Immunization with Replication-Defective Mutants of Herpes Simplex Virus Type 1: Sites of Immune Intervention in Pathogenesis of Challenge Virus Infection

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Replication-defective mutants of herpes simplex virus type 1 (HSV-1) were used as a new means to immunize mice against HSV-1-mediated ocular infection and disease. The effects of the induced immune responses on pathogenesis of acute and latent infection by challenge virus were investigated after corneal inoculation of immunized mice with virulent HSV-1. A single subcutaneous injection of replication-defective mutant virus protected mice against development of encephalitis and keratitis. Replication of the challenge virus at the initial site of infection was lower in mice immunized with attenuated, wild-type parental virus (KOS1.1) or replication-defective mutant virus than in mice immunized with uninfected cell extract or UV-inactivated wild-type virus. Significantly, latent infection in the trigeminal ganglia was reduced in mice given one immunization with replication-defective mutant virus and was completely prevented by two immunizations. Acute replication in the trigeminal ganglia was also prevented in mice immunized twice with wild-type or mutant virus. The level of protection against infection and disease generated by immunization with replication-defective mutant viruses was comparable to that of infectious wild-type virus in all cases. In addition, T-cell proliferative and neutralizing antibody responses following immunization and corneal challenge were of similar strength in mice immunized with replication-defective mutant viruses or with wild-type virus. Thus, protein expression by forms of HSV-1 capable of only partially completing the replication cycle can induce an immune response in mice that efficiently decreases primary replication of virulent challenge virus, interferes with acute and latent infection of the nervous system, and inhibits the development of both keratitis and systemic neurologic disease.

Herpes simplex virus type 1 (HSV-1) or HSV-2 infections cause significant disease in humans, commonly manifest as orolabialis or genital infection, respectively. HSV infection is also associated with diseases of greater morbidity: keratoconjunctivitis resulting from recurrent ocular infection and encephalitis are typically caused by HSV-1; disseminated disease of the newborn can result from birth to an HSV-2-infected mother (47). The pathogenesis of HSV infection is complex, consisting of a primary infection at the site of exposure, transport of virus into the nervous system where acute and latent infection ensues, and periodic reactivation of latent virus leading to recurrent disease. Thus, an immune response effective in reducing and ameliorating disease must have the capacity to control primary infection and to prevent recurrent disease by blocking establishment of latent infection (35). Despite its considerable medical importance, no effective vaccine yet exists to prevent HSV infection. Moreover, the issues of how and at what stages of pathogenesis a vaccine-induced immune response may act to block infection by challenge virus are poorly understood but are crucial to the identification of a potentially effective vaccine.

Adoptive transfer studies in animals have indicated that either HSV-specific antibody or T cells can mediate protection against lethal HSV infection, but that their functions may differ (reviewed in reference 28). HSV-immune T cells transferred into T-cell-immunosuppressed mice (32, 33) or nude mice (14) mediate clearance of virus from the primary site of infection.

Likewise, B-cell-suppressed mice are still able to clear infectious virus following inoculation into the skin (13, 40). Passively administered, HSV-specific neutralizing antibodies appear to mediate protection principally by decreasing spread of virus to and within the nervous system (14, 16, 27, 39). Effective vaccination, resulting in the capacity to control primary epithelial infection and prevent nervous system colonization, may thus require elicitation of a variety of immune responses.

Many attempts have been made to generate protective immune responses, including immunization with glycoprotein subunits, HSV glycoproteins expressed via vaccinia virus or adenovirus vectors, or live and live attenuated virus (reviewed in reference 1; 6, 12, 24). Often the ability to protect animals against lethal infection has been used as an indicator of a potentially successful vaccine. However, protective efficacy of glycoprotein vaccines in humans, as measured by decreased rate of transmission or amelioration of primary infection, has not been borne out in clinical trials (26). Some studies have specifically addressed protection from HSV-1-mediated disease. Subcutaneous (s.c.) immunization of mice with live attenuated (36, 44) or UV-inactivated (42) virus or with purified gD in adjuvant (7) confers protection from encephalitis and at least partial protection from keratitis following corneal challenge. Results of studies using footpad administration of purified gB in adjuvant (17) or genetically engineered attenuated virus (24) suggest that reduction of latent infection of the nervous system may also be possible.

