MINIREVIEW

The Immune Response to Ocular Herpes Simplex Virus Type 1 Infection

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Herpes simplex virus type 1 (HSV-1) is a prevalent microbial pathogen infecting 60% to 90% of the adult world population. The co-evolution of the virus with humans is due, in part, to adaptations that the virus has evolved to aid it in escaping immune surveillance, including the establishment of a latent infection in its human host. A latent infection allows the virus to remain in the host without inducing tissue pathology or eliciting an immune response. During the acute infection or reactivation of latent virus, the immune response is significant, which can ultimately result in corneal blindness or fatal sporadic encephalitis. In fact, HSV-1 is one of the leading causes of infectious corneal blindness in the world as a result of chronic episodes of viral reactivation leading to stromal keratitis and scarring. Significant inroads have been made in identifying key immune mediators that control ocular HSV-1 infection and potentially viral reactivation. Likewise, viral mechanisms associated with immune evasion have also been identified and will be discussed. Lastly, novel therapeutic strategies that are currently under development show promise and will be included in this review. Most investigators have taken full advantage of the murine host as a viable working *in vivo* model of HSV-1 due to the sensitivity and susceptibility to viral infection, ease of manipulation, and a multitude of developed probes to study changes at the cellular and molecular levels. Therefore, comments in this review will primarily be restricted to those observations pertaining to the mouse model and the assumption (however great) that similar events occur in the human condition.

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Humoral and Cellular Immunity to Herpes Viruses

General Concepts. Humans and animals infected by a herpes virus develop immunity in the form of circulating antibody and cell-mediated immunity, including T helper, T cytotoxic, and memory T lymphocytes (1–7). This constellation of specific immune factors serves to protect the infected organism from disseminated viral disease and death (8–10). Thus, the acquired immune response to herpes viruses such as herpes simplex virus type 1 (HSV-1) is relatively effective in protecting the organism from morbidity or even mortality due to this virus (10–13).

However, herpes viruses, including HSV-1, become latent in the infected host and can undergo reactivation and cause recurrent disease even though the host may have intact, innate, and acquired immune defenses (1, 3, 4). Recurrent herpetic infection is not uncommon in immune, healthy humans and can be induced to recur in immune, healthy animals (5–8). Understanding the paradox of how the acquired immune response protects against disseminated viral disease on the one hand and yet is not completely effective in preventing against recurrent viral infection is a goal that still eludes us.

In the paragraphs that follow, the ocular route of HSV-1 infection, the development of immunity subsequent to ocular infection, and the role of humoral and cellular immunity in HSV-1 ocular disease, both acute and recurrent, will be discussed particularly with reference to the conundrum of the coexistence of protective immunity and recurrent infection in the same individual.

Innate Resistance Mechanisms at the Ocular-Surface. Herpes viruses reaching the surface of the eye from an external source are initially suspended in the ocular tear film. Although largely consisting of water, the tear film contains a number of substances that have antiviral activity

(14). Several tear proteins, including lysozyme, immunoglobulin A antibody, complement, lactate dehydrogenase, amylase, and peroxidase, may act to prevent viral infection of the ocular surface. The interferons, particularly the alpha and beta types, are also present in the tears during a viral infection of the ocular surface (15–20). The key feature of ocular surface innate resistance is the constant washing of the ocular surface by tears produced by the lacrimal gland. Thus, molecules, cells, and infectious agents reaching the ocular surface are washed away rapidly and effectively. However, even though this first line of defense is effective, it is not an impenetrable barrier.

The outermost cells of the cornea are endstage epithelial cells that are no longer capable of replication (21). These cells are constantly being replaced by cells beneath them. As the outermost epithelial cells lose their cell-to-cell connections, they are flushed away in the tears. The intact corneal epithelium is a very effective physical barrier to infection by microorganisms. In addition to acting as a physical barrier, the outermost epithelial cells of the cornea are presumably poor hosts for herpes viruses because these cells are terminally differentiated and not capable of replication (21). It is evident that the herpes viruses replicate best in metabolically active cells (22–24). When there has been a breach in the corneal surface epithelial cells, viruses such as HSV-1 can infect the underlying cells, which are metabolically active. Even in such a circumstance, however, the virus must attach to the epithelial cells, penetrate, and undergo replication. In the presence of sufficient endogenous interferons, this process may be blocked. Therefore, the sum and substance of the innate resistance mechanisms at the ocular surface is that they are very effective in preventing primary or secondary herpes viral infections.

