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Mechanisms of Immunization with a Replication-Defective Mutant of Herpes Simplex Virus 1

LYNDA A. MORRISON[1] and DAVID M. KNIPE[2]

Department of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, Massachusetts 02115

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We have investigated the mechanisms by which subcutaneous immunization of mice with a replication-defective mutant of herpes simplex virus 1 protects against infection of the eye and latent infection of the trigeminal ganglion following corneal challenge. First, we have shown that immunization reduces the number of trigeminal ganglion neurons in challenged animals that express the latency-associated transcript. This indicates that the reduction in the incidence of latent infection by challenge virus is likely due to immune mechanisms and not saturation of the potential sites of latent infection by the immunizing mutant virus itself. Second, the duration of protective immunity against acute infection, keratitis, and latent infection was similar in mice immunized with replication-defective or -competent virus; thus, the replication-defective mutant virus is able to induce durable immunity apparently without spread in the host. Third, although the mutant virus showed no evidence of replication *in vivo,* it was present in footpad tissue in an infectious form for several days. This surprising observation raises the possibility that continued infection events by input virus over an extended period of time may have a boosting effect on the developing immune response which could explain, at least in part, the capacity of these replication-defective mutant viruses to elicit a robust and durable immunity despite their inability to spread within the host.

INTRODUCTION

Herpes simplex virus (HSV) is a common human pathogen that causes significant morbidity in its various disease manifestations, which include encephalitis, keratitis, and disseminated disease of the newborn (Roizman, 1991; Whitley, 1996). In addition to acute infection which initiates at a mucosal or epithelial surface, the virus can establish a latent infection of sensory neurons innervating the infected mucosa. Once latency is established in the nervous system, the virus is apparently impervious to elimination by the host immune response. Periodic reactivation from latency leads to recurrent episodes of disease that can be debilitating, as in the case of keratitis, the second most common cause of corneal blindness (Whitley, 1996). Because of the multiple stages of HSV infection, effective vaccination will require the induced immune response to intervene in the establishment of latent infection as well as reduce the severity of acute disease. Third, the immunity must be long-lasting.

Numerous approaches to immunization against HSV have been tried, including inactivated virus, live attenuated virus, glycoprotein subunits, and heterologous virus vectors expressing HSV glycoproteins (reviewed in Burke, 1992). HSV glycoprotein formulations elicit both humoral and cellular immune responses, but these have been shown to decline with time in both animal studies (Meigner *et al.,* 1987) and human trials (Mertz *et al.,* 1984) and have been nonprotective in humans (Mertz *et al.,* 1990; Corey, 1991). Recombinant HSV glycoproteins have also been employed with potent adjuvants and are protective in animal model systems against acute infection and establishment of latent infection (Dix and Mills, 1985; Manservigi *et al.,* 1990a, 1990b; Langenberg *et al.,* 1995; Mishkin *et al.,* 1991) and reduce the number of ganglia expressing the latency-associated transcript (Burke *et al.,* 1991). Both adenoviruses and vaccinia viruses have been used as vectors to express HSV glycoproteins as a form of live virus vaccine (Burke, 1992; Gallichan *et al.,* 1993). The vaccinia vectors have been shown to elicit immune responses in mice that can reduce disease (Cantin *et al.,* 1983; Paoletti *et al.,* 1984; Cremer *et al.,* 1985; Weir *et al.,* 1989; Blacklaws *et al.,* 1990) and latent infection (Cremer *et al.,* 1985; Blacklaws *et al.,* 1990). In addition, durable immunity was elicited in mice by a vaccinia virus vector expressing HSV gD (Rooney *et al.,* 1988), as would be expected of a live-virus vaccine (Burke, 1992). The utility of vaccinia vectors may be limited by their poor immunogenicity in vaccinia-immune individuals (Cooney *et al.,* 1993; Hammond *et al.,* 1992).

Replication-impaired mutant HSV strains have also been examined for their capacity to induce protective immunity against HSV (Nguyen *et al.,* 1992; Morrison and Knipe, 1994; Farrell *et al.,* 1994). These viruses have the advantage of expressing virus proteins in an endogenously synthesized form to stimulate broader immune

[1] Current address: Department of Molecular Microbiology and Immunology, Saint Louis University School of Medicine, 1402 S. Grand Ave., St. Louis, MO 63104.

[2] To whom correspondence and reprint requests should be addressed. Fax: (617) 432-0223. E-mail: dknipe@warren.med.harvard.edu.

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responses, while possessing the safety of an inactivated vaccine. One form of replication-impaired virus lacks a viral gene product essential for progeny virus production (Gao and Knipe, 1989; Rice and Knipe, 1990). These mutants express viral proteins in infected cells but cannot replicate in the host, and must be propagated *in vitro* on a cell line that complements the defective gene product. We had previously observed that mice immunized twice subcutaneously with *d*301, a replication-defective virus containing a deletion in the gene encoding ICP8 (Gao and Knipe, 1989), mount an immune response similar to that seen in mice immunized with the wt parental strain, KOS1.1 (Morrison and Knipe, 1994). When challenged 1 month later by corneal inoculation of a virulent HSV-1 strain, the mice were completely protected from development of keratitis and encephalitis and from latent infection of the nervous system (Morrison and Knipe, 1994). A second form of mutant HSV-1 lacking gH, and therefore capable of undergoing a single round of replication in infected cells, has been shown to protect against disease in a mouse skin model (Farrell *et al.,* 1994) and a guinea pig genital model (McLean *et al.,* 1994).

Little is known about the mechanisms by which the replication-defective mutant viruses induce protective immunity against latent infection by challenge virus or, in general, how live viruses induce protective immunity against persistent infection. The studies in this report address three questions regarding these mechanisms. First, does immunization with replication-defective mutant HSV reduce establishment of latent infection by the challenge virus or merely its ability to reactivate? A related issue is whether the mutant virus establishes latency in the neurons and interferes with establishment of latent infection by the challenge virus, as hypothesized for the live attenuated SIV vaccine viruses in T lymphocytes (Rud *et al.,* 1994). Second, is the immunity induced by the replication-defective mutant virus durable? Live viruses are known to elicit long-lived immunity in the host while killed or subunit vaccines elicit a less durable immunity. Replication-defective mutant viruses share properties with each of these two forms of vaccine in that they infect cells and express endogenous viral antigens like a live virus but fail to spread within the host similar to an inactivated or subunit vaccine. Thus, it is not apparent how durable the immune responses to replication-defective mutant viruses would be. Third, how can a replication-defective virus induce an immune response that is comparable to that induced by a virus that spreads within the host?

