Effect of immunization on herpes simplex virus type 1 latent infection in the trigeminal ganglion

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Abstract

Purpose. To quantify and characterize immune protection from herpes simplex virus (HSV) latent infection in mice following corneal challenge.

Methods. Mice immunized or mock-immunized and boosted in the flank with an HSV replication-deficient mutant were challenged by corneal inoculation with wild type (wt) or thymidine kinase-negative (TK-) HSV. At specified times post challenge, trigeminal ganglia were assayed for in vitro reactivation, latent and acute viral load (using quantitative PCR), acute infection, and cellular infiltration (hematoxylin and cosin stained sections).

Results. With wt HSV challenge infection, immunization led to reduced reactivation, significantly less latent and acute viral DNA, and no acute viral replication in ganglia, and rapid infiltration of inflammatory cells. Immunization had little effect on viral load following challenge with replication-conditional TK- mutant virus.

Conclusion. These results indicate that immune protection from latent HSV infection in mouse trigeminal ganglia following ocular infection can act under these experimental conditions to block acute viral replication in ganglia and is directed to antigenic targets within the ganglia.

Keywords: corneal infection; HSV latency; HSV vaccines; immune protection; viral load

Introduction

The normal host immune response to herpes simplex virus (HSV) involves every arm of the immune system and rapidly clears productive infection, with development of lasting, specific immunity.1,2 Concomitantly, HSV infection invariably results in the deposit of a latent form of virus in sensory neurons localized in ganglia. Because episodic recurrences at or near the site of the primary infection arise from reactivation of the latent infection despite development of specific immunity against HSV, latent infection represents the reservoir for virus and disease transmission.

An appropriate protective vaccine against HSV must not only prevent primary infection and disease but also block latent infection and/or reactivation. Specifically, an adaptive, cell-mediated immune response directed to both mucosal surfaces and sensory neurons is desirable.3,4 Types of experimental vaccines developed in animal models include killed virus, subunit vaccines with various adjuvant preparations, chimeric vector viruses expressing HSV proteins,4 genetically modified live HSV viruses, and DNA vaccines.5 Live virus vaccines express the widest array of antigens and may be expected to generate immunity against the greatest number of viral epitopes expressed at a natural route of entry and within the nervous system.

Several immunization studies have shown reduction of latent infection as measured by reactivation following ganglionic explant and viral load. Passive immunization with pooled human immunoglobulin reduced latent infection in mice.6,7 Immunization with virus subunit glycoprotein vaccines reduced latent infection in guinea pigs8 and mice.9 Immunization with attenuated HSV mutants conferred protection from disease and lethality10,11 and latency12 after wt HSV challenge in mice. Protection from latency was conferred by the replication-deficient HSV mutant d301 was demonstrated by a 5-fold reduction in in vitro reactivation...
frequency and by an 80-fold reduction in the number of latency-associated-transcripts (LAT)-positive neurons detected by in situ hybridization. Immune protection from latency in other systems has been demonstrated by a decrease in or complete absence of in vitro reactivation. An important caveat in interpreting measures of latency is that in vitro reactivation is a qualitative indicator of reactivation potential rather than a quantitative measure of latent infection. Sensitive methods of detecting and defining latency are warranted.

How prior immunity decreases latent infection has not been described. In the naive host, latent virus engages host immune components in ongoing interactions. Cytokines and T cells persist in ganglia during latency and may play a significant role in regulating reactivation. Because latent HSV resides in neurons in the peripheral nervous system, specialized, tissue-specific immune mechanisms may be involved. In the immunized host, protection from latency may be secondary to containment of the challenge infection at the periphery. However, it is possible that a rapid, specific immune response is targeted to sites of latent infection within the ganglia. To address these questions, mice were immunized or mock-immunized and boosted in the flank with an HSV replication-deficient mutant, d301, challenged by corneal inoculation with wild type (wt) or TK- HSV, and at specified times post challenge, trigeminal ganglia were assayed for in vitro reactivation, latent and acute viral load (using quantitative PCR), acute infection, and cellular infiltration (hematoxylin and eosin stained sections).