We have used a new strategy for vaccination against HSV that utilizes replication-defective mutants of HSV-1. These viruses bear mutations in the essential genes encoding infected cell protein 8 (ICP8) or ICP27. The mutants express the protein products of α and β or α , β , and γ_1 genes, respectively,

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in cells that they infect (9, 34). However, they do not replicate and can be propagated only in cells that have been stably transformed with the gene that they lack. Several regulatory proteins encoded among the α and β genes are known targets of cell-mediated immune responses to HSV-1 (22, 23, 43), suggesting the immunogenic potential of the replication-defective mutants. Intraperitoneal immunization with the ICP8 or ICP27 mutant has, in fact, been shown to protect mice from lethal infection following intraperitoneal challenge and to elicit HSV-specific antibody and T-cell proliferative responses (31). We have examined the potential of replication-defective mutants of the HSV-1 KOS strain to induce immune responses protective against not only lethal infection but also HSV-induced eye disease, and acute and latent neurologic infection, in a mouse model. The routes of immunization and challenge used are relevant to vaccination for prevention of human infection. In addition, we have begun to examine the mechanisms by which the immune response induced by vaccination intervenes in HSV pathogenesis.

MATERIALS AND METHODS

Viruses. The mP strain and the attenuated, wild-type (wt) parental KOS1.1 strain of HSV-1 were propagated and assayed on Vero cell monolayers as previously described (18) and stored as infected cell extracts. For use in control animals or cultures, extract of uninfected Vero cells (control cell extract) was prepared by procedures analogous to those used for preparation of infected cell extracts. A portion of the KOS1.1 virus stock was inactivated by exposure of infected cell extracts at a concentration of 10^8 PFU/ml to 254-nm UV light irradiation for 15 min at a distance of 5 cm. This treatment decreased infectivity to 10^4 PFU/ml. Viral protein expression in BALB/3T3 cells exposed to inactivated virus (multiplicity of infection [MOI] prior to inactivation = 10) could not be detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of lysates of cells labelled with [35 S]Met from 2 to 10 h postinfection, as previously described (34). Reovirus from infected L-cell extracts was similarly inactivated at a concentration of 3×10^8 PFU/ml.

Three engineered mutant viruses derived from the KOS1.1 strain were used. The d301 virus (9), containing a deletion in the ICP8 gene, was propagated and titrated on S-2 cells expressing ICP8. The n504r virus (34), bearing a nonsense mutation in the ICP27 gene, was propagated on V27 cells expressing ICP27. The amount of viral DNA detected in cells by slot blot hybridization after a 1-h adsorption of mutant virus is not greater than the amount in cells adsorbed with the same number of PFU of wt virus (10, 34), indicating that the viral DNA content of wt and mutant virus preparations was equivalent for a given infectious dose. The mutant virus n12 (38) was kindly provided by N. DeLuca and was propagated on the ICP4-expressing cell line E5. The n12 virus contains a nonsense mutation in the ICP4 gene. The mutant virus stocks were demonstrated to contain <1 PFU of wt virus per 10^7 PFU of replication-defective mutant virus by (i) infectious center assay and (ii) an amplification assay. For the former assay, Vero cell monolayers were infected with mutant virus at an MOI of 100 and then were trypsinized, diluted 20-fold, and reseeded with uninfected Vero cells in medium 199 containing 75 U of varicella-zoster virus-immune globulin (human) per liter, for a total of 1.7×10^6 cells per 25-cm² flask. Vero cell monolayers infected with 2×10^3 PFU of KOS virus and reseeded after 20-fold dilution with uninfected cells were prepared in parallel as a control for plating efficiency. For the amplification assay, Vero cell monolayers were infected with mutant virus at an

MOI of 100, collected and sonicated after 2 days of culture at 37°C, and diluted and assayed for plaque formation on fresh Vero cell monolayers.