MATERIALS AND METHODS

Cells and viruses

The wild-type (wt) KOS1.1 (Knipe *et al.,* 1982) and microplaque (mP) (Hoggan and Roizman, 1959) strains of HSV-1 were grown on Vero cell monolayers. The *d*301 strain is a replication-defective mutant of the KOS1.1 strain bearing a deletion in the UL29 gene encoding ICP8 (Gao and Knipe, 1989). This virus expresses products of α, β, and $\gamma 1$ gene classes in infected normal cells but cannot replicate and must be propagated on a cell line, S2, which is stably transformed with the UL29 gene. Stocks of all viruses were prepared by inoculating Vero or S2 cell monolayers at low multiplicity of infection (0.1 PFU/cell) and incubating at 34° in 199 medium containing 1% FCS. Cells were collected when rounded and detaching and pelleted at 300 *g* for 10 min at 4°. Cell pellets were resuspended in culture supernatant plus an equal volume of sterile nonfat milk (0.9×), frozen at −80°, thawed, and disrupted by sonication (Heat Systems, Farmingdale, New York) three times for 30 sec each on ice. Lysates were clarified by centrifugation at 300 *g* for 10 min, and aliquots were stored frozen at −80°. Lysate of uninfected Vero cell monolayers was prepared as a control (control cell lysate). Partially purified KOS1.1 was prepared by harvesting the culture medium of infected Vero cell monolayers, subjecting the supernatant to low speed centrifugation to remove detached cells, and then pelleting the virus at 27,000 *g* for 45 min at 4°. Virus pellets were resuspended in 199 medium containing 15% glycerol, sonicated for 15 sec, and stored at −80°. Wild-type KOS1.1 and mP strains were titered on Vero cells, and *d*301 was titered on S2 cells. Although titered on Vero cells, KOS1.1 grows equally well on Vero and S2 cells (Gao and Knipe, 1991). Because inactivated virus particles are not effective for induction of immune responses in this system (Morrison and Knipe, 1994), the number of viral genomes capable of entering cells is a more valid comparison of different viral stocks than particle:PFU ratios. Cells exposed to equal numbers of PFU's of KOS1.1 wt and *d*301 contain approximately equal amounts of viral DNA at 1 hr postinfection (hpi) (Gao and Knipe, 1993). Thus, the two viral stocks seemed to be comparable in terms of genomes capable of entering host cells.

Animals and inoculations

Female BALB/c mice, 6 weeks of age, were purchased from the National Cancer Institute (Fredericksburg, MD) or Taconic (Germantown, NY) and were acclimated 1 week before use. The mice were housed in accordance with institutional policies and National Institutes of Health guidelines (Committee on Care and Use of Laboratory Animals, 1985). For immunization, hair on the left rear flank of each mouse was trimmed and 2×10^6 PFU of *d*301 or wt KOS1.1 virus, or an equivalent amount of control cell lysate, were inoculated by subcutaneous (s.c.) injection of a 20-μl vol using a 26-gauge needle. Secondary immunizations were given 4 weeks later in identical fashion.

At 1, 4, or 7 months after the final immunization, groups

of six to nine mice were anesthetized and challenged by inoculation onto the scarified corneas of 1×10^5 PFU/eye of the HSV-1 mP strain as previously described (Coen *et al.,* 1989), except that the volume of the inoculum was 5 μl.

Clinical observations and keratitis scoring

Mice were observed daily in the period postchallenge for clinical signs of encephalitis: ruffled fur, hunched posture, ataxia, and anorexia. Keratitis scores, representing both severity and extent of disease, were determined for each eye of challenged mice as described previously (Morrison and Knipe, 1994).

Assays of acute and latent infection

To assess acute replication at various times after corneal challenge, the eyes of each mouse were individually swabbed with moistened cotton-tipped applicators. The two swabs from each mouse were placed together in vials containing 1 ml assay medium (PBS containing 0.1% glucose and 1% FCS) and stored frozen at −80° until time of assay. Viral titers were determined by standard plaque assay on Vero cells (Knipe and Spang, 1982).

To assess replication *in vivo* of mutant and wt virus, groups of mice were injected s.c. in the footpad with 1×10^6 PFU of *d*301 or KOS1.1 virus. At 5 min after inoculation, footpad tissue was collected from a pair of animals to assess the amount of recoverable virus. Then at 4 hr and 1, 2, and 3 days after inoculation, pairs of mice were sacrificed and footpad tissue was collected into vials containing 1 ml assay medium and stored at −80°. Viral titers in the tissues were determined by plaque assay on S2 cells after samples were thawed and disrupted by sonication for 30 sec each.

For assessment of reactivating virus indicative of latent infection, trigeminal ganglia were removed from animals 28 days after corneal challenge and the tissue was enzymatically dissociated by incubation in trypsin and collagenase as previously described (Kennedy *et al.,* 1980; Leib *et al.,* 1991), except that after 30-min incubation at 37°, the tissue was pelleted and half of the dissociation medium was removed and replaced with serum-free medium, followed by another 30-min incubation. Dissociated ganglionic cells were added to Vero cell monolayers in 6-well tissue culture dishes (1 ganglion/well). Wells were observed for the presence of cytopathic effect (CPE) after 11 days of incubation at 37° and 5% CO_2. Wells with no apparent CPE were collected, sonicated, and cultured on fresh Vero cell monolayers to look for plaque formation.

For assessment of latency-associated transcript (LAT) expression in the trigeminal ganglia indicative of latent infection, trigeminal ganglia removed from mice immunized twice with *d*301 or control cell lysate and then challenged 1 month later were frozen and serially sectioned at 8 μm using a cryostat microtome. Every other section was collected and hybridized with a ^{35}S-labeled DNA probe, pIPH (Leib *et al.,* 1989), to detect LAT. *In situ* hybridization was performed as previously described (Haase *et al.,* 1984; Stroop *et al.,* 1984; Leib *et al.,* 1989).

Statistical analyses

Fisher's exact test with *P* values in 1 tail were used to determine the significance of differences in the proportion of latently infected trigeminal ganglia between mice inoculated with control cell lysate and those immunized with *d*301 or KOS1.1. The significance of differences in the number of LAT$^+$ neurons per section was determined by the two sample *t* test for independent samples.

