus-encoded proteins. The  $\alpha$  (immediate-early) genes are expressed first (16, 36). The products of  $\alpha$  genes are required for expression of the next set of genes, which are termed  $\beta$  (early) genes. The gene products of the  $\beta$  genes encode products required for DNA synthesis. Expression of the  $\gamma_1$  class of late genes can be detected before DNA replication, but the  $\gamma_2$  late gene products are barely detected in the absence of DNA replication (36).

Several viral proteins have been shown to regulate expression of HSV-1 genes. The ICP4 gene product is essential for  $\beta$  and  $\gamma$  protein expression (10). The ICP27 gene product is required for  $\gamma$  protein expression and viral DNA replication (26, 35). Separate functions of ICP27 activate  $\gamma_1$  and  $\gamma_2$  gene

expression because the *n504* mutant allows  $\gamma_1$  but not  $\gamma_2$  gene expression (35). The major DNA-binding protein (ICP8), a  $\beta$  product, is also an essential viral gene product because it is required for viral DNA replication (12, 32, 33) and late viral gene expression (12). Mutant viruses lacking ICP8 function express immediate-early and early viral gene products (12).

Although late glycoproteins of HSV are produced in great quantities in cell culture as a result of lytic infections and these proteins may be an important target of antiviral antibodies (3, 14, 18, 20), early regulatory proteins are also recognized by T cells (23, 25). Although T cells appear to be important in reactivation of herpesviruses, it is not clear what epitopes are seen *in vivo*. It is conceivable that proteins which are made in less quantity or at an earlier stage of infection may be more important in inducing T-cell immunity (23).

Live virus infection has been demonstrated to induce long-lived T-cell immunity. However, live virus vaccines, even those based on attenuated virus, are often shunned because of the possibility of (i) reversion to virulence or (ii) dissemination of even avirulent vaccines in unusual hosts (30). Therefore, we have examined the ability of live viruses that are unable to replicate to induce immune responses.

## MATERIALS AND METHODS

**Mice.** Female BALB/c mice were purchased from Taconic Laboratory, Germantown, N.Y., and were used at 6 to 12 weeks of age. Mice were injected with virus (or medium alone) intraperitoneally (i.p.) in 0.5-ml volumes. In accordance with our Institutional Animal Care and Use Committee, in order to avoid 50% lethal dose measurements and the unnecessary sacrifice of animals, a previously established dose ( $5 \times 10^7$  PFU of the mP strain of HSV-1) at which 80 to 100% of mice would die was used in these experiments (40).

**Viruses.** HSV-1 strains KOS 1.1 and mP were propagated on Vero cells as described previously (33). The ICP8 gene deletion mutant virus *d301* was described previously (33) and was propagated and titrated on S-2 cells, which express the

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ICP8 protein from a resident gene upon viral infection (33). The mutant virus *n504R* has a nonsense mutation in the ICP27 gene and was described previously (35). This virus was propagated and titrated on V27 cells, which express the ICP27 protein from a resident gene upon HSV infection (35). The *d301* and *n504* mutants fail to replicate on Vero cells or other normal cells and thus are considered to be replication defective. They can, however, replicate on these special cells, which provide the complementing gene product. Wild-type virus gives very similar titers on Vero, S-2, or V27 cells. Thus, the administration of equal numbers of PFUs of these different viruses ensures that approximately similar numbers of viral particles are being administered, as observed previously (33, 35). The ICP4 gene deletion mutant virus *d120* was a gift of Neal DeLuca (10). Vesicular stomatitis virus (VSV) (Indiana strain) was obtained from Robert Kauffman (Harvard Medical School) and was passaged on L cells as described previously (15). Virus stocks were stored at  $-70^{\circ}\text{C}$ , and a new aliquot of stock virus was used for each experiment. UV-irradiated HSV-1 and UV-irradiated VSV were prepared by irradiating the virus at  $0^{\circ}\text{C}$  by using a 30-W UV source (G30T8; General Electric) for 45 min at a distance of 5 cm. Psoralen-inactivated virus was obtained from Lee Biomolecular (San Diego, Calif.). UV irradiation reduced viral titers by 5 to 6 log units, while psoralen-inactivated virus had no evidence of live virus when tested by plaque assay.