Acquired Immunity and Its Role in Preventing Ocular Infection. During a primary viral infection of the corneal epithelium, an inflammatory reaction occurs (25). This inflammation is characterized by an infiltrate of polymorphonuclear leukocytes (PMNs), macrophages, and a scattering of other mononuclear cells, including lymphocytes and, perhaps, a few natural killer (NK) cells (7, 26–33). The PMN response, properly speaking, falls in the category of an innate or nonadaptive immune response to primary and subsequent viral infection. There is considerable evidence to show, however, that the infiltration of PMNs is intimately linked to the adaptive response and facilitates the development of the acquired antigen-specific immune response (20, 30–32, 34, 35). Thus, the PMNs and macrophages present in the acute inflammatory lesion in the corneal epithelium not only serve to scavenge virus-infected cells, but some of these cells produce chemokines that attract lymphocytes into the area and, as well, some of these cells express major histocompatibility complex (MHC) molecules containing herpes viral antigenic epitopes, which are recognized by T cells entering the lesion (7, 36).

Regarding initiation of the acquired immune response in the cornea and the processing and presentation of viral antigens, it is likely that antigen-presenting cells such as Langerhans cells and macrophages engulf virus and virus-infected cells locally in the cornea and then transport these antigens to regional lymph nodes and possibly through the blood to the spleen, where they present these antigens to viral antigen-specific T lymphocytes (37–41).

In this regard, it is evident that a highly localized herpes viral infection such as occurs in the corneal epithelium results in the establishment of systemic immunity in the infected organism (5, 7). Numerous studies over the past 40 years have shown that animals and patients infected with HSV-1 develop both cellular and humoral immunity, which is measurable in the blood and secondary lymphoid tissues such as the spleen and lymph nodes.

The cornea of the eye is an immunologically privileged site by virtue of its avascularity and dearth of lymphatic vessels (42). Acquired immunity, which is developed following a primary corneal infection, was originally thought not to persist in the cornea in the form of resident, antigen-specific T lymphocytes. However, recent reports have isolated HSV-reactive T lymphocytes from corneas of patients with a history of herpetic keratitis (43, 44). Not only are there T cells residing in the tissue proximal to the original site of infection, but also memory T and B lymphocytes specific for viral antigens circulate in the blood and lymph armed for the next encounter with the virus. Animals and humans who have been either immunized with herpes viral antigens or infected by herpes virus have circulating titers of antiviral antibody primarily of the IgG isotype. And, at the ocular surface, secretory IgA antibody secreted into the tears from the conjunctival associated lymphoid tissue and produced by plasma cells in the lacrimal gland should bind to infectious virus, prevent its infection of epithelial cells, and transport the virus away in the tear flow (45). Indeed, the presence of IgA isotype anti-HSV antibodies has been demonstrated in tears (45–47).

Regarding the establishment of cell-mediated immunity following primary corneal infection by HSV-1, it has been amply demonstrated that cell-mediated immunity in the form of CD4+ T helper lymphocytes and CD8+ cytotoxic T lymphocytes, which are antigen-specific for viral HSV-1 antigens, are generated during a primary corneal infection in patients and in experimental animals (5, 7, 28). Numerous studies in the 1960s and 1970s demonstrated that patients who develop ocular infection by HSV had circulating peripheral blood T lymphocytes reactive with viral antigens in the lymphocyte transformation assay (5, 7). Presumably, a fraction of these cells persist in patients and in animals as a long-lived memory population that can provide a rapid response to the threat of viral reinfection or viral reactivation. As mentioned above, antigen-specific T lymphocytes that may be present in the infected corneal epithelium in small numbers during primary infection do not remain there once the infection has resolved (5, 7). Presumably these antigen-specific T cells migrate to regional lymph nodes and some enter the blood and lymph where they circulate. Numerous

histopathological studies of human corneas and experimental infections with herpes virus in animals in which immunochemical staining has been performed indicate that the presence of lymphoid cells in the infected cornea is a transient phenomenon. CD4+ and CD8+ T cells are found, as are macrophages and increased numbers of corneal Langerhans cells, during the acute phase of the infection (7, 28, 29, 36, 48, 49). This cellular infiltrate wanes as the infection resolves and the cornea returns to its normal cellular architecture.