Inoculations. Female 6-week-old BALB/c mice were purchased from the National Cancer Institute (Frederick, Md.), housed in accordance with institutional and National Institutes of Health guidelines (4), and acclimated for 1 week before use. The BALB/c strain was selected because of its susceptibility to keratitis (8) and to latent infection with HSV (11). Mice were immunized with 2×10^6 PFU of virus, or an equivalent amount of UV-inactivated KOS (UV-KOS) or control cell extract, in a 20- μ l volume s.c. near the base of the tail, using a 26-gauge needle. In some cases, a group of mice was immunized and 2 weeks later was boosted s.c. with 2×10^6 PFU of the same virus. Four weeks after primary immunization, all mice were challenged by inoculation of 2×10^5 PFU of HSV-1 strain mP per eye after corneal scarification as previously described (3) except that the volume of the inoculum was 5 μ l. This dose produces encephalitis in 100% of nonimmune BALB/c mice and represents 10 to 30 times the minimum dose required to cause symptomatic encephalitis (29). In one experiment, mice were immunized with 5×10^5 PFU of KOS, n12, d301, or equivalent control cell extract and challenged as described above with 10^5 PFU of mP per eye.

Clinical observations. Mice were observed daily after immunization and challenge. Fur on the flank and at the base of the tail of some mice was shaved to permit examination of the skin at the inoculation site. Eyes were examined for evidence of blepharitis and keratitis by using an ophthalmoscope. Each eye received a separate score for severity (scale of 0 to 4+) and extent (0 to 100% in 25% gradations) of disease, and the mean keratitis score for each group of mice was determined by the formula $\Sigma \text{severity} \times \text{extent}/n \text{ eyes}$.

Assays of acute and latent infection. Quantitation of virus in tear film at various times after challenge as a measure of acute replication in the eye and determination of reactivatable virus in trigeminal ganglia by explant cocultivation with Vero cell monolayers were performed as previously described (3). One half of the sonicated culture volume (0.9 to 1.1 ml) was added to fresh monolayers for titer determination as in the standard plaque assay. The Fisher exact test for comparison of proportions was used to determine statistical significance of the data. For quantitation of acute replication in the eye and trigeminal ganglion, tissue samples were frozen in 1 ml of assay medium (phosphate-buffered saline containing 0.1% glucose and 1% fetal calf serum), thawed, and disrupted by sonication, and viral titer was determined by standard plaque assay.

Assay of T-cell proliferative responses. Four weeks after immunization or challenge, splenocytes from two mice per group were collected and pooled. The mononuclear cell fraction was obtained by sedimentation through Ficoll-Hypaque (LSM; Organon Teknika, Durham, N.C.) and depleted of B cells by magnetic separation as described elsewhere (31). Remaining cells were incubated at 2×10^5 cells per well in 96-well flat-bottom culture plates. UV-KOS virus (or an equivalent amount of control cell extract) was added at an MOI corresponding to 1 prior to inactivation, for a total volume per well of 200 μ l of RPMI 1640 supplemented to contain 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U of penicillin per ml, 100 μ g of streptomycin per ml, 1% nonessential amino acids, 1 mM sodium pyruvate, and 5 μ M 2-mercaptoethanol. Cultures were incubated in quadruplicate in a 7% CO₂ atmosphere for 4 to 5 days. Assays were then pulsed with 1 μ Ci of [3 H]dT per well for 8 h and harvested onto glass fiber filters. Filters were counted in Opti-fluor O for 1 min in a beta scintillation counter.

Assay of antibody titers. Blood was obtained from the tail veins of eight mice per group 18 days after primary immunization and 7 days after challenge, when antibody responses are at maximal levels (29). Samples from each group were pooled, and serum was collected after clot retraction. Serial twofold dilutions of serum in assay medium, or medium alone, were mixed with an equal volume of KOS virus at approximately 10^3 PFU/ml and incubated for 2 h at 4°C. Two hundred microliters of the mixture was then adsorbed to Vero cell monolayers, and a standard plaque assay was carried out in duplicate. Serum from an HSV-hyperimmune mouse and preimmune serum served as positive and negative controls, respectively. The neutralizing antibody titer was defined as the serum dilution yielding $\geq 50\%$ reduction in the number of plaques.

RESULTS

We investigated the potential of immunization with replication-defective mutants of the HSV-1 KOS1.1 strain to (i) protect mice against local and systemic disease, using ocular infection with HSV-1 as a model, and (ii) block infection at specific stages of viral pathogenesis. BALB/c mice were immunized by a single s.c. injection with KOS1.1, replication-defective mutant virus, or an equivalent amount of UV-KOS or control cell extract. The mice were challenged 4 weeks later by administration of an encephalitis-producing dose of virulent HSV-1 mP strain to the corneas following scarification.