**Assays of T-cell response.** Immune spleen cells were obtained from mice injected i.p. 3 to 4 weeks earlier with  $10^6$  PFU of either the ICP4-, ICP27- or ICP8-deficient HSV-1 mutant or the parental HSV-1 KOS 1.1 strain (virus titer was determined prior to inactivation in the case of inactivated viruses). Negative controls were phosphate-buffered saline (PBS)-injected mice. Spleen cells were depleted of erythrocytes and polymorphonuclear leukocytes by Ficoll-Hypaque gradient sedimentation. B cells were depleted by immunodepletion, using J-11d2 supernatants (6, 42). Splenocytes were incubated with J-11d2 supernatants for 30 min at  $4^{\circ}\text{C}$  and were then washed and further incubated with goat anti-rat antibody-coated latex-polymer beads with a magnetic core (Advanced Magnetics, Cambridge, Mass.). J11-d2-positive cells bound to the magnetic beads were then removed by a magnet (BioMag Separator; Advanced Magnetics). While decreasing the B cells background, this depletion did not eliminate all antigen-presenting cells, and good T-cell activity was seen (6). In experiments in which spleen cells were depleted of  $\text{CD4}^+$  cells, depletion was accomplished with the rat anti-mouse CD4 antibody GK1.5 (American Type Culture Collection). After depletion, the remaining spleen cells (now  $>98\%$  T cells) were washed and incubated at  $10^5$  cells per well in quadruplicate in a total volume of 0.2 ml in 96-well round-bottom culture plates (Nunc, Roskilde, Denmark). Responder cells were stimulated with UV-irradiated HSV-1 KOS 1.1 or VSV. Control cultures without added viruses were prepared in parallel. Medium used was Dulbecco modified Eagle medium (Hazleton) supplemented with 5% bovine calf serum (HyClone Laboratories) heat inactivated at  $56^{\circ}\text{C}$  for 1 h, 100 U of penicillin (GIBCO) per ml, 100 U of streptomycin per ml, 1 mM sodium pyruvate (GIBCO), 0.1 mM nonessential amino acids (GIBCO),  $10^{-4}$  mM 2-mercaptoethanol (Sigma), and 2 mM glutamine (GIBCO). The cells were incubated for 3 or 5 days in a 10%  $\text{CO}_2$  at  $37^{\circ}\text{C}$  before being pulsed with 1  $\mu\text{Ci}$  of  $[^3\text{H}]$ thymidine (New England Nuclear) for 6 h and harvested by using a Skatron cell harvester. The samples were counted for 1 min in a scintillation counter (Beta Trac 6895; TM Analytic).

**Antibody assays.** Serum samples were analyzed by enzyme-linked immunosorbent assay (ELISA) for the presence of HSV-specific antibodies. The protocol was adapted for murine serum from that described by Kahlon and Whitley (18). Microtiter plates (Linbro/Titertek) were coated with 0.1 ml of a 1:50 dilution of a  $10^7$  PFU of HSV-1 (grown in Vero cells) in PBS overnight. Serum samples of mice immunized with viruses i.p. 3 weeks previously were obtained individually through retro-orbital bleeding. Microplates coated with HSV antigen were washed three times and incubated with 100  $\mu\text{l}$  of serum diluted 1:100 and then threefold overnight at room temperature. Plates were then washed again and incubated with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G2a at a 1:250 dilution (Southern Biotechnology) for 3 h at  $37^{\circ}\text{C}$ . Thirty minutes after the addition of 1 mg of phosphatase substrate (Sigma 104) per ml, the reaction was stopped by the addition of 75  $\mu\text{l}$  of 3 N NaOH. The plates were read with an ELISA reader at 405 nm. For all experiments, the standard was pooled serum from HSV-1-immunized mice. The negative control consisted of pooled naive mouse serum. Mouse sera were run individually, and data are presented as means and standard errors of the means.