The benefit to the organism of having memory T lymphocytes specific for viral antigens at some distance away from the tissue originally infected and the site at which recurrent infection is likely to occur is not immediately apparent. It would seem more beneficial to the organism to have the memory T lymphocytes immediately at hand in the tissue threatened by viral reinfection. However, numerous animal studies have shown that secondary or recurrent infection of the cornea results in a rapid and intense infiltration of mononuclear cells, including antigen-specific T lymphocytes, macrophages, and NK cells (28, 36, 50, 51). The rapid mobilization of memory cells, followed by their replication and stimulation to secrete interferon and other cytokines provides a rapid response mechanism following viral recurrence (15, 17–20, 52–54). Furthermore, it is evident that recurrent viral infection of the cornea is prevented from spreading to adjacent tissues by virtue of response of the memory T cells present in the regional lymph nodes and those circulating in the blood. Thus, recurrent corneal infection seldom spreads beyond the borders of this tissue.

The NK cell mechanism of resistance should not be overlooked in any consideration of herpes viral infection. It has been amply demonstrated in patients and in experimental animals that NK cells and macrophages are key mediators of viral resistance (55–63). Thus, separate from, and without the direct intervention of antigen-specific T lymphocytes, NK cells and macrophages mediate an important component of resistance to herpes virus and resolution of the herpes viral infection of a tissue such as the cornea.

Paradoxically, there is a circumstance of herpes viral infection of the cornea in which cell-mediated immunity appears to be immunopathogenic. In a distinct disease entity known as herpetic stromal keratitis (HSK), there is evidence to indicate that there is a chronic low level viral infection of corneal stromal keratocytes in the area subtending the corneal epithelium (25, 47). (Note that this observation has not been consistently observed and, therefore, may only occur in select individuals by means that are not fully understood, but may be either host- or viral strain-specific.). The expression of viral antigens by the stromal keratocytes may provide a chronic stimulus to antigen-specific T lymphocytes (48, 49). It is thought that the T lymphocytes recognize viral antigens on the keratocytes and kill by a cytotoxic mechanism. HSK is typified by its chronicity, lasting for weeks, months, or even longer, by its unresponsiveness to standard topical antiviral therapy, and by the relative efficacy of topical steroid therapy in treating this condition (25). These observations all point to a cell-mediated immunopathogenic mechanism for this disease process. These conclusions are supported by numerous studies in experimental animals, particularly in thymus-deficient mice and severe combined immune-deficient mice (48, 49, 63–70). HSK is a unique clinical entity and is distinctly different from herpetic keratitis or short-term infection of the corneal epithelium, which responds to antiviral therapy and in which use of corticosteroids is contraindicated (25).

In summary, there are multiple innate and adaptive immune mechanisms that act to prevent sight-threatening infection of the corneal epithelium. These immune mechanisms are effective, but not absolute in terms of preventing infection and, in particular, from recurrent viral infection following reactivation in the nervous system. Subsequent sections of this review will deal with the role of immunity in the establishment, maintenance, and reactivation of viral infection.

Ocular Infection, Latency, and Reactivation. The surface of the eye consists of several layers of epithelial cells that when infected by HSV-1, become inflamed in a disease process termed herpetic keratitis (25). Histopathologically, this acute infection of the ocular surface epithelium is characterized by the destruction of epithelial cells and the infiltration of acute inflammatory cells, including polymorphonuclear leukocytes, macrophages, and lymphocytes. In humans and animals, herpetic keratitis is usually a self-limiting process that resolves within 7 to 10 days after the initial infection (25). During this initial infection, however, two key events take place that impact the infected organism for the rest of its life.