Determination of neutralizing antibody titers

Blood was collected from the tail veins of groups of five to seven mice at various times after immunization. Sera from each group were pooled and stored at −20°. When all serum samples had been collected, complement-dependent and -independent neutralizing antibody titers were determined by a CPE inhibition assay. Sera were serially diluted in twofold steps beginning at 1:4 in DME supplemented to contain 10% FCS, 20 m*M* HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 m*M* glutamine. Aliquots of 25 μl of each dilution were mixed with an equal volume of partially purified virus in medium alone or containing complement (1:4 dilution of rabbit low-tox H, Cedarlane, Hornby, Ontario, Canada) for final concentrations of 2×10^3 PFU/ml of virus and 1:8 dilution of complement. Mixtures containing complement were incubated at 37° for 80 min and mixtures lacking complement were incubated at 4° for 80 min. Each was then mixed with an equal vol of a suspension of trypsinized Vero cells at 4×10^5/ml, and 100 μl were added to individual wells of 96-well flat-bottom tissue culture plates, six replicates per sample, for final concentrations of 100 PFU/well and 2×10^4 cells/well. After 3 days of incubation at 37° and 5% CO_2, wells were scored for the presence of CPE. Titers represent the reciprocal of the final serum dilution preventing CPE in 50% of wells as approximated by the method of Reed and Muench (1938). Complement mixed with virus in the absence of HSV-specific serum antibody or in the presence of normal mouse serum had no effect on infectivity.

Assays of T cell proliferation

HSV-specific T cell proliferative responses of splenocytes from two pooled spleens for each immunization group were determined at various times after immunization, as described previously (Morrison and Knipe, 1994), except that partially purified virus and control supernatant of uninfected cells were used as the *in vitro* stimuli.

TABLE 1

Reduction in Number of LAT$^+$ Neurons in Trigeminal Ganglia of Immunized Mice[a]

Immunization group	Ganglion	Number of sections examined	Number of LAT$^+$ neurons	LAT$^+$ neurons per section	Mean ± SD
Cell lysate	1	19	520	27.37	19.26 ± 11.48
	2	14	156	11.14	
*d*301	1	30	11	0.37	0.25 ± 0.1
	2	30	2	0.07	
	3	29	7	0.24	
	4	27	8	0.29	
	5	27	7	0.26	
	6	24	7	0.29	

[a] Mice were immunized twice with 2×10^6 PFU of *d*301 or an equivalent amount of uninfected cell lysate and then underwent corneal challenge. One month later, trigeminal ganglia were collected and tissue sections were hybridized to a probe for LATs.

RESULTS

Mechanism of protection against latent infection by challenge virus

We had shown previously that immunization of mice with replication-defective mutant viruses reduces the incidence of latent infection of the trigeminal ganglia by the wt challenge virus as assayed by reactivation following explant cocultivation (Morrison and Knipe, 1994). This reduction could be due to decreased establishment of latent infection or to decreased frequency of reactivation. To distinguish between these two possibilities, we measured the numbers of trigeminal ganglion neurons expressing the LAT, a marker of latent infection (Stevens *et al.,* 1987; Croen *et al.,* 1987), in animals immunized twice with the replication-defective mutant virus *d*301 or inoculated with control cell lysate and challenged with virulent virus by corneal inoculation. At 28 days postchallenge, the trigeminal ganglia were removed from the animals and tissue sections from the ganglia were processed for *in situ* hybridization using a probe specific for LAT (Leib *et al.,* 1989). The control animals showed approximately 20 LAT-positive neurons per section (Table 1) while the *d*301-immunized animals showed a LAT-positive neuron in only about one in four sections (Table 1). These results allowed two conclusions about the mechanism of protection against latent infection. First, immunization with *d*301 reduced the number of LAT-positive neurons ($P < 0.01$) and therefore likely reduced the number of neurons that are latently infected by challenge virus. Thus, the protective effect was not simply a reduction in the ability of the latently infected neurons to reactivate, the parameter measured previously (Morrison and Knipe, 1994). Second, as evidenced by the lack of LAT-positive neurons, *d*301 did not spread from the site of immunization and establish latent infection in the trigeminal ganglion neurons, thereby saturating the reservoir of sites for latent infection in the trigeminal ganglion. Instead, the mechanism of protection seemed to be based on the immune response of the host to the *d*301 mutant virus.

Duration of the protective immunity induced by a replication-defective virus

Because replication-defective viruses possess properties of both inactivated or subunit vaccines and live virus vaccines, it was unclear how durable the immune responses to a replication-defective mutant virus would be. Of particular interest to infection with HSV is protection against latent infection because reduction in latency is crucial to lessening the severity of recurrent disease and the possibility for transmission. We could not measure the duration of responses to inactivated virus because UV-inactivated virus had not protected against latent infection by challenge virus or elicited detectable neutralizing antibody titers in our previous studies (Morrison and Knipe, 1994). As a consequence, we compared the durability of the immune response induced by *d*301 and its replication-competent parental wt virus, KOS1.1 in protecting against disease and acute and latent infection, as outlined in Fig. 1. Groups of BALB/c mice (which are susceptible to HSV-mediated keratitis and latent infection (Harbour *et al.,* 1981; Foster *et al.,* 1986)) were immunized s.c. in the rear flank with 2×10^6 PFU of *d*301, KOS1.1, or an equivalent amount of control cell lysate, and were immunized again 1 month later. Sera were collected after each immunization to assess primary and secondary HSV-specific, neutralizing antibody responses. At 1, 4, and 7 months after the second immunization, sera were again collected, and a cohort of mice was sacrificed to assess the HSV-specific, T cell proliferative response. Subsets of the mice were then challenged by inoculation of virulent HSV-1 onto scarified corneas in an amount equivalent to 5 to 15 times the dose required to produce encephalitis in 50% of the mice. The eyes of the mice were swabbed daily through 4 days postchallenge to determine viral titer in the tear film as a measure

