Therapeutic vaccination with vhs\(^{-}\) herpes simplex virus reduces the severity of recurrent herpetic stromal keratitis in mice

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When herpes simplex virus (HSV) infects the eye, the potential for harm is great. The resulting inflammation of the corneal stroma, known as herpetic stromal keratitis (HSK), may lead to permanent scarring and vision changes. Indeed, HSV is the most common cause of infectious corneal blindness in the United States (Pepose et al., 1996). After primary ocular infection, the virus becomes latent within sensory neurons innervating the cornea. From this location, HSV periodically reactivates and again replicates in the eye, causing additional HSK lesions. Signs of recurrent ocular HSV infection in humans include focal stromal opacities and neovascularization of the cornea (Pepose et al., 1996). The accumulation of such lesions over time leads to corneal clouding and blindness, and thus recurrent HSK presents the greatest danger to normal sight.

HSK is thought to be immune-mediated, with key participation by CD4\(^{+}\) T cells (Russell et al., 1984; Niemialtowski & Rouse, 1992). Post-infection vaccination may change immune responses from pathological to protective, and is therefore a reasonable approach to disease control. Desirable characteristics of a therapeutic vaccine for HSK include the ability to suppress virus reactivation and/or virus-induced corneal lesions, but reports of effective therapeutic vaccines for recurrent ocular herpes infections are scant (Walker et al., 1998; Keadle et al., 1997; Pivetti-Pezzi et al., 1999; Nesburn et al., 1998). In two such studies using different animal models, post-infection vaccination decreased spontaneous and inducible ocular virus shedding (Walker et al., 1998; Nesburn et al., 1998). In other work, vaccine treatment decreased the incidence and duration of recurrent herpetic keratitis or reduced the length of virus shedding (Keadle et al., 1997; Pivetti-Pezzi et al., 1999; Nesburn et al., 1998). The severity of resulting corneal stromal disease in all cases was unaffected.

Recently, HSV mutants deficient in virion host shutoff (vhs) activity have been examined for efficacy as vaccines for ocular herpes (Walker et al., 1998; Geiss et al., 2000; Walker & Leib, 1998). Vhs\(^{-}\) mutants are attenuated for both replication and pathogenesis following primary infection of the mouse eye (Strelow & Leib, 1995). Overexpression of immunogenic viral proteins, coupled with normal host MHC class I expression in infected vhs\(^{-}\) cells, could account for the reduced virulence of vhs\(^{-}\) mutants in vivo (Strelow & Leib, 1995; Kwong & Frenkel, 1987; Tiggles et al., 1996), as well as the immunogenicity of vhs\(^{-}\) vaccine strains. Accordingly, prophylactic vaccination of mice with vhs\(^{-}\) virus decreased challenge virus replication in the cornea, acute and latent infection of the trigeminal ganglia, blepharitis and keratitis (Geiss et al., 2000; Walker & Leib, 1998). In previously infected mice, vaccination with vhs\(^{-}\) virus

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**Virion host shutoff (vhs)-deficient herpes simplex virus (HSV) was tested as a therapeutic vaccine in a mouse model of UV light-induced recurrent herpetic stromal keratitis. Four weeks after primary corneal infection, mice were vaccinated intraperitoneally with vhs\(^{-}\) vaccine or control. Four weeks after vaccination, the eyes of latently infected mice were UV-B irradiated to induce recurrent virus shedding and disease. Post-irradiation corneal opacity in latently infected, vhs\(^{-}\)-vaccinated mice was significantly reduced compared to control-vaccinated mice (\(P = 0.007\) to 0.035). The incidence and duration of recurrent virus shedding were the same in both groups. Antibody titres were increased (\(P = 0.05\) and delayed type hypersensitive responses were unaffected by vhs\(^{-}\) vaccination. Combined with studies using different vaccination timing and vhs\(^{-}\)-genotypes, these data suggest that deletion of vhs is a useful strategy in the development of a therapeutic HSV vaccine, and that temporal and genetic factors influence vaccination outcome.**

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protected against inducible HSV reactivation and replication in the cornea (Walker et al., 1998).

A model of recurrent HSK has been developed in which the eyes of mice latently infected with HSV are exposed to UV-B irradiation (Shimeld et al., 1989; Laycock et al., 1991). Irradiated eyes subsequently shed virus into the tears and develop corneal lesions that clinically and histologically mimic the human disease (Pepose et al., 1996; Miller et al., 1996). Because one of the principal clinical signs of recurrent HSK in humans is opacification of the cornea, we investigated whether therapeutic vaccination of latently infected mice with a vhs− HSV vaccine virus would protect them from developing corneal opacity after UV-induced virus reactivation.