One of these key events is that the infecting virus enters nerve terminals in the corneal epithelium and is transferred by retrograde flow to neuron cell bodies in ganglia whose neurons innervate the ocular surface. These neuron cell bodies reside in the superior cervical and trigeminal ganglia (71). In the neurons the virus enters into viral latency, an inactive state in which the virus may remain throughout the life of the organism (10–12, 71–74). It is also possible, and not all that uncommon, that the virus may reactivate from its latent state and cause recurrent disease in the organ or tissue where the infection first occurred (Fig. 1).

The second key event that occurs during acute viral infection of the cornea is the establishment of acquired immunity to a large array of viral antigenic epitopes (5, 7). Animals given an experimental corneal infection with HSV-1 become seropositive for antibodies to this virus within 7 to 10 days of the initial infection (25). Similarly, during this time, cell-mediated immunity is also developed (26, 28, 64). Humans and animals that have undergone ocular infection by HSV-1 have long-standing immunity to the virus in the form of memory cells and serum neutralizing antibodies to the virus.

How, then, is it possible that the virus can reactivate from latency and cause recurrent disease in the presence of

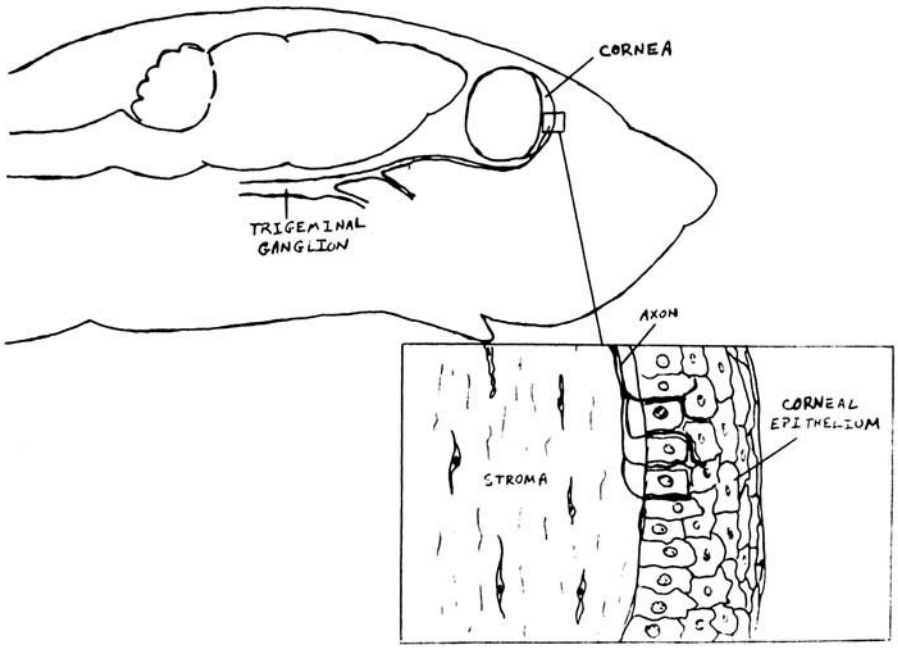

Figure 1. Anatomic relationship between the trigeminal ganglion and the eyeball. The ganglion sits underneath the brain and has several branches that lead to oral-facial structures, including the eye. Some of the neural fibers innervate the cornea and branch profusely throughout the corneal epithelium. Inset, schematic showing an axon in the corneal stroma branching and innervating amongst the corneal epithelial cells. The corneal epithelium has the highest density of nerve endings of any cell layer in the body.

humoral and cellular immunity directed against viral antigenic epitopes? When HSV-1 infects the epithelial cells of the cornea, viral replication takes place for a period of 5 to 7 days. Subsequently, the resistance mechanisms of the infected organism are marshaled and the viral infection is prevented from continuing. During the 5- to 7-day period of viral replication, some viral particles gain entry to the neurites that interdigitate between the corneal epithelial cells (Fig. 2). These viral particles, once inside of the nerve fibers, are relatively protected from host immune mechanisms (11). The viral particles, or a fraction of them, are transported by retrograde flow to the cell bodies of neurons in the sensory ganglia such as the trigeminal ganglion (69). It is intuitively obvious that viral particles inside neurites and in the cell bodies of neurons are relatively protected from the host immune response. It also seems clear that during a primary infection when the infected organism has only innate immune mechanisms with which to resist the virus, viral infection of corneal epithelial cells proceeds relatively unimpeded and during this stage some viral particles gain entry to neurites. As the infection proceeds and host immunity is garnered, the virus inside the neurites remains protected, is transported to the neuron cell bodies in the ganglia, and occasionally into the central nervous system where the virus remains relatively protected from host defense factors (72–74).