Clinical observations and protection from neurologic disease. The mice showed no systemic reaction to immunization, and only one of the mice immunized with wt virus had mild erythema at the site of inoculation. Beginning 5 days after challenge, control cell extract-inoculated mice uniformly showed signs of encephalitis: hunched posture, ruffled fur, ataxia, changes in responsiveness, weakness, and anorexia. The symptoms became profound by day 7 in all control cell extract-inoculated mice. Deaths occurred by days 9 to 11, with mortality of 50%. Surviving mice recovered by 17 days postchallenge. Mice immunized with the replication-defective *n12* mutant also developed signs of encephalitis after challenge, but the symptoms were mild and transient, and all mice recovered by 9 days postchallenge. In contrast to control cell extract- or *n12*-immunized mice, all mice immunized with KOS, UV-KOS, *d301*, or *n504r* remained healthy throughout the course of the experiments. Thus, immunization of mice with replication-defective mutant virus *d301* or *n504r*, but not *n12*, fully protected them against HSV-induced encephalitis.

Protection from local eye disease. Following corneal challenge, a few mice in all groups showed evidence of purulent conjunctivitis, occasionally accompanied by mild blepharitis. This reaction resolved within 4 days in all mice except those that received control cell extract prior to challenge. In these mice, blepharitis often progressed, and the severity of disease generally correlated with the condition of the eye. All mice were examined for development of HSV-induced keratitis every second day from days 1 to 11 postchallenge and every third day thereafter through day 20. Mean keratitis scores are shown in Fig. 1A. The eyes of mice immunized with KOS, UV-KOS, or replication-defective mutant virus *n504r* or *d301* showed only minor changes: some patchy infiltration, dendritic figures, and/or epithelial irregularities. Mice inoculated with control cell extract, however, developed signs of corneal disease by 5 days postchallenge that rapidly progressed to severe disease in the majority of eyes. These findings indicate that the immune response generated by immunization with replication-defective mutant virus *d301* or *n504r* was highly efficacious in preventing HSV-induced keratitis. Immunization with the rep-

FIG. 1. Herpes keratitis scores of immunized mice receiving corneal challenge with HSV-1. The eyes of all immunized mice were examined at 2- to 3-day intervals after corneal challenge for development of keratitis. Keratitis scores represent the mean scores of all eyes examined in each group (experiment 1 [A], $n = 16$; experiment 2 [B], $n = 10$ to 16). (A) Mice immunized with control cell extract (●), KOS (□), UV-KOS (○), *n504r* (◇), *d301* (▽), or *d301* with boost (Δ); (B) mice immunized with control cell extract (●), KOS (□), *d301* (▽), or *n12* (◇).

lication-defective mutant *n12* did not prevent the development of keratitis, though in most cases the disease was milder than that of mice immunized with control cell extract (Fig. 1B). Because the *n12* mutant synthesizes a truncated ICP4 protein and expresses only α gene products (38), this result indicates that the products of α genes alone are insufficient to elicit complete protection against either eye or neurologic disease. This result also suggests that the immunity observed following immunization with *n504r* or *d301* results from de novo protein expression in infected cells in addition to the stimulus created by virions and infected cell proteins contained in the inoculum.

To confirm that the protection afforded by immunization with replication-defective mutant virus was not generated solely by virions and infected cell protein contaminants in the infected cell extracts, mice were immunized with partially purified virion preparations consisting of extracellular virus concentrated by centrifugation. In this case, mice immunized with UV-inactivated KOS virions developed disease equivalent in severity and time of onset to disease in mice inoculated with control uninfected cell supernatant, although no mortality was observed (29). The degree of protection from disease was unchanged, however, for mice immunized with KOS, *n12*, or

FIG. 2. Virus titers in the tear film of immunized mice during the period of acute replication after corneal challenge. Values are the mean virus titers $\pm$ standard errors of the means of the eye swabs of all mice (eight) per group. The inoculum dose of 4×10^5 PFU per mouse is indicated at the zero time. Shown are results for mice immunized with control cell extract (●), KOS (□), UV-KOS (○), *n504r* (◇), *d301* (▽), and *d301* with boost (Δ).

d301 partially purified virions. This result indicates that de novo expression of viral proteins upon infection with the replication-defective mutants *in vivo* is essential for the high level of protective immunity achieved by these viruses.