## RESULTS

**Lack of morbidity with use of replication-defective viruses.** The major DNA-binding protein, ICP8, is an essential viral gene product required for viral DNA replication. Similarly, ICP27 is an essential viral gene product required for late gene expression. To study the ability of replication-defective viruses to stimulate immunity, mice were injected with live HSV or replication-defective mutant viruses which were defective for either ICP8 (*d301*) or ICP27 (*n504*). Challenge of mice with up to  $10^8$  PFU of these replication-defective viruses had no apparent effect on the mice. Littermates injected with  $10^7$  PFU of the parental KOS 1.1 strain were all killed.

**Induction of antibody responses by replication-defective viruses.** To determine whether the replication-defective viruses were capable of inducing antibody responses, we bled mice 2 weeks postchallenge and measured HSV-specific antibodies. Interestingly, the antibody responses, while present, were markedly lower than those seen after stimulation with parental (KOS 1.1) strain (Fig. 1). As expected, more antibody was induced by infection with the ICP27 mutant than by infection with the ICP8 mutant. Similar results were noted both 2 and 4 weeks postinfection in an independent experiment. To investigate whether expression of early ( $\beta$ ) proteins was necessary for induction of antibody responses, we challenged mice with  $10^6$  PFU of a virus strain which could not produce either early or late proteins (the ICP4 deletion mutant *d120*). Challenge with the ICP4-deficient strain, while inducing a response greater than that seen with a medium control, was below that seen with the other mutants (Fig. 1). Antibody titers remained elevated in all groups for at least 3 months after immunization.

**Induction of T-cell responses by replication-defective viruses.** To investigate the ability of the various mutant viruses to induce T-cell immunity, we measured the responses of splenic T cells from challenged mice to viral antigens *in vitro*. Mice received  $10^6$  PFU of either live wild-type (KOS 1.1) virus or a replication-defective mutant (ICP4, ICP8, or ICP27). Three weeks later, T cells from mice of each group were incubated with UV-irradiated HSV. To control for the effect of nonspecific T-cell stimulation, we also evaluated the

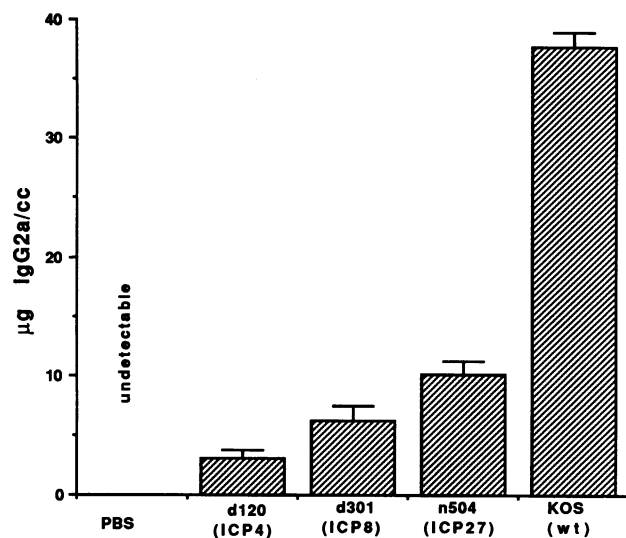


FIG. 1. Induction of an antibody response by  $\alpha$  (immediate-early) and  $\beta$  (early) viral protein-deficient mutants. BALB/c mice (eight per group) were challenged i.p. with either  $10^6$  PFU of the ICP4-, ICP27-, or ICP8-defective mutant or with the same dose of wild-type (wt) HSV-1 (KOS 1.1 strain) in PBS (negative control). Two weeks later, HSV-specific immunoglobulin G2a (IgG2a) in serum was measured individually by ELISA. Data are presented as means with standard errors of the means. This experiment is representative of four experiments performed.