At some point during the establishment of the primary infection and entry of the virus into the nervous system, the HSV-1 enters a state of latency in selected neurons (71). During latency, it is presumed that there is little or no viral replication, production of viral glycoproteins, or expression of viral protein antigens on the latent cell membrane (72). Thus, latent HSV-1 resides in the neurons shielded from the

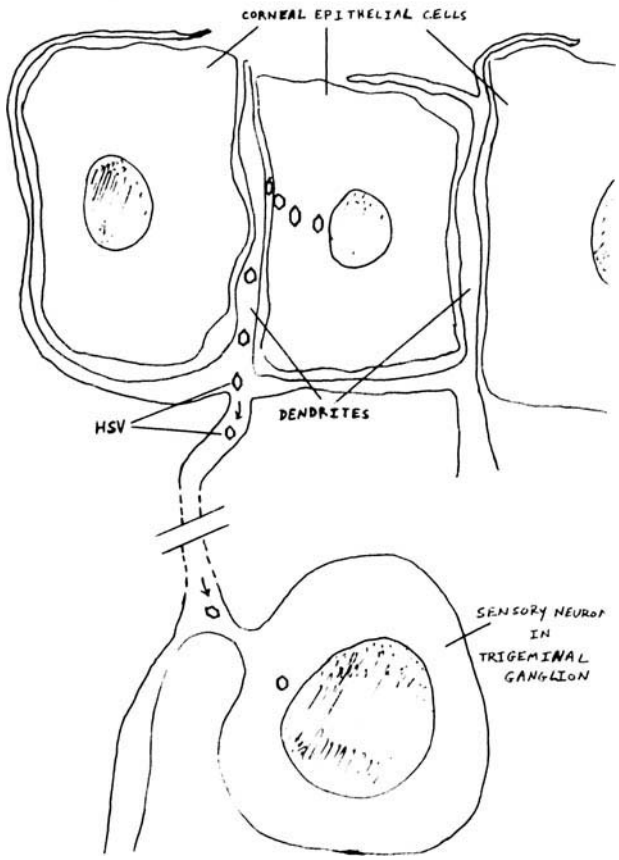

Figure 2. Schematic showing the magnified view of the relationship between sensory neurons in the trigeminal ganglion and corneal epithelial cells. Virus replicating in an epithelial cell enters the nerve terminals and is transported by retrograde axoplasmic flow to neurons in the trigeminal ganglion. The majority of the sensory neurons survive the entry of HSV-1 and serve as hosts for the viral genome during latency. Virus undergoing reactivation in a sensory neuron is transported back to the corneal epithelium by anterograde movement, where recurrent infection may occur.

hostile immune processes that would render it noninfectious and eliminate it from the host. Some investigators have proposed that the host immune response is necessary for the establishment of viral latency (75–77). Thus, the presence of a functioning B lymphocyte system and antiviral antibodies and/or the capacity of the infected organism to generate cell-mediated immunity are thought by some investigators to be necessary for the enforcement of the establishment of viral latency. However, since viral latency can be maintained in the neurons of mice deficient in both B and T cells, it is concluded by some that cellular transcription factors alone can enforce the latent state (72, 78–80).

Immunity, Latency, and Reactivation. Twenty years ago it was shown that HSV-1 could enter into a latent state in mice in the absence of a primary acquired immune response to viral antigens (81). Mice were simultaneously infected and given an injection of rabbit antiserum to HSV-1. The rabbit antiserum was cleared from the mice within 2 months, during which time the animals developed viral latency. These animals failed to mount a humoral antibody response and remained seronegative until the viral reactivation was induced. The viral latency was maintained in these animals for an indefinite period of time until reactivation was provoked by traumatizing the epithelial site originally infected. This study is one of the earliest to establish that an acquired immune response is not necessary for the establishment and maintenance of viral latency. In more recent experiments using immune deficient mice, it has been shown that HSV-1 can establish latency in ganglionic neurons of mice lacking functional B and T lymphocytes (78–80, 82). These studies clearly indicate that once HSV-1 is in the nervous system in neurons, it is relatively inaccessible to the host immune response. In this location, then, the virus can exist in a latent state without suffering immune destruction.