Stages in viral pathogenesis: acute replication in the eye. Virus replication at the primary site of infection was measured on days 1 to 4 after challenge by determining viral titer in the tear film. Mice immunized with the replication-defective mutant *d301* or *n504r* had lower titers of virus in eye swabs on day 1 after challenge and significantly lower titers on day 2 than did mice immunized with control cell extract ($P < 0.05$, KOS, *d301*, and *d301* boost; $P < 0.02$, *n504r*) or UV-KOS (Fig. 2). At 3 days postchallenge, eye swab titers of mice in all groups except UV-KOS had dropped, and by 4 days, virus shedding from the eyes of mice in all groups had subsided. No difference could be discerned between viral titers in the eye swabs of KOS-, *n504r*-, or *d301*-immunized mice days 1 to 4 after challenge. Thus, immunization with replication-defective mutant viruses significantly decreased shedding of virus from the corneal surface during at least part of the period of primary replication.

Latent infection of the trigeminal ganglia. Four weeks after challenge, when no infectious virus remains in trigeminal ganglion tissue (19), the establishment of latent infection by wild-type challenge virus was evaluated by explant cocultivation assay of the trigeminal ganglia of four randomly selected mice from each group. All cultures of ganglia from mice receiving control cell extract contained large numbers of plaques indicative of reactivated virus (Table 1). All but one trigeminal ganglion from mice immunized with UV-KOS also harbored latent virus, despite the low incidence of disease in these mice. In contrast to the control cell extract group, reactivated virus was found only sporadically in cultures of ganglia from mice immunized with KOS, *d301*, or *n504r* ($P < 0.05$), and no reactivated virus was found in the culture of any ganglion from mice immunized twice with *d301* ($P < 0.02$; Table 1). Significantly, these results indicate that immunization with replication-defective mutant viruses not only confers protection against encephalitis and corneal disease but also can reduce or even prevent latent infection by high-dose challenge with a virulent strain of HSV-1.

Acute replication in the eyes and trigeminal ganglia of mice

TABLE 1. Reduction of latent virus^a in trigeminal ganglia of mice immunized with replication-defective mutants

Immunogen	No. of ganglia yielding virus ^b /no. of eyes infected	% Protection from latency ^c
Cell extract	8/8	0
UV-KOS	7/8	13
KOS	3/8	63
<i>n504r</i>	1/8	88
<i>d301</i>	4/8	50
<i>d301</i> boost	0/8	100

^a Trigeminal ganglia were removed from immunized mice that had received corneal challenge 30 days earlier. Presence of latent virus was assessed by explant cocultivation assay.

^b Ganglia were minced and cocultured with Vero cell monolayers. After 5 days, cultures were collected, sonicated, and inoculated onto fresh monolayers to test for plaque formation.

^c Defined as percent reduction in number of positive ganglia compared with control cell extract.

immunized with *d301*. Replication in the eye and nervous tissue of mice immunized twice with replication-defective mutant virus *d301* was further assessed to determine the extent of protection against acute as well as latent infection. Groups of mice were immunized twice with KOS, *d301*, or control cell extract, and eye and trigeminal ganglion tissues were collected on days 1 to 5 after corneal challenge. The titer of virus in eye tissue of mice from all three groups was high 1 to 2 days postchallenge. The titer then dropped precipitously in mice immunized twice with KOS or *d301* but remained elevated in mice inoculated with control cell extract (Fig. 3), corroborating our observations of reduced viral titer in tear film of immunized mice. In the trigeminal ganglion, a typical increase in viral titer occurred 2 to 5 days after challenge in mice inoculated with control cell extract (Fig. 4). No virus was detected in trigeminal ganglia of mice immunized twice with KOS or *d301*, however, demonstrating that prior exposure to replication-defective mutant virus at a distant site can prevent acute as well as latent ganglionic infection. These results suggest that the preexisting immune response acts rapidly to intervene in viral pathogenesis at the initial site of infection and to prevent secondary infection of the nervous system.