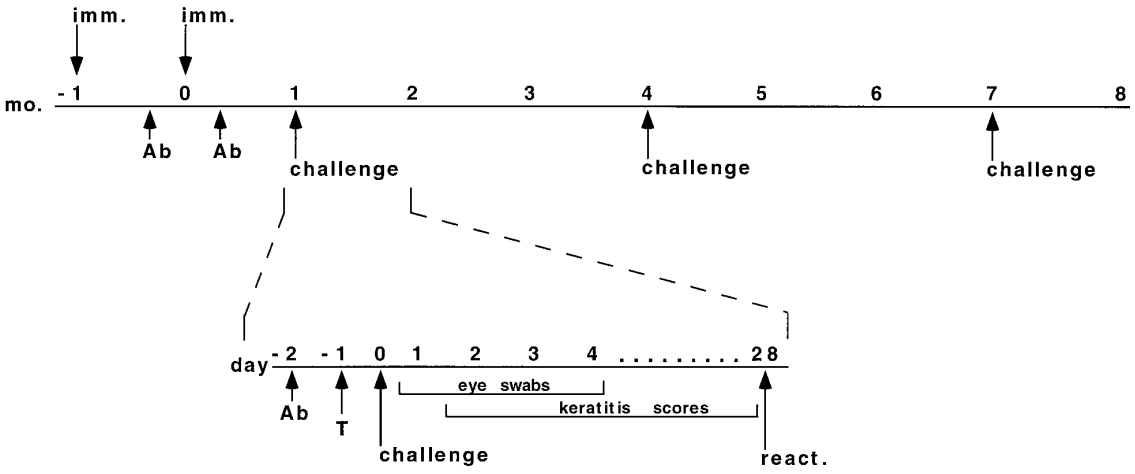

FIG. 1. Schematic representation of the experiment designed to test duration of immune responses and protection. The upper horizontal time line shows the full time course of the experiment. The lower horizontal line shows expanded view of procedures surrounding each challenge at 1, 4, or 7 months after the final immunization. Imm., s.c. immunization; Ab, serum collection for determination of neutralizing antibody titers; T, assessment of splenic T cell proliferative responses in cohort animals; challenge, corneal inoculation of challenge virus; eye swabs, daily sampling of tear film for determination of viral titer; keratitis scores, periodic examination of eyes for signs of disease (also indicates time period of evaluation for signs of encephalitis); react, explant and coculture of dissociated trigeminal ganglia for determination of latent infection.

of acute replication. The mice were also monitored at regular intervals for signs of encephalitis and keratitis. Surviving mice were sacrificed 28 days after challenge to assess the presence of latent infection in the trigeminal ganglia.

Duration of immune responses. Sera collected from five or six mice of each immunization group were tested for HSV-specific neutralizing antibody in a CPE inhibition assay. Titration of complement-dependent neutralizing antibodies (Fig. 2A) showed a primary antibody response 21 days after the first immunization with mutant or wt virus. The titers were augmented greatly and to similar extents by secondary immunization with the mutant or wt virus. The titers in both mutant and wt virus-immunized mice declined by 60–75% during the ensuing 3 weeks but then remained constant through the following 7-month period (Fig. 2A). Complement-independent neutralizing antibody titers of these same sera were lower but were also similar for wt and mutant-infected mice and remained roughly constant for 1–7 months after immunization (Fig. 2B).

To examine the durability of a T cell memory response, we examined T cell proliferative responses at various times after immunization. We detected a strong T cell proliferative response from splenocytes of either mutant or wt virus-immunized mice at 1, 4, and 7 months after immunization (Fig. 3). The response was HSV-specific in that control supernatant placed in culture stimulated only low levels of incorporation compared to responses to HSV-infected lysate (Figs. 3A and 3B), except that back-

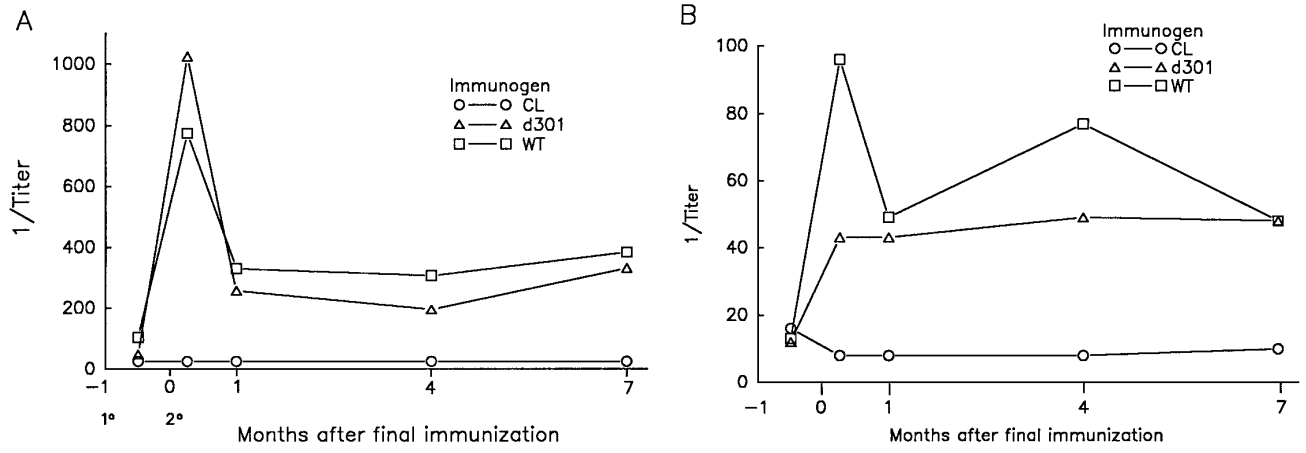

FIG. 2. Neutralizing antibody titers in sera from immunized mice. Sera were collected from groups of six mice at the indicated times after immunization, and HSV-specific neutralizing antibody titers of pooled sera were determined in a CPE inhibition assay. Titers shown represent a statistical approximation of the reciprocal of the highest serum dilution giving 50% inhibition of CPE. CL, control cell lysate. (A) Complement-dependent titers; (B) complement-independent titers.