It should be possible to harmonize the seemingly conflicting results regarding the role of immunity in the establishment and maintenance of latency. For example, the report that antiviral antibody plays an important role in the maintenance of latent herpetic infections in the trigeminal ganglion and the latter studies that revealed that antibody had an important role in the neuroinvasiveness of HSV-1 seem to contradict the results showing that viral latency could be established in the absence of antiviral antibody (75, 76, 81). Furthermore, it was noted that latency was stable in the continued absence of host immunity (81). This latter finding, along with the more recent observations regarding the establishment of viral latency in ganglionic neurons in severe combined immune-deficient mice, suggests that a humoral immune response is not an absolute requirement for the establishment and maintenance of viral latency (78–80, 82). Harmonization of these seemingly conflicting results with recent findings indicating that CD8+ T lymphocytes are required for the maintenance of viral latency can be achieved by invoking a role for various cytokines in the establishment and maintenance of the latent state (83, 84).

As discussed elsewhere in this review, several cytokines, including the interferons and interleukin 6 (IL-6), are important mediators that affect primary acute viral infection of the eye and the establishment and maintenance of viral latency in the neural ganglia. Given the redundancy of various defense mechanisms in the vertebrate immune system, it seems reasonable to conclude from the experiments conducted to date using animals deficient in various immune factors, including antibodies, B lymphocytes, T lymphocytes, natural killer cells, and macrophages, that these immune-deficient animals have other innate immune mechanisms such as interferon and perhaps other cytokines that serve to protect the integrity of the organism so as to minimize the effect of viral infection. Thus, even in mice with severe combined immune deficiency, it is likely that interferons alpha and beta, and certainly IL-6 are produced in quantities adequate to modulate the spread and severity of a viral infection. It has been shown that there is a persistent cytokine response in the trigeminal ganglia of mice latent for HSV-1 (85–88). Cytokines such as IL-6 and various interferons bind to plasma membrane receptors, transducing signals into the neurons and other susceptible cells. One or more of the signal transduction events may render the cells resistant to viral replication. Similarly, cytokines and cellular transcription factors may act in concert to maintain viral latency and suppress viral reactivation. It may be that these cytokines and the mononuclear cells that produce them are responsible for the maintenance of viral latency by such a mechanism (Fig. 3). Maintenance of viral latency in neurons in mice with severe combined immune deficiency may well be regulated and maintained by a cascade of cytokines that act to maintain the latent state.

We conclude that the early literature on the role of cellular and humoral immune factors in regulating the establishment of viral latency and maintaining the virus in a latent state can best be appreciated in light of the recent findings regarding the central role of various cytokines in limiting the spread of the initial viral infection and in suppressing viral reactivation in latent neurons.

Strategies of Escaping Immune Detection by HSV-1

As part of the co-evolutionary process of HSV-1 and its host, the virus has developed a number of mechanisms to escape immune surveillance/recognition that, in part, explain its prevalence of the virus in society. Since HSV-1 is obligated for replication inside the host cell, the innate and cellular immune systems are primarily involved in eliminating the virus. One component of the cellular immune system responsible for viral clearance are cytotoxic T lymphocytes (CTLs) that recognize virally infected cells through viral peptides expressed in association with class I major histocompatibility complexes (MHC class I) on the surface of infected cells. However, HSV-1 downregulates MHC class I expression (89, 90) through the high-affinity binding of the HSV-1 immediate early gene product ICP47 to the trans-