T-cell proliferative responses. Given the importance as-

FIG. 3. Virus titers in eye tissue of immunized mice during the period of acute replication after corneal challenge. Values are the mean virus titers $\pm$ standard errors of the means in individual eyes ($n = 4$ to 6) from mice immunized and boosted with control cell extract, *d301*, or KOS and then challenged with HSV-1 mP.

FIG. 4. Virus titers in trigeminal ganglion tissue of immunized mice during the period of acute replication after corneal challenge. Titers of individual ganglia ($n = 4$ to 6) are represented by symbols for mice immunized and boosted with control cell extract, *d301*, or KOS prior to challenge. The mean value of titers from cell extract-immunized mice is indicated by a dashed line. Three PFU = limit of detection in the plaque assay.

cribed to T-cell-mediated immunity in controlling HSV infection, we examined the HSV-specific T-cell proliferative responses in immunized mice 4 weeks after challenge with mP. Strong HSV-specific proliferative responses to inactivated KOS virus were mounted by splenic T cells from mice in all groups (Table 2). Vero cell extract (present in all viral preparations) elicited a minimal response from splenocytes of all groups except *d301*-immunized mice which had been boosted and thus had received three exposures to antigen prior to assay. Heterologous virus antigen also did not induce proliferation. In addition, T cells from naive mice did not proliferate in response to HSV (29), demonstrating that HSV antigen does not have mitogenic activity. These results indicate that mice immunized with the replication-defective mutants and challenged with virulent HSV-1 mount an HSV-specific T-cell proliferative response in culture that is equivalent to that of mice immunized with wt KOS virus. The strong HSV-specific response noted in control cell extract-inoculated mice that

TABLE 2. T-cell proliferative responses of immunized mice after challenge with HSV-1

Group immunized with ^a :	Mean [³ H]thymidine uptake (cpm) $\pm$ SD in response to ^b :		
	Uninfected cell extract	Reovirus antigen	HSV antigen
Cell extract (survivors of challenge)	1,435 $\pm$ 1,024	1,123 $\pm$ 1,024	49,261 $\pm$ 11,800
KOS	726 $\pm$ 270	396 $\pm$ 101	24,582 $\pm$ 3,027
<i>n504r</i>	5,204 $\pm$ 1,580	2,525 $\pm$ 1,196	29,350 $\pm$ 3,757
<i>d301</i>	3,734 $\pm$ 1,181	2,197 $\pm$ 436	29,931 $\pm$ 4,487
<i>d301</i> boost	7,910 $\pm$ 2,088	3,907 $\pm$ 1,669	28,512 $\pm$ 3,769
UV-KOS	1,428 $\pm$ 877	867 $\pm$ 462	25,876 $\pm$ 4,838

^a Mice were immunized with the indicated uninfected or virus-infected cell extracts, and all groups were challenged 4 weeks later. The mononuclear cell fraction of two pooled spleens, depleted of B cells, was prepared from each group 4 weeks postchallenge.

^b Mean of quadruplicate cultures on day 5 of culture. Data shown are representative of two independent experiments performed with similar results.

TABLE 3. Primary T-cell proliferative responses of immunized mice

Group immunized with ^a :	Mean [³ H]thymidine uptake (cpm) $\pm$ SD in response to ^b :	
	Uninfected cell extract	HSV antigen
Cell extract	845 $\pm$ 595	1,593 $\pm$ 327
<i>d301</i>	2,044 $\pm$ 708	15,107 $\pm$ 3,584
<i>d301</i> boost	2,996 $\pm$ 533	17,444 $\pm$ 581

^a Mice were immunized with the indicated uninfected or virus-infected cell extracts. The mononuclear fraction of two pooled spleens, depleted of B cells, was prepared from each group shown 4 weeks after immunization (or final immunization of boosted mice).

^b Mean of quadruplicate cultures on day 4 of culture.

survived challenge with mP most likely is stimulated by continual exposure to high levels of HSV antigen during their protracted illness in the 4-week period between challenge and assay.

We have also observed that in the absence of challenge, a single s.c. dose of *d301* efficiently stimulates T-cell proliferation against HSV that is increased upon secondary exposure to antigen (Table 3). Partially purified virus used to immunize mice was also capable of inducing an HSV-specific proliferative response (29). Notably, splenocytes from unchallenged, control cell extract-inoculated mice did not respond to HSV antigen in culture (Table 3).