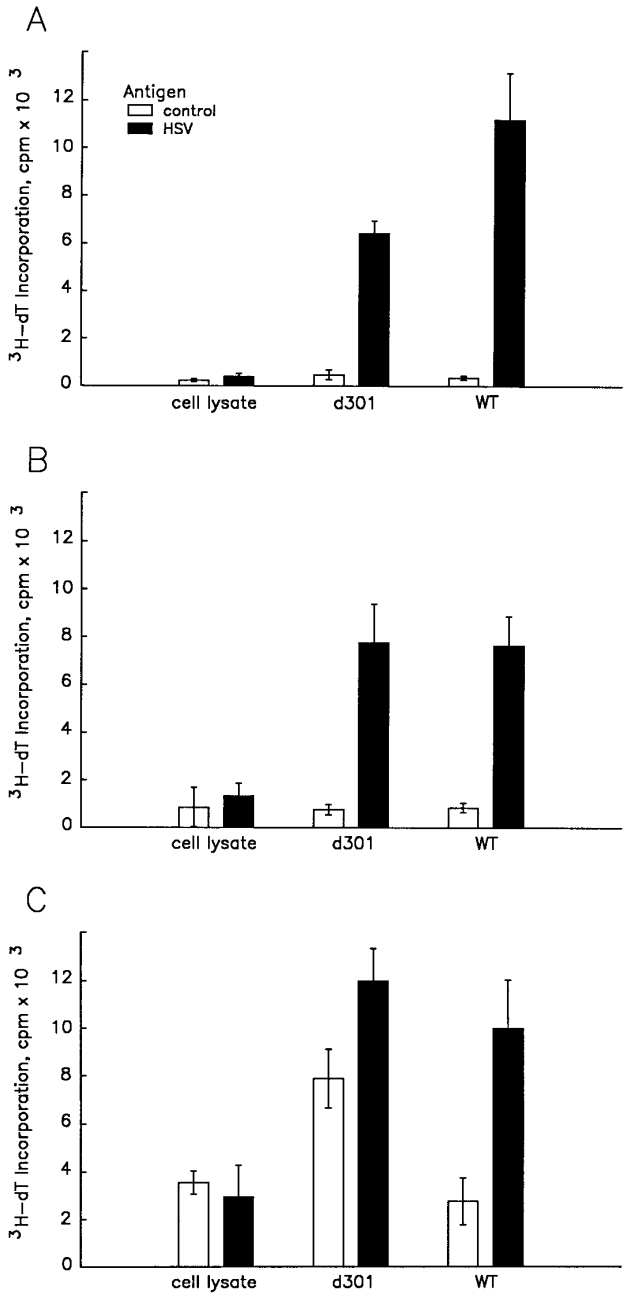

FIG. 3. T cell proliferative response in immunized mice. Splenocytes from two mice per group were collected and pooled and depleted of B cells at (A) 1, (B) 4, and (C) 7 months after immunization. The remaining mononuclear cells were placed in culture in quadruplicate with partially purified, UV-inactivated HSV or control supernatant. Values represent the mean cpm ± SD of [^{3}H]thymidine on Day 4 of culture.

ground proliferation was much higher at 7 month (Fig. 3C). Furthermore, the proliferative response of splenocytes from mutant virus-immunized mice was maintained at a level nearly equivalent (1 month) or equivalent (4 and 7 months) to that seen in mice immunized with wt virus. These results indicated that mice immunized with replication-defective virus maintained neutralizing antibody and T cell proliferative responses as well as mice immunized with wt virus through at least 7 months after immunization, and that HSV-specific immunity existed in the mutant virus-immunized mice at each time of challenge.

Duration of protection against acute viral replication in the eye. We assessed whether the immune response elicited by immunization with replication-defective virus maintained over time the capacity to reduce viral replication at the initial site of infection in the eye. Less virus was shed in the tear film of mice immunized with mutant virus than in mice inoculated with control cell lysate when both groups of mice were challenged 1 month later (Fig. 4A). This capacity of the immune response to reduce viral titer in the eye was maintained in mice challenged at 4 or 7 months after the final immunization (Figs. 4B and 4C). Differences in viral eye titer of 1 to 2 log between control and immunized mice were apparent as early as 1 day after infection. Mice immunized with mutant or wt virus showed similar reductions in eye titer at 1 and 4 months, and the reduction was strikingly equivalent at 7 months (Fig. 4).

Duration of protection against encephalitis and keratitis. By 5 days postchallenge, mice inoculated twice with control cell lysate uniformly developed signs of encephalitis when challenged at 1, 4, or 7 months after immunization: 88, 100, and 86% of the mice eventually succumbed to infection at the three times, respectively. In contrast, mice immunized twice with replication-defective or wt virus showed no signs of encephalitis whether challenged at 1, 4, or 7 months after immunization. Severe keratitis with stromal lesions also was observed in mice inoculated with control cell lysate, with mean keratitis scores reaching 3+ after challenge at all three times (Fig. 5). Immunization with mutant or wt virus completely protected mice challenged at 1 or 4 months from development of stromal disease (Figs. 5A and 5B) and protected most mice challenged at 7 months (Fig. 5C). However, at 7 months, focal lesions of the cornea were seen in three of the eyes of both mutant and wt virus-immunized mice beginning 14 days after challenge. In 2/14 or 1/12 eyes from mutant or wt-immunized mice, respectively, these lesions increased in severity to 3–4+. Histologic examination of the lesions revealed heavy infiltration by mononuclear cells compared to unaffected eyes (data not shown). Whether damage to the corneas was mediated by viropathologic or immunopathologic mechanisms could not be determined. Thus, there was less acute replication of challenge virus in the eyes of mutant virus-immunized mice and lower incidence of disease compared with control mice at all times although there was some evidence of waning protection by 7 months in both wt and mutant virus-immunized mice.

Duration of protection against latent infection. We assessed whether the immune response to replication-defective virus maintained over time its capacity to decrease latent infection of the nervous system by chal-