Materials and methods

Cells and viruses

The KOS strain of HSV was propagated and assayed on Vero cell monolayers as previously described. The tk gene deletion mutant, dlsactk, was derived from the HSV KOS strain, and has been previously described. The HSV KOS1.1 ICP8 deletion mutant, d301, and the complementing cell lines S2 and V827 used for its propagation, have been previously described. The d301 stock and the control Vero cell extract were prepared as previously described.

Animal inoculations

Six-week-old female BALB/c mice were purchased from Taconic Farms (Germantown, NY) and housed in accordance with institutional and NIH guidelines on the care and use of animals in research. Procedures were approved by the Institutional Animal Use Committee of Harvard Medical School. Immunization of mice was performed as described previously. Briefly, fur on the left rear flank was trimmed and the animals were injected subcutaneously with 2 × 10⁶ PFU of d301 or an equivalent volume of control cell lysate. Identical immunizations were repeated two weeks later. Two weeks after the second immunization, mice were challenged with wt HSV strain KOS or dlsactk, or mock-challenged with virus growth medium, by inoculation onto scarified corneas as previously described.

Quantitative PCR

At the indicated times post challenge, the animals were sacrificed, and trigeminal ganglia were removed, rapidly frozen in dry ice, and stored at −80°C. Each ganglion was digested with proteinase K, followed by heat inactivation as previously described. Aliquots were taken in duplicate for PCR analysis of viral (tk gene) and cellular (mouse adipsin gene) sequences. Viral RNA standards were prepared from HSV virion DNA and quantified by competition with mass standards, as previously described, then serially diluted and combined with uninfected ganglia digests. Cellular DNA standards were made from uninfected ganglia digests. PCR reactions, using primers tk1 and tk2 or MT1 and MT2 for the tk and mouse adipsin genes, respectively, and thermal cycling parameters using a Perkin-Elmer Cetus DNA Thermal Cycler were as described previously. Reaction products were separated on 12% non-denaturing polyacrylamide gels and stained with SYBR-Gold fluorescent nucleic acid stain (Molecular Probes, Eugene, OR). Gels were viewed on a UV light box. Specific products were identified by expected length and by comparison with positive and negative controls included with every set of reactions. Gels images were recorded on a Fluor-S Multimager (Bio-Rad Laboratories, Hercules, CA), and the specific product bands were quantified using Molecular Analyst software (Bio-Rad Laboratories).

In vitro reactivation

At 30 days post challenge, ganglia were dissociated and assayed for their ability to reactivate, as described previously. Briefly, individual ganglia were harvested into serum-free medium on ice, which was replaced with serum-free medium containing 0.125% trypsin (Irvine Scientific, Santa Ana, CA) and 0.2 mg/ml collagenase Type I-A (Sigma-Aldrich, St. Louis, MO), then incubated for 1h at 37°C with gentle agitation. The dissociated cells were collected by centrifugation, washed with culture medium containing 10% fetal calf serum (Life Technologies, Rockville, MD), overlaid onto Vero cell monolayers, and incubated at 37°C in 5% CO₂. The cultures were supplemented with medium every three days and were observed daily for evidence of cytopathic effect (CPE) for up to twelve days.

Assays of acute infection

Eye swabs were collected and viral titers of mouse eye swabs were determined as described previously. Viral titers were determined by plaque assay after ganglia had been placed in 1 ml of assay medium, frozen, thawed, and disrupted by a 30 second sonication as previously described.
Histochemistry

Trigeminal ganglia and portions of the root entry zone and nerve bundles (posterior and anterior boundaries, respectively) were dissected from the skull. Pairs of ganglia from individual mice were pooled. Each pair was fixed overnight in Zinc Formal-Fixx (Shandon Lipshaw, Pittsburg, PA) containing 25 mM betaine (Sigma-Aldrich, St. Louis, MO) at room temperature, then dehydrated in graded alcohol, cleared with xylene, infiltrated with paraffin (Paraplast, Fisher Scientific, Medford, MA) using standard protocols and embedded ventral sides down. Slides were pretreated with 3-aminopropyl triethoxy silane (Sigma, St. Louis, MO). Transverse 6 micron sections were collected sequentially and heat-fixed at 60°C for 1 h. Slides were deparaffinized, stained using a standard Mayer’s hematoxylin and eosin (H&E) procedure and mounted with VectaMount permanent mounting medium (Vector Laboratories, Burlingame, CA). Stained sections were visualized with a Zeiss Axioplan 2 microscope, and images were captured with a Hamamatsu ORCA digital camera using Improvision Openlab software.