Neutralizing antibody titers. The titer of neutralizing antibody contained in serum collected from mice after immunization and after challenge was determined in a complement-independent plaque reduction assay (Table 4). A neutralizing primary antibody response was detected in serum of mice immunized once with KOS or the replication-defective mutant virus *d301* or *n504r* but not in serum of mice immunized with UV-KOS or control cell extract. The titer was greatly augmented by immunizing twice with *d301*. Neutralizing antibody titers in the sera of all mice increased upon secondary exposure to HSV in the form of challenge virus. Thus, appreciable titers of neutralizing antibody existed in mice immunized with the replication-defective mutant viruses as well as those immunized with KOS prior to corneal challenge.

DISCUSSION

We have shown that immunization of mice with the replication-defective mutant strains of HSV-1, *d301* and *n504r*, prevents the development of encephalitis and keratitis induced by corneal challenge with virulent HSV-1. Significantly, immunization with these replication-defective mutant viruses also reduced or prevented latent infection of the trigeminal ganglia by the challenge virus compared with mice immunized with control cell extract or UV-inactivated virus. Replication-defective mutant viruses were as effective as attenuated wt virus in

TABLE 4. HSV-specific neutralizing antibodies induced by immunization with replication-defective mutants

Time of serum collection	Antibody titer in mice immunized with ^a :					
	Cell extract	UV-KOS	KOS	<i>n504r</i>	<i>d301</i>	<i>d301</i> boost
After immunization	≤ 8	≤ 8	16	16	16	128
After challenge	≤ 8	64	64	64	64–128	256

^a Inverse of the serum dilution yielding 50% reduction in plaque number.

eliciting immune responses that reduced acute infection of the eye and prevented viral spread to, and acute as well as latent infection of, the trigeminal ganglia. Equivalent T-cell proliferative and neutralizing antibody responses were elicited by immunization with KOS or replication-defective mutant virus, and antibody titers were higher than those induced by immunization with UV-inactivated virus. These results demonstrate that immunization with replication-defective mutants of HSV-1 can efficiently elicit a T-cell- and B-cell-mediated immune response and that the response generated reduces primary replication of virulent challenge virus at a distant site, decreases acute and latent infection of the nervous system, and protects against eye disease and systemic neurologic disease.

The observation that immunization with replication-defective mutant virus reduced or prevented latent infection by virulent challenge virus is especially noteworthy. Prevention of lethal infection has been demonstrated in many studies of immune protection against HSV-1. Additional studies have revealed that immunization with UV-KOS (42) or purified gD in adjuvant (7) can reduce development of keratitis following corneal challenge. Few studies have addressed the establishment of latent infection in the nervous system, though the recurrent disease associated with reactivation from latency can be debilitating (e.g., corneal deterioration) and provides an opportunity for transmission. Results of two previous studies using corneal challenge with an HSV-1 strain had suggested that immunization with purified gB (17) or genetically engineered viruses (24) could reduce latent infection of the trigeminal ganglia. Our observations demonstrate more definitively that complete protection against establishment of latent infection can be achieved by s.c. immunization with replication-defective mutant viruses.

The means by which reduction of latent infection occurs are not yet defined, but our data are consistent with the possibility that both established cell-mediated and neutralizing antibody responses play roles in optimal protection against HSV-mediated acute disease and latent neurologic infection. Many studies have linked immunization-stimulated cellular immune responses and protection (2, 37, 45) and specifically have demonstrated a role for cell-mediated immunity in viral clearance from the skin (14, 21, 30). In our studies, a decrease in replication at the primary site of infection is likely to contribute to reduction of latent infection by diminishing the total amount of virus available for transport into the nervous system. Clearly, the preexisting immune response in replication-defective mutant-immunized mice was able to control primary replication in the eye within 48 h. T cells specific for α or β gene products (22, 23, 43) may limit primary replication through cytolysis of infected corneal keratocytes, thereby reducing the amount of infectious progeny virus available for uptake into sensory nerve fibers, in turn diminishing acute and latent infection of the nervous system. Some component of the immune response, either immune T cells or specific antibody, also may intervene directly in virus colonization of the nervous system, since even the initial waves of virus replicating in eye tissue evidently were prevented from reaching the trigeminal ganglia. Indeed, it has been shown that passively transferred anti-HSV antibody apparently can inhibit spread of virus to and within the nervous system (14, 16, 27, 39). In addition, CD8⁺ T cells appear to mediate control of acute HSV replication in the peripheral nervous system (41). It is likely that a variety of immune mediators induced by immunization with the replication-defective mutants have the concerted effect of reducing viral infection of the nervous system.