responses to an unrelated virus, VSV. Splenic T cells from mice immunized with VSV did not proliferate in response to HSV (while the same cells did proliferate in response to VSV) (Fig. 2 and 3). Stimulation above the background level was seen in splenocytes from mice challenged with each of the replication-defective viruses (Fig. 2). While our results indicate that immunization of mice with the mutant viruses led to a level of stimulation less than that induced by the wild-type parental KOS 1.1 strain, evidence for substantial T-cell reactivity is seen with all nonreplicative viruses. Similar responses were seen when spleen cells were stimulated for either 3 (Fig. 2) or 5 (Fig. 3) days *in vitro*. Previous experiments had indicated that no T-cell proliferation was obtained from T cells of mice immunized by heat-inactivated or UV-irradiated virus (30a, 39). Furthermore, immunization with psoralen-inactivated virus also did not stimulate T-cell responses (Fig. 3). The responding T cell was  $CD4^+$ , as *in vitro* depletion of spleen cells with anti- $CD4$  antibodies eliminated the response (Fig. 3).

**Induction of protective immunity by replication-defective viruses.** To assess the ability of replication-defective viruses to induce immunity to lethal HSV disease, we inoculated mice with  $10^6$  PFU of live ICP4, ICP27, or ICP8 mutant virus. Three to six weeks later (depending on the experiment), all mice were challenged with a lethal dose ( $5 \times 10^7$  PFU) of a virulent strain of HSV-1 (mP). In three separate experiments, all mice infected with  $10^6$  PFU of the ICP8 or ICP27 mutant were protected against lethal disease, while 80 to 100% of mock-infected mice died. To compare the protective effects of the various mutant viruses, subsequent experiments were performed with all three mutant viruses (d120, d301, and n504). While only one of nine control (PBS-injected) mice survived, survival with ICP27 or ICP8 mutant preimmunization was 100%. Even the ICP4 mutant (d120), which allows for expression of only the immediate-

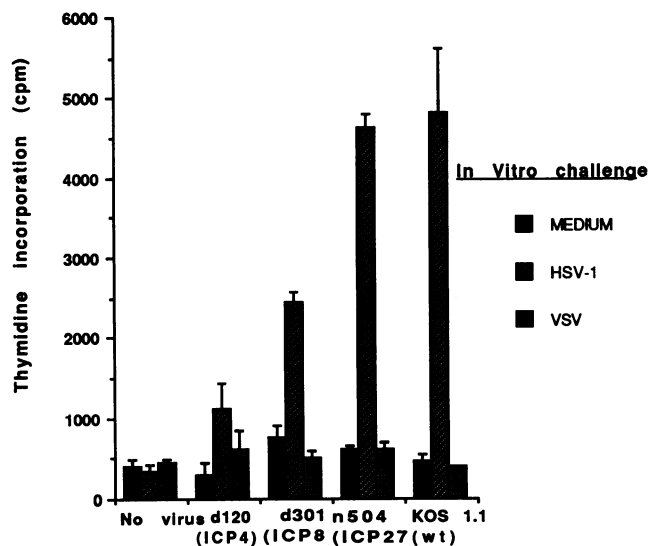


FIG. 2. Evidence that immunization with nonreplicative mutant viruses leads to T-cell responses. T cells from mice (four per group) immunized with ICP4-, ICP27-, or ICP8-defective HSV-1 were incubated with UV-irradiated HSV-1 or VSV at a multiplicity of infection of 1 for 3 days as described in Materials and Methods. T cells from unimmunized mice (as a negative control) and wild-type (wt) (KOS 1.1)-primed T cells (as a positive control) were analyzed in parallel. Data for [ $^3H$ ]thymidine uptake are presented as means with standard errors of the means. Results of one assay, representative of three performed, are shown.

early genes, protected the majority of mice. In contrast, immunization with psoralen-inactivated virus did not protect mice against lethal challenge (Fig. 4).