Results

Effect of immunization on in vitro reactivation

It has been shown previously that immunization with d301 reduced in vitro reactivation following challenge with the HSV microplaque strain. However, microplaque is a virulent strain, and latency studies are hampered by high mortality of mock-immunized control mice. Although increased survival of mock-immunized mice following challenge with a virulent strain can be achieved by pretreatment with acyclovir or immunoglobulin, our objective was to compare immunized with naive mice. Therefore, to study the effects of immunity on latent infection and maintain an adequate number of mock-immunized control mice, we challenged mice with the less virulent wt HSV strain KOS. Mice were immunized with d301 or mock-immunized in the flank, boosted, and challenged with wt HSV at 10^5 or 10^6 PFU per eye. Four weeks later, ganglia from each group were enzymatically dissociated, cultivated on Vero cell monolayers, and monitored for CPE for at least ten days. In vitro reactivation was reproducibly lower in immunized mice at both challenge doses (Fig. 1), confirming the previous studies with microplaque and characterizing this system with KOS.

Effect of immunization on the latent viral load

Latent viral load in trigeminal ganglia was analyzed using quantitative PCR (qPCR) (Fig. 2) following challenge with wt HSV. Ganglia from mock-challenged and unchallenged mice (negative controls) yielded no detectable HSV-specific PCR product. All ganglia from mice challenged with wt HSV yielded quantifiable HSV-specific PCR product indicating that HSV latent infection was established in both non-immune and immune mice. Immunization with d301 in the flank significantly reduced latent viral load in trigeminal ganglia following challenge infection with wt HSV in the eye (Student’s unpaired t-test, P < 0.05) (Fig. 3A). Furthermore, reduced latent viral load correlated with reduced in vitro reactivation (Fig. 3B).

Effect of immunization on acute viral load in ganglia

Reduced latent viral load observed in immunized mice challenged with wt HSV could have been a consequence of reduced amounts of viral DNA deposited during establishment of latent infection or of depletion of latent viral load over time. To distinguish between these possibilities, viral DNA in ganglia was quantified by PCR at one to six days post challenge with wt HSV (Fig. 4). All ganglia tested at day one post challenge contained viral DNA, and the immunized and control group DNA levels were indistinguishable. On day two, mock-immunized mouse ganglia contained higher levels of viral DNA compared to immunized mouse ganglia. By day three, viral DNA in the mock-immunized mice was 100-fold greater than immunized ganglia, whereas viral DNA in the d301-immunized mice had reached a plateau amount that was comparable to the latent viral load measured at day thirty. Thus, prior immunization significantly reduced the amount of viral DNA that accumulated in ganglia.

Effect of immunization on acute viral replication in eye and ganglia

Reduced acute viral load observed in immunized mice challenged with wt HSV could have been a consequence of reduced seeding of the ganglia, for example by curtailing the primary infection in corneas, or by reduced viral replication in ganglia. To address these possibilities, we assayed viral replication in cornea (PFU/eye swab) and ganglia (PFU/
ganglion) following challenge with either $10^7$ or $10^8$ PFU wt HSV per eye. In immunized compared to control mice, both challenge doses resulted in moderately reduced replication in corneas (Fig. 5A) and, more drastically, little to no detectable replication in ganglia (3 ganglia out of 11 tested contained a few PFU at day 2 only) (Fig. 5B). These results reproduced those following challenge of $d301$-immunized BALB/c mice with $10^8$ PFU of the virulent HSV microplaque strain, verifying that prior immunity, and not virus strain or challenge dose, accounted for the block to acute replication in ganglia.