Another clue to the mechanism of protection afforded by immunization with replication-defective mutant viruses may lie

in the observation that immunization with UV-KOS did not reduce eye swab titers or diminish latent ganglionic infection. This result lends support to the conjecture of a relationship between virus titer at the site of primary replication and the number of latently infected cells in the nervous system, but further experiments are needed to test the validity of this relationship. Unlike wt and replication-defective mutant viruses, inactivated virus can express only a limited array of endogenously synthesized viral proteins. Thus, inactivated virus may not efficiently induce CD8⁺ T cells which are likely to be active at the primary site of challenge virus replication. In addition, the primary titer of neutralizing antibody in mice immunized with UV-KOS was lower than that of mice immunized with wt or replication-defective mutant virus and may have been insufficient to prevent spread of challenge virus to the nervous system. These possibilities are currently under investigation.

Although eye swab titers and latent infection were not reduced in mice immunized with the UV-inactivated, infected cell extract, symptomatic eye and neurologic disease were prevented. Residual infectious virus present in the UV-KOS inoculum (200 PFU) cannot account for this protection because immunization with an equivalent dose of wt KOS virus is not sufficient to protect mice from either keratitis or encephalitis (29). Keratitis and encephalitis also are not prevented by immunization with partially purified, inactivated virions (29). Consequently, the amount of total viral protein present in inocula of UV-inactivated, infected cell extracts must be adequate to prevent disease but not to preclude latent infection. One important implication of these results is that the threshold amount of challenge virus replication required for latent infection is much lower than the threshold required to produce clinical disease, and thus latent infection is a more sensitive measure of the efficacy of vaccination.

The replication-defective mutants varied in their protective capacities. Clearly, immunization with the *n12* mutant was less efficacious than immunization with *d301* or *n504r* in protecting against keratitis or encephalitis. The *n12* mutant may induce an immune response of lower magnitude and/or narrower spectrum than that induced by the *d301* or *n504r* mutant because *n12* expresses only the five α gene products (38).

We have demonstrated in a mouse model that immunization with the replication-defective mutant viruses *d301* and *n504r* can generate an immune response protective against HSV-1 infection and disease at a distant site of challenge. These observations indicate that neither amplification of the viral genome nor viral replication is apparently required for effective vaccination, a result which contradicts existing dogma about live virus vaccines (46). Our study employs a route of inoculation useful for human vaccination and examines keratitis and encephalitis, two major medical problems associated with HSV-1 infection. Some reports have indicated that keratitis may result not solely from viral infection but also from destruction of corneal tissue by T cells responding to the infection (5, 25). In this regard, it is important to note that an immunopathologic response apparently was not induced by immunization with the replication-defective mutant viruses. As a potential vaccine strategy, the replication-defective mutant viruses offer several advantages. (i) They are replication defective and therefore cause self-limiting infections which do not readily establish latency as detected by *in situ* hybridization for latency-associated transcript RNA (20) or maintenance of viral DNA as detected by PCR (15). (ii) Virus preparations that contain no detectable contaminating wt virus can be made. (iii) The *d301* and *n504r* mutants permit a significant portion of the HSV gene expression cascade to occur, making numerous

HSV proteins available to stimulate immunity. Potentially, immune responses generated by vaccination with these viruses sufficiently mimic in magnitude and spectrum the immune responses to natural infection that effective control of viral replication and spread can occur. In particular, the endogenously synthesized viral proteins may elicit a broader array of cell-mediated responses than would exogenous glycoprotein subunits (1). (iv) Expression of HSV proteins does not require a second virus vector such as adenovirus or vaccinia virus, and efficient immune responses are generated without use of adjuvant. Lastly, construction of analogous mutants of HSV-2, other herpesviruses, or possibly even human immunodeficiency virus may be a useful strategy for promoting immunity against infection by these viruses as well.

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