All investigations conformed to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Six-week-old National Institutes of Health (NIH) inbred mice (Harlan Olac, Oxford, UK) were infected on the scarified right cornea with $1 \times 10^5$ p.f.u. HSV-1 McKrae, with concurrent administration of anti-HSV serum to protect corneas from damage during primary infection (Laycock et al., 1991). A latency rate of 80–100% is expected (Keadle et al., 1997). Four weeks after primary infection, latently infected mice were vaccinated intraperitoneally with a cell lysate stock containing $2 \times 10^6$ p.f.u. of vhs− strain UL41NHB (vhs deletion mutant; two experiments) or BGS41 ( lacZ insertion into the vhs locus; one experiment) in 200 μl volume. Latently infected control mice received the same amount of uninfected cell lysate (control vaccine). Cell lysate stocks of vhs− mutants UL41NHB and BGS41 were prepared as described and viruses were propagated on Vero cells (Geiss et al., 2000; Strelow & Leib, 1995). Four weeks after vaccination, clear eyes of latently infected, vhs− and control-vaccinated mice, and age-matched uninfected mice were exposed to 250 ml/cm² UV-B irradiation (90 s) in order to stimulate recurrent virus shedding and disease. Before (day 0), and on days 1 to 7 post-UV-B irradiation, eyes were swabbed, and the swab material was cultured on Vero cells for 4 days to detect recurrent virus shedding (reactivation). After irradiation, a masked observer scored stromal opacity on a scale from 0 (clear) to 4 (total opacity with no posterior view) using a binocular-dissecting microscope (Laycock et al., 1991).

First, we observed that corneal opacity of both groups of latently infected mice was greater than that of mock-infected, UV-B-irradiated animals (UV-B control) on days 7 through 35 post-irradiation (Fig. 1; $P < 0.001$ to $P = 0.035$, t-test), indicating that irradiation itself caused significantly less severe opacity than recurrent virus infection. Second, corneal opacity in latently infected, control-vaccinated mice significantly exceeded that of vhs− vaccinees on days 14 through 35 post-irradiation ($P = 0.007$ to $0.035$, t-test). The incidence of stromal opacity in each group did not vary on any observation day (85%, vhs− vaccine; 100%, control vaccine). Third, the proportion of eyes shedding virus (average 56%, vhs− vaccine; 59%, control vaccine) and the number of virus-shedding days per mouse (average 2.8 days) were equivalent between vaccine groups over three separate experiments. Finally, all eyes shedding virus developed HSK. Latently infected, non-shedding eyes also developed opacity, but UV-induced opacity could not be differentiated from virus-stimulated disease because of the potential for virus reactivation in deep, swab-inaccessible layers of the corneal stroma (Miller et al., 1996). The results indicated that systemic therapeutic vaccination with vhs− HSV significantly decreased the severity of recurrent HSK without affecting virus shedding.

To assess virus-specific immune responses after vaccination, a sampling of vhs− and control-vaccinated mice was bled 2 weeks after vaccination for determination of HSV-specific IgG levels using ELISAs as previously described (Geiss et al., 2000). Briefly, serial 2-fold dilutions of serum from each mouse were incubated for 2 h in duplicate wells of a 96-well plate coated with purified HSV-1 glycoproteins. Biotinylated goat antimouse IgG and streptavidin-HRP were subsequently added in a colorimetric assay to determine specific IgG amounts based on comparison to standards with known HSV-specific IgG content. As shown in Fig. 2(A), serum antibody titres in vhs− vaccinated mice were greater than those in control-vaccinated mice ($P = 0.05$, t-test of geometric means), indicating that pre-existing virus-specific immunity was augmented by vhs− vaccination.
In addition to serum antibody determinations, delayed type hypersensitivity (DTH) responses were measured at the time of UV-B irradiation (Fig. 2B). Accordingly, just before corneal UV-B irradiation, mice from each group (excluded from further experiments) were injected in the right rear foot pad with $5 \times 10^6$ pfu. UV-inactivated HSV-1 McKrae strain in 30 µl of medium (Keadle et al., 1997). The left rear footpad was injected with the same amount of virus-free tissue culture medium. Footpad swelling was measured with a micrometer (Mitutoyo Manufacturing, Tokyo) immediately before and 24 h after injection. HSV-specific footpad swelling was determined by the formula (right footpad swelling at 24 h − right footpad swelling before injection) − (left footpad swelling at 24 h after injection − left footpad swelling before injection). While both groups of latently infected mice had significant DTH responses compared to naive controls (Fig. 2B; $P = 0.02$ and 0.003, t-test), vhs $^-$ vaccination did not significantly change DTH responses compared with control vaccination.