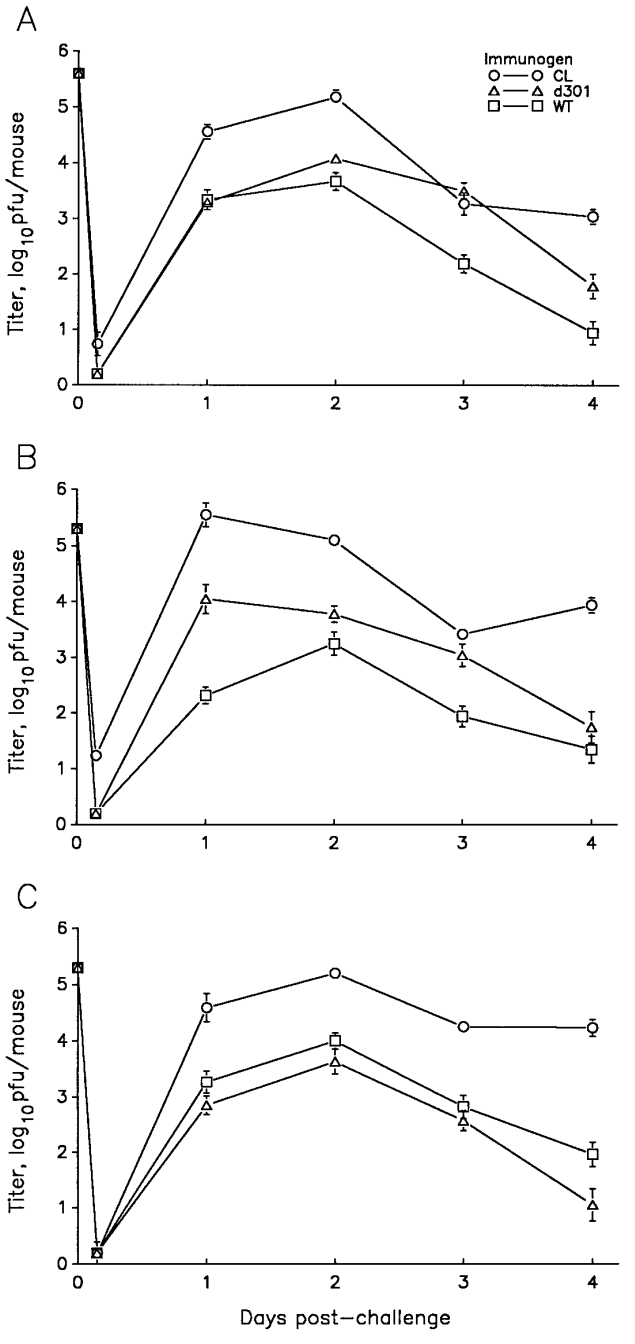

FIG. 4. Reduction of challenge virus replication in the eye. Mice were challenged at: (A) 1, (B) 4, or (C) 7 months after final immunization. Samples of tear film were collected daily, and titer of infectious virus as a measure of acute replication was assessed by standard plaque assay. All samples contained infectious virus, and the values shown represent the mean titer of samples from five or six mice per group ± SEM.

lenge virus. Twenty-eight days after challenge, the trigeminal ganglia of surviving mice were assayed for the presence of latent, reactivatible virus by tissue explant, dissociation, and coculture with permissive cell monolayers. The dissociation assay was used because it is regarded as the most sensitive means of assessing latent infection with the capacity for reactivation (Leib *et al.,* 1991; Jacobson *et al.,* 1993). When challenged at 1 month after immunization, none of the ganglia from wt virus-immunized mice and only 17% of ganglia from mutant virus-immunized mice contained reactivating challenge virus. Cultures of all ganglia from surviving mice inoculated with control cell lysate showed evidence of

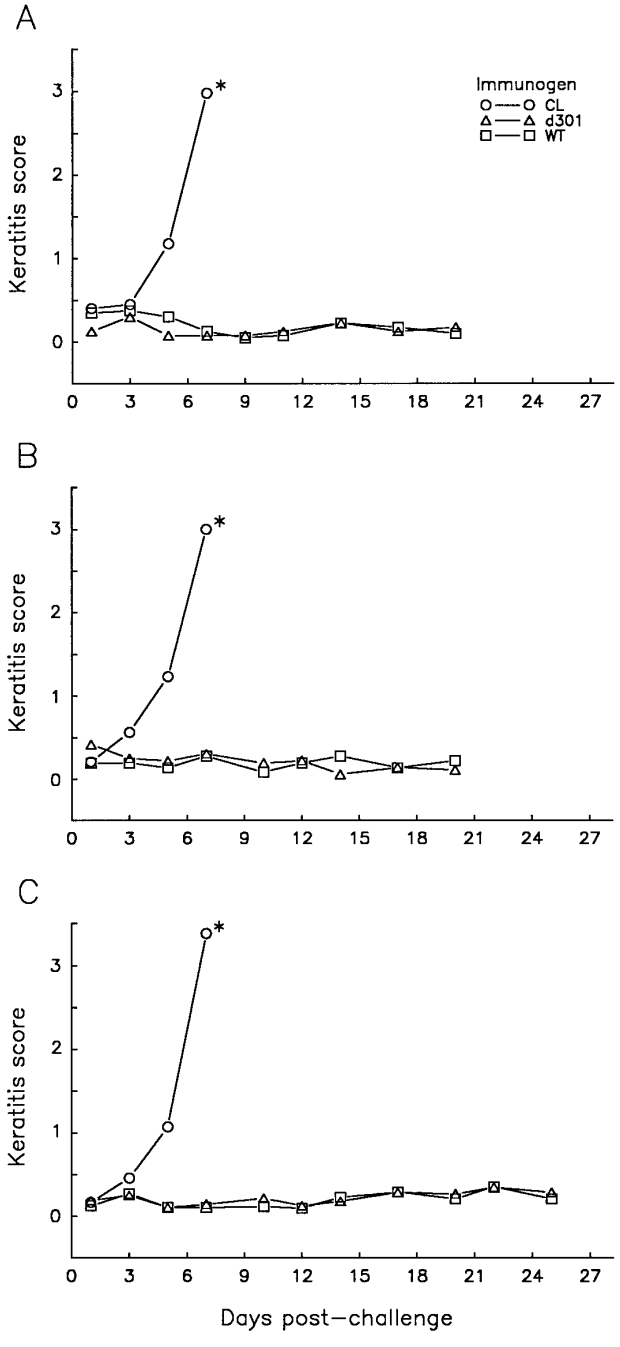

FIG. 5. Prevention of HSV-mediated keratitis in mutant virus-immunized mice. The eyes of groups of mice challenged at: (A) 1, (B) 4, or (C) 7 months after the final immunization were evaluated for signs of keratitis at the indicated times postchallenge. Values represent the mean score of all eyes in each immunization group. The asterisk indicates death of the majority of mice.

TABLE 2

Reduction of Latent Virus in Trigeminal Ganglia

Immunization group	Fraction of ganglia with reactivating virus[a] from mice challenged at the indicated time after immunization: 1 month	4 months	7 months
Expt 1			
Cell lysate	2/2	n.d.[c]	2/2
*d*301	1/6 ($P = 0.107$)[b]	0/10	5/12 ($P = 0.231$)
KOS1.1 wt	0/6 ($P = 0.036$)	1/10	2/12 ($P = 0.066$)
Expt 2			
Cell lysate	10/12	10/10	
*d*301	1/8 ($P = 0.003$)	2/8 ($P = 0.002$)	
KOS1.1 wt	1/8 ($P = 0.003$)	2/8 ($P = 0.002$)	

[a] Assayed by cocultivation of dissociated ganglia.