To further address whether viral replication in ganglia was a key target for immune protection from latent infection, immunized and mock-immunized mice were challenged with the replication-conditional mutant virus, dlsactk. dlsactk replicates relatively efficiently in mouse cornea but not detectably in ganglia. We predicted that if immune protection from latent infection was targeted to the primary corneal infection, then dlsactk DNA in ganglia would be reduced in immunized mouse ganglia, whereas if immune protection was targeted to viral replication in ganglia then dlsactk DNA in immunized and naive mouse ganglia would be similar. Naïve and immunized mice challenged with dlsactk were comparable to immunized mice challenged with wt HSV for replication in corneas and for accumulation of viral DNA in ganglia (Figs. 4 and 5). Thus, immune protection from latent infection measured as reduced latent or acute viral load correlated with the ability of the challenge virus to replicate in ganglia. These results suggest that the main effect of immune protection under these experimental conditions was to block acute replication in ganglia.

**Rapid inflammatory cell infiltration in immunized mouse ganglia**

If the main target of immune protection from latent infection was acute viral replication in the ganglia, then the presence of immune effectors would be predicted in ganglia shortly after challenge infection. We therefore harvested ganglia at three days post challenge for histological examination. The time course of appearance of inflammatory cell infiltration in ganglia following infection of naïve mice correlates with the end of viral replication in ganglia, from 10 to 15 days p.i. Accordingly, in mock-challenged mice and mock-immunized mice challenged with wt HSV, H&E stained ganglia (Fig. 6A and B) and optic nerve tracts extending from the ocular branch of the ganglia (Fig. 6D and E) appeared normal at three days. In contrast, immunized mice challenged with wt HSV exhibited abundant cellular infiltration in a very limited area, specifically the anterior portion of the ocular branch of the trigeminal ganglia (Fig. 6C), and the optic nerve tracts were heavily infiltrated with small, round, intensely stained cells (Fig. 6F). Thus, in immunized mice,
ocular challenge induced rapid inflammatory cell infiltration in ganglia and associated optic nerve tracts.

**Discussion**

*In vitro* reactivation, latent viral load and acute viral load were reduced and acute viral replication was blocked in ganglia of immunized mice following corneal challenge with wt HSV, at challenge doses of $10^7$ and $10^8$ PFU/eye, confirming and extending prior studies that suggested that the mechanism of immune reduction of latent infection was primarily to block acute viral replication in ganglia. Challenge with replication-conditional mutant dlsactk in naive and immunized mice addresses the question in a new way. Viral load with dlsactk was similar to that following challenge with wt HSV in immunized mice, a finding that corroborates identification of acute replication in ganglia as a key stage of immune protection from latent infection.

HSV can establish latency in immune-compromised mice, normal mice with host-strain variations in immune status, and mice immunized with serum antibodies or HSV subunit vaccines or live, replication-defective or attenuated virus mutants. Although some studies report vaccine strategies that provide complete protection from latency measured by *in vitro* reactivation, our quantitative PCR assay revealed that all ganglia, naive and immunized, challenged with $10^7$ or $10^8$ PFU/eye, contained viral DNA. This may be in part because the cornea is avascular and highly innervated, and a more effective block might be found following cutaneous challenge of an immunized host. For example, intranasal inoculation of mice with a TK mutant of the virulent HSV 2 strain 186-syn-1 in immunized mice reduced latent viral load by several orders of magnitude. Nevertheless, in the mouse eye model, the seeding of ganglia with viral genomes appeared to be synchronous with primary replication in the eye and independent of the host immune system. Thus, realistic goals of immune protection from latency are protection from damage to and
spread within the nervous system, and protection from reactivation.

The d301 immune-related block to acute replication was first documented in mice challenged with 10^5 PFU/eye of the virulent microplaque strain of HSV^29^ and reproduced here in mice challenged with the lab-attenuated KOS strain at 10^5 or 10^5 PFU/eye. Thus, the block to replication in ganglia did not depend on virus strain, relative virulence, or challenge dose. Viral replication in the eye was shortened by two to three days in immunized mice. To determine whether the shortened time course of infection in eye reduced the amount of viral genomes available to seed ganglia, naïve and immunized mice were challenged with replication-impaired dlsactk. This mutant replicates in the eye but not in ganglia^26^ and seeds ganglia at the same rate and to the same extent as wt HSV for up to 48 hours after eye infection. We found that prior immunity shortened the time course of dlsactk eye infection to the same extent as that of wt HSV. The amount of dlsactk DNA in ganglia was similar in naïve and immunized mice, and similar to the amount of wt HSV DNA in immunized mice. Thus, viral replication in ganglia, not entry of viral DNA into sensory ganglia, was targeted by prior immunity.