The data in this study show that immunotherapy of recurrent HSK can be accomplished using a single dose of a live-attenuated HSV-1 vaccine. Since corneal pathology resulting from HSK is thought to be an immune-mediated process, we evaluated representative protective (antibody) and destructive (DTH) immune responses. Higher antibody levels were detected in mice vaccinated with vhs $^-$ HSV (Fig. 2A). A change in the type or amount of HSV-specific antibodies could account for some disease amelioration in this group, possibly by limiting virus spread or decreasing the amount of antigen in corneas available for local induction or activation of pathological responses (Shimmeld et al., 1990).

T cell-mediated DTH activity has been associated with deleterious effects on corneal clarity during primary (Lausch et al., 1985) and recurrent (T. L. Keadle, unpublished data) ocular HSV infection. In this work, however, DTH levels were unchanged by vaccination (Fig. 2B), suggesting that systemic suppression of DTH activity was not responsible for vaccine-induced protection against recurrent HSK. Even so, evidence indicates that cell-mediated responses, including cytotoxic T cell activity and interferon-γ (IFN-γ) production, play a central role in controlling recurrent HSV infections (Deshpande et al., 2000; Stanberry et al., 2000), and CD8 $^+$ T cells can block HSV-1 reactivation from latency in sensory neurons (Liu et al., 2000). Thus, the effect of vhs $^-$ vaccination on other forms of cell-mediated immunity requires investigation.

As reported by us (Keadle et al., 2001) and others (Stumpf et al., 2001), a mix of T helper 1 (IFN-γ) and T helper 2 (interleukins 4, 10) cytokines is present in mouse corneas throughout the course of recurrent HSK (Keadle et al., 2001). A vaccine with protective effects might alter the balance between such cytokines, favouring, for example, production of IL-10 with its attendant palliative effects on corneal opacity (Tumpey et al., 1994; Daheshia et al., 1997). We are currently testing this hypothesis with regard to therapeutic vhs $^-$ vaccination.

In recent work from our laboratory, a lacZ $^+$ vhs $^-$ vaccine strain protected mice from recurrent HSK when administrated 4 months after primary ocular infection (Keadle et al., 2002). Combined with the present work, these data confirm the efficacy of therapeutic vhs $^-$ vaccination in controlling the severity of recurrent herpetic corneal lesions. To our knowledge, these are the first reports of therapeutic vaccines with a positive influence on the severity of recurrent HSK. Although different vaccination schedules (4 months vs 4 weeks post-infection) yielded similar amelioration of virus-induced corneal opacity, recurrent virus shedding was reduced only in mice receiving vhs $^-$ vaccination 4 months after primary infection (Keadle et al., 2002). Earlier studies also indicated that vaccination at 4 months with vhs $^-$ virus decreased the reactivation rate (Walker et al., 1998). These findings suggest that the therapeutic effects of vhs $^-$ vaccines on recurrent virus
sheding are time dependent, whereas the effects on recurrent HSK are not. To determine the clinical usefulness of vhs− vaccines, further studies of the temporal effects on vaccine efficacy are clearly needed.

In addition to temporal effects, it is possible that differences in the vhs− virus itself play a role in vaccine efficacy. Thus, evidence suggests that lacZ− vhs− viruses are not as pathogenic in vivo as gene-deleted vhs− viruses (Smith et al., 2002), and may elicit β-galactosidase-specific immune responses that alter HSV responses in a bystander fashion (Brubaker et al., 1996). This might explain differential effects on corneal opacity of lacZ− vhs− (Walker et al., 1998) and lacZ+ vhs− (Keadle et al., 2002) vaccination at 4 months post-infection as reported in other work. Hence, both the type and timing of vhs− vaccination may affect clinical outcome and should be important considerations in vaccine design.

Despite their attenuation, vhs− mutants retain the potential to replicate and cause disease in vaccinated hosts (Strelow & Leib, 1995). Such an adverse outcome may be avoided through the creation of new, immunogenic and replication-defective modified live virus vaccines (Geiss et al., 2000). Deletion of vhs function augments protective immunity in vaccinees, and may be an appropriate mutation to be used in combination with other virus defects that negate replication, for therapeutic vaccination.

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