[b] Compared to control cell lysate-inoculated mice by Fisher's exact test.

[c] Not determined.

reactivating virus (Table 2, Expt 1), consistent with previous observations (Morrison and Knipe, 1994). Mice challenged at 4 months after immunization also were apparently protected from latent infection of the trigeminal ganglion by challenge virus: 0 and 10% of ganglia from mutant and wt virus-immunized mice, respectively, contained reactivating virus. These data from the 4-months time point could not be compared with time-matched controls, however, because all mice inoculated with control cell lysate died as a result of challenge virus infection. A separate experiment in which more control mice survived the challenge confirmed the apparent protection seen at 1 and 4 months (Table 2, Expt 2). At 7 months, all control ganglia but only 17% of ganglia from mice immunized with wt virus contained reactivating challenge virus. Although the mice immunized with wt virus showed a reduction in latent infection, the reduction was not statistically significant. Reduction in the incidence of reactivation was observed also in mutant virus-immunized mice in which 42% of trigeminal ganglia showed reactivation, but this difference was also not statistically significant. These reactivation data indicate that protection against latent infection was maintained for at least 4 months following immunization with either mutant or wt virus.

In vivo replication phenotype of mutant virus. The observation that a replication-defective virus effectively stimulated long-term immunity analogous to live, replication-competent virus by all parameters tested was somewhat surprising. We investigated the possibility that the replication-defective virus was as effective as wt virus in stimulating long-term immunity because its genetic defect was somehow complemented *in vivo,* rendering it replication-competent. Mice were inoculated with mutant or wt virus in the footpad (as a form of s.c. immunization), and footpad tissue was collected at various times to determine viral titer. Titers of wt virus rose on Days 1–3 postinoculation, but titers of mutant virus declined progressively in footpads of inoculated mice (Fig. 6). In addition, mutant virus is undetectable within 1 day after inoculation onto the cornea while wt virus titers increase by 4 logs (Babu *et al.,* 1996). These observations support the contention that the mutant virus, *d*301, is replication-defective *in vivo* as well as in tissue culture (Gao and Knipe, 1989), and that its parent, the KOS1.1 strain, does replicate *in vivo* even though it is attenuated for virulence.

A second important observation from these experiments was that in contrast to the cornea, where infectious replication-defective virus is rapidly cleared (Babu *et al.,* 1996), the mutant virus was recovered in an infectious form in the footpad tissue for the 3-day interval of one experiment (Fig. 6A) and up to 6 days after inoculation in a second experiment of longer duration (Fig. 6B). Titration on Vero cells of Day 1 and Day 3 samples from *d*301 mutant-infected mice showed no plaques (results not shown), confirming that the virus persisting in the footpads was truly input mutant virus. The persistence of the mutant virus in an infectious form may prolong the period of time during which infection and expression of viral gene products occurs, thereby increasing the host immune response to the replication-defective virus even if the virus does not spread from the primary cells it infects.

DISCUSSION

This work has addressed the mechanisms by which a live virus induces protection against a persistent infection and, specifically, how a replication-defective mutant of HSV induces protection against latent infection by challenge HSV. We have shown that immunization with the mutant virus reduces the number of trigeminal ganglion neurons expressing the LAT transcript, arguing that the number of neurons latently infected by challenge virus was reduced by a mechanism other than establishment of latency in those neurons by the immunizing virus. Second, the duration of immunity induced by the mutant virus at the chosen dose appeared to be nearly equivalent to the replication-competent parental virus, demonstrating that the immunity induced by a replication-defective mutant virus is durable like live viruses even though, like killed virus vaccines, the mutant virus cannot spread. Third, the mutant virus showed no evidence of replication *in vivo* but was present in an infectious form for several days in the mouse footpad, raising the possibility that primary infection continues over an extended period of time.

Mechanism of protection against latent infection

Our experiments show that animals immunized with the replication-defective mutant virus and challenged with virulent HSV-1 bear approximately 80-fold fewer

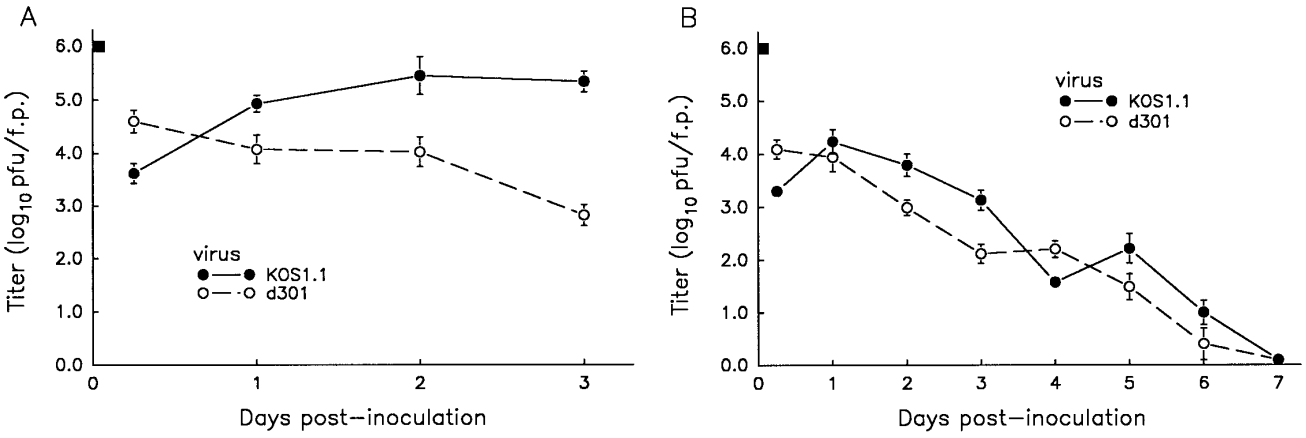

FIG. 6. *In vivo* replication of KOS1.1 but not *d*301 in footpad tissue. Groups of mice were injected with 1×10^6 PFU per footpad of KOS1.1 or *d*301, and footpad tissue was dissected from subsets of the mice at the indicated times. Infectious virus in the tissue homogenate was titrated by standard plaque assay on S-2 cells. Values represent mean titer of four tissue samples per group ± SEM. A and B show two separate experiments carried out for different periods of time.