## DISCUSSION

The studies presented in this report demonstrate that replication-defective HSV mutant strains can induce both humoral and cellular immunity and protect against subsequent infection with wild-type virus. Cellular immunity is especially important in protection against infection by HSV, probably both in limiting virus spread at a site of productive infection and in maintaining virus in a latent infection (44). Thus, any agent which is capable of inducing anti-HSV cellular immunity is a potential anti-HSV vaccine. In contrast to the observations with replication-defective mutant viruses presented in this report, inactivated viruses are incapable of inducing cellular immunity to HSV (38). The replication-defective mutant viruses used in this study are intermediate in nature between an attenuated live virus (29) and an inactivated virus vaccine in that these mutant viruses can infect cells and carry out a substantial portion of the replicative cycle and thus are living, but they cannot produce progeny virus.

Studies with VSV have shown that immunization with defective interfering particles results in the generation of cellular immunity at levels less than that induced by whole virus (5). Defective interfering particles are able to stimulate  $CD4^+$  cells but not  $CD8^+$  cells, which may be important in T-cell immunity (5). Interestingly, immunization of swine with live VSV results in substantial levels of protective

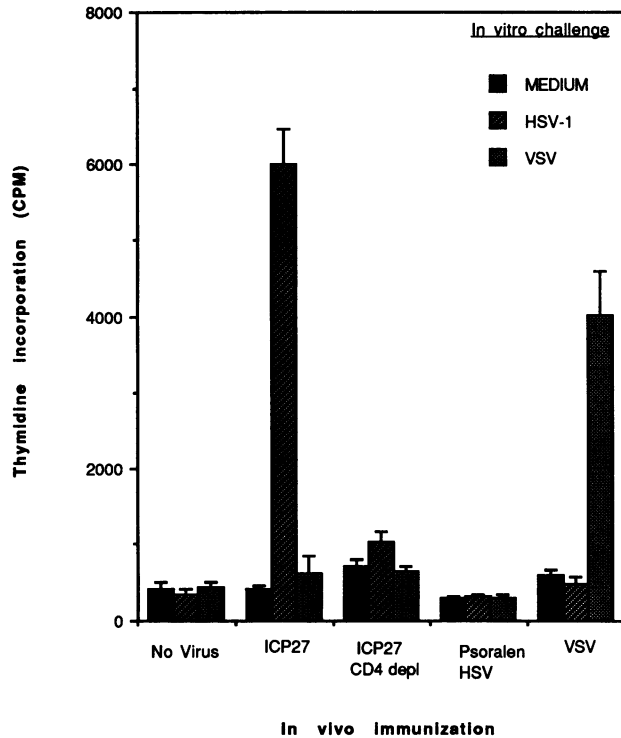


FIG. 3. Stimulation of CD4<sup>+</sup> T cells by mutant virus. Mice were immunized with psoralen-inactivated virus (four mice per group), VSV (two mice per group), or ICP27 (five mice per group). Another group of spleen cells from the ICP27-immunized mice were also depleted (depl) of CD4<sup>+</sup> cells prior to culture. Cells were cultured for 5 days prior to pulsing with [<sup>3</sup>H]thymidine as described in Materials and Methods.

immunity (34), suggesting that whole virus vaccines may be protective and that noninfectious particles may be suboptimal inducers of immunity.

The induction of cellular immunity by these mutant viruses is presumably due to the expression of viral gene products in the infected cells, processing of these viral polypeptides, and their subsequent presentation to helper T cells. The *n504* ICP27 gene mutant virus appeared to elicit greater humoral and cellular immunity than did the *d301* ICP8 gene mutant virus or the *d120* ICP4 gene mutant virus. This finding is consistent with the ability of the *n504* mutant virus to express nearly normal amounts of  $\alpha$ ,  $\beta$ , and  $\gamma 1$  late viral gene products (35), thereby expressing a wider range of viral gene products than does *d301*, which expresses  $\alpha$  and  $\beta$  viral gene products and more limited amounts of  $\gamma$  viral gene products (12). The *d120* mutant virus, which expresses the fewest number of viral gene products, elicited the lowest level of T-cell response.