The immune-related block to acute replication in ganglia may have resulted from the action of cytokines and/or a rapid cell-mediated immune response. Both type 1 and type 2 interferons (INFα/β and IFNγ) can restrict HSV replication in vitro^53-55^ and in vivo. A rapid cell-mediated response would require antigen recognition within the ganglia. Are
HSV antigens produced in ganglia following wt HSV challenge of d301-immunized mice. Both dlsactk and the parental wt HSV strain KOS express productive cycle transcripts between 24 and 48 hours in naïve mice, and KOS generates infectious virus by 48 hours. In a pilot experiment, ganglia were processed for immunohistochemical detection of HSV antigens. HSV antibody-specific stain was apparent at a few foci at day two in both naïve and immunized mouse ganglia (unpublished observations). H&E stained sections of ganglia showed pronounced cellular infiltration in the ophthalmic division and along optic nerve tracts in immunized mouse ganglia by three days after corneal challenge (this report). Taken together, this provides strong evidence that HSV proteins are produced and recognized as antigens in ganglia of immunized mice.

What antigens might be recognized in the acute ganglia infection of immunized mice? Abundant expression of late genes is dependent on the viral DNA synthesis stage of viral replication. The block to viral replication in ganglia that was accompanied by a block to accumulation of viral DNA is akin to that seen in infections with TK- viruses, suggesting that abundant late gene expression was not crucial for immune recognition in ganglia. Immunization with the live-defective virus, d301, would have permitted presentation of IE, E and some L gene products. Challenge infection in the eye may have primed the immune response to IE and/or E genes, stimulating a more rapid response in the ganglia. ICP40 and ICP27 stimulate CTL responses in vitro, and ICP47 stimulates lymphoproliferation. Immunization with the HSV-2 ICP27 protects immunocompetent mice from zosteriform lesions following wt HSV-2 challenge, and this protection is dependent on CD4+ T cells, which are important in protection from latency following challenge of d301-immunized mice with wt HSV. Thus, expression of IE, E, or leaky L genes could account for rapid antigen recognition within the microenvironment of the ganglia.

Viral replication is associated with spread within the nervous system. By blocking replication, spread is likely curtailed. A previous report showed that the number of LAT+ neurons was 80-fold greater in naïve mouse ganglia than in immunized mouse ganglia at thirty days after challenge with microplaque at 10^7 PFU/eye. Thus, immunity may constrain viral genomes to the few neurons that innervate the cornea.

In vitro reactivation assays have been used extensively to define and measure HSV latency in naïve mice. Under controlled conditions, cytopathic effect (CPE) arising after a characteristic lag period in tissue culture is a definitive indicator that tissue had been latently infected. However, failure to induce CPE in tissue culture cannot be interpreted definitively, as it may result from the absence of latent genomes or the presence of inhibitor(s) of reactivation. We found that reactivation correlated with latent viral load (Fig. 3B), perhaps due to an increased probability of reactivation with more viral genomes. However, reactivation also showed an inverse correlation with immune status and, surprisingly, with challenge dose (naïve: 100% or 87% at challenge doses 10^7 or 10^6 PFU/eye, respectively; immunized: 45% or 3.5% at challenge doses 10^5 or 10^6 PFU/eye, respectively), consistent with a possible role of suppression of reactivation due to immune effectors such as CD8+ T cells or CD4+ T cells. Thus, decreased reactivation in immunized animals could be the result of multiple mechanisms, including reduced viral load and immune suppression of reactivation.

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Figure 6. Immunized and control mouse trigeminal ganglia three days post challenge with 10^7 PFU wt HSV. H&E stained 6-micron sections, 40x. A, B, and C: anterior ocular branch of the trigeminal ganglia; D, E, and F: optic nerve tract adjoining the ocular branch of the trigeminal ganglia. A and D: mock-infected (scarified cornea inoculated with virus-free medium); B and E: mock-immunized control mice, corneally challenged with 10^7 PFU wt HSV; C and F: immunized mice, corneally challenged with 10^7 PFU wt HSV.

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