LAT-positive neurons than animals immunized with uninfected cell extract and then challenged. We conclude that immunization with the mutant virus reduces the number of latently infected cells and not merely the ability of the latent virus to reactivate. It is conceivable that in the immunized animals the challenge virus can establish latency but not express LAT. *In situ* hybridization techniques have not been able to detect HSV DNA during latent infection, but a few recent studies have detected HSV DNA during latent infection through the use of *in situ* PCR techniques (Ramakrishnan *et al.,* 1994; Gressens *et al.,* 1994; Gressens and Martin, 1994; Mehta *et al.,* 1995). These papers differ in their conclusions regarding the latently infected cells in that one reports that the number of HSV DNA-positive neurons is 50- to 100-fold higher than the number of neurons expressing LAT (Ramakrishnan *et al.,* 1994) while another concludes that the distribution of LAT-positive cells approximates the distribution of HSV DNA-positive cells (Mehta *et al.,* 1995). We are currently attempting to establish a reliable *in situ* PCR method to address the question of whether immunization reduces the number of neurons containing HSV DNA. If the number of latently infected cells is truly reduced by immunization, the question remains as to whether the viral genome is prevented from reaching the neuronal cell body or whether it reaches the neuronal cell body but cannot establish a latent infection.

It has been proposed that live, attenuated viruses can protect against the establishment of persistent infection by a more virulent virus by occupying the cellular sites of latency and interfering with subsequent infection (Rud *et al.,* 1994). Although it seemed unlikely that a replication-defective mutant could populate trigeminal ganglion neurons after inoculation in the rear flank, our experiments rule out a form of latency involving LAT expression by the mutant virus in the trigeminal ganglion by demonstrating that very few trigeminal ganglion neurons in the animals immunized with the mutant virus and then challenged express LAT at 28 days after challenge. Furthermore, LAT-positive neurons were not observed in dorsal root ganglia following inoculation of *d*301 into the footpad (Morrison and Knipe, unpublished results). Therefore, the protection induced by the mutant virus is not the consequence of saturation of the latent infection sites by a less virulent virus but rather appears to involve host immune mechanisms. Other studies have, in fact, demonstrated that specific subsets of T cells are required for protection against latent infection by the challenge virus (L. A. Morrison and D. M. Knipe, manuscript in preparation).

Duration of immunity induced by a replication-defective mutant virus

The capacity to provide long-lasting, ideally life-long, protection is an important attribute of an effective vaccine-induced immune response. Historically, live viral vaccines have induced a more long-lived immunity than killed virus vaccines. A major concern about replication-defective viruses has been that they might not induce a durable immunity because they could not spread to critical sites during infection of the host, much like killed virus vaccines. Nevertheless, at the dose used in mice in this study, the duration of immunity induced by the mutant virus seemed comparable to the replication-competent parental virus. The mice in this study were immunized as young adults at 6 and 10 weeks of age, and immunity was demonstrated to last until the mice are nearly 11 months old. For virgin, female BALB/c mice this protection covers approximately 2/3 of their adult lifespan (Russell, 1966). The durability of immunity induced in mice by the replication-defective HSV mutant appears to approximate that of vaccinia vectors expressing HSV glycoprotein D (Rooney *et al.,* 1988; Wachsman *et al.,* 1992) and may exceed that induced by glycoprotein sub-

units (Meignier *et al.,* 1987) where immunity against intracerebral challenge waned with increasing time after immunization.

It is interesting to note that in mice challenged 7 months after immunization, disease prevention and effects on acute replication remained largely unchanged from earlier times, but reduction of latent infection was less dramatic. These results suggest two things. First, protection against latent infection may be the most sensitive indicator of the efficacy of long-term protection against HSV infection. Consequently, even by the most sensitive criterion, immunization with *d*301 was effective long-term in reducing challenge virus infection. Second, while immune functions operating to reduce primary replication in the eye are still intact, those necessary to prevent virus spread to the nervous system and/or establishment of latent infection may not be operating as effectively at late times after immunization. Conceivably, one component of the immune response may have a shorter life span. It is likely to be a T cell component because neutralizing antibody titers remained steady through 7 months after immunization. We also observed some breakthrough in protection against keratitis in a few of the mice challenged after 7 months, suggesting that some regulatory check on the activity of $CD4^+$ T (Newell *et al.,* 1989) cells may be lost over time. The question of long-term maintenance of various T cell subsets and their roles in latent infection and disease merits further detailed analysis.

In vivo behavior of the replication-defective mutant virus

The similar immune responses to the replication-defective and -competent viruses at the dose used raised the possibility that there might be replication of the mutant virus *in vivo,* but measurement of virus titers in footpad tissue after subcutaneous inoculation in the footpad (this report) or in eye tissue after corneal inoculation (Babu *et al.,* 1996) showed no evidence for an increase in mutant virus indicative of replication. Surprisingly, mutant virus is present in the footpad tissue in an infectious form for several days. This may be in the form of free virus particles or as virus particles sticking to cells and retaining infectivity, as documented for HIV with follicular dendritic cells in lymph nodes (Heath *et al.,* 1995). Regardless of the mechanism, this raises the possibility that there is a prolonged series of primary infection events by the input mutant virus that could serve to boost the host immune response much as spread of live virus is thought to boost the host immune response. This may help to explain why, at the dose used, the replication-defective mutant virus induces an immune response similar to the replication-competent virus. Nevertheless, other mechanisms including prolonged antigen expression in the mutant virus-infected cells could also explain the capacity of the mutant virus to elicit strong, durable immunity in the absence of spread within the host. Further dose studies are needed to better compare the immune responses induced by these two types of virus, but our results are consistent with studies demonstrating that other replication-defective mutant viruses, including canarypox vectors (Cox *et al.,* 1993) and vaccinia virus vectors (Sutter *et al.,* 1994), elicit immunity similar to replication-competent viruses. The survival of mutant virus in an infectious form *in vivo* and continued primary infection may provide a way to reconcile current observations on the vaccine capabilities of replication-impaired mutant viruses with dogma about viral vaccines which holds that live forms of viral vaccines effectively stimulate immunity as a consequence of virus replication and spread (White and Fenner, 1986; Mims *et al.,* 1993).

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