The target for anti-HSV T-cell immunity is not well defined. The  $\alpha$  ICP4 and ICP27 proteins are recognized by CTL from certain strains of mice (1, 23). Both mutant viruses express these gene products, and although *n504* expresses a slightly truncated form of ICP27, this form of ICP27 should retain the potential CTL epitope identified by Banks et al. (1). Human CTL clones have been shown to recognize gD or gB (46). Murine T lymphocytes have been shown to recognize gB, gC, or gD (7, 17, 24, 27, 28, 37, 45). The *n504* mutant should express more gD and gB than does the *d301* mutant. Neither mutant is likely to express very

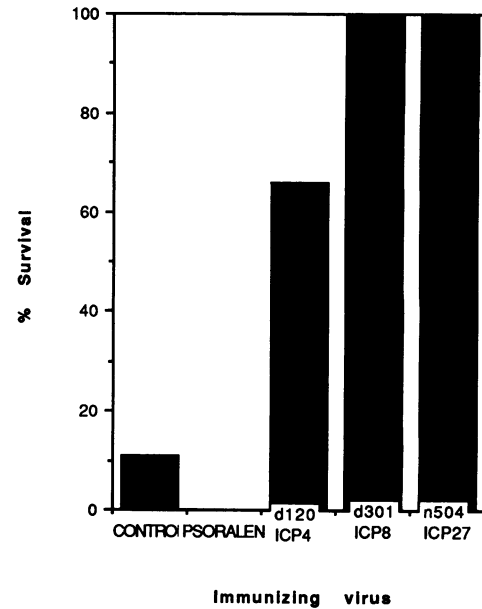


FIG. 4. Evidence that immunization with nonreplicative HSV-1 mutants leads to protection against a lethal challenge. Groups of BALB/c By mice were immunized i.p. with  $10^6$  PFU of either the ICP4 (six mice), ICP27 (six mice), or ICP8 (six mice) deletion mutant or an equivalent dose of psoralen-inactivated virus in PBS (five mice). The control group (nine mice) was injected i.p. with PBS. Six weeks later, all mice were challenged i.p. with a lethal dose of  $5 \times 10^7$  PFU of HSV-1 (mP strain). Most mice died between days 7 and 11. Survival rates were recorded 4 weeks postchallenge. This experiment is representative of four experiments performed.

significant levels of gC. Therefore, in general, the magnitude of the immune responses to these mutant viruses is consistent with the number of viral gene products expressed. This study suggests that the magnitude of the cellular immune response to HSV is proportional to the number of different viral gene products expressed by the infecting virus. This observation has implications for the design of vaccines against HSV.

None of the mutant viruses used in this study can replicate in cells that do not express the complementing viral gene product. Thus, it is unlikely that these viruses spread further than the initial cells that they infect. This consideration has two implications for the pathogenesis of these viruses. First, the viral antigens are expressed only in the cells that are initially infected, and these cells have to be capable of presenting the antigens for an immune response. Second, the viruses are not likely to spread to the nervous system. Consistent with this notion, no latent infection can be detected in studies using in situ hybridization to detect the latency-associated transcript in trigeminal ganglion tissue from animals inoculated with the mutant virus by corneal scarification (19a).

The fact that these replication-defective viruses are able to induce immune responses which are capable of protecting mice against lethal infection suggests a new general strategy for vaccine design. Many virus replicative cycles involve a programmed order of steps in viral replication in order to produce infectious progeny virus particles. If a regulatory gene mutation that allows expression of some or nearly all of the viral gene products but does not allow completion of the viral life cycle can be introduced into a viral genome,

immunity may be elicited against the viral gene products. The processing of the viral gene products should then allow the generation of a cellular immunity. However, the lack of spread of these viruses should provide a very safe vaccine. This approach should be applicable to any virus with a complex replicative cycle such as HSV.

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