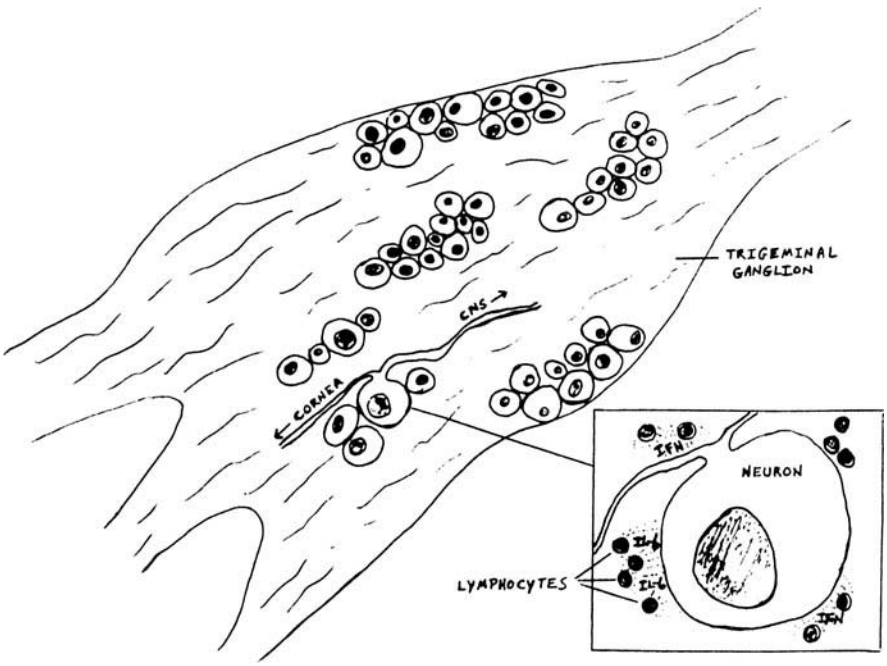

Figure 3. Schematic of the microarchitecture of the trigeminal ganglion. Clusters of neurons are scattered throughout the ganglion interspersed among the nerve fibers traversing through this tissue. During a primary infection, a ganglionitis composed of polymorphonuclear leukocytes (PMNs) and mononuclear cells, including lymphocytes, is seen. Once viral latency is established, the PMNs are no longer present, but many of the lymphocytes remain for months after the initial infection. Inset, magnified schematic of a sensory neuron around which are cytokine-producing lymphocytes, particularly T lymphocytes. Some of these lymphocytes produce cytokines, which help maintain the virus in a latent state. Viral reactivation may result from the loss of these lymphocytes in the ganglion and/or their discontinuation of production of the inhibitory cytokines.

porter associated with antigen processing molecule (91). This association prevents peptide interactions with the MHC groove, resulting in improper folding and exclusion of transportation across the endoplasmic reticulum (91). In nervous tissue, this process also occurs, but by an ICP47-independent mechanism (92). Even in those cells that still express MHC class I at levels that may be recognized and targeted by HSV-1-specific CTLs, the virus has developed additional means of evasion. Recent results suggest HSV-1-infected cells are resistant to CTL-induced, Fas-mediated apoptosis (93, 94). Coupled with the suppression of MHC class I expression, HSV-1 has evolved an elaborate means of escaping CTL detection and elimination of virally infected cells. HSV-1 has also been found to target destruction of activated $CD4^+$ T cells (95) and CTLs through induction of apoptosis via T cell receptor-mediated upregulation of CD95 (96). In addition, HSV-1-infected peripheral blood mononuclear cells have been found to secrete transforming growth factor β1 (TGF-β1) upon infection (97). Since TGF-β1 has been shown to downregulate IFN-γ-induced MHC class II expression (98) and MHC class II expression drives $CD4^+$ T cell activation, the presence of TGF-β1 would favor the virus and act as another means of eluding immune detection. Similar to cell-mediated immunity, the humoral immune system also plays a role in controlling HSV-1 infection (99, 100). Therefore, it is not surprising that HSV-1 had developed a means of antagonizing the humoral response by binding complement, which may ultimately lead to a reduced B cell memory response to the virus (101). While humoral and cell-mediated immunity participate in controlling HSV-1 replication, the activation of these immune pathways is delayed with the innate immune response taking up the bulk of the anti-viral blockade within the first two to three rounds of viral replication. Therefore, components of innate immunity, including type I IFNs, are natural targets for disruption by HSV-1-encoded proteins. Type I IFNs (IFN-α and -β) interfere with viral transcription, translation, and assembly of viral proteins (102). Recently, it has been found that HSV-1 ICP0 mutants are sensitive to the effects of type I IFN (103, 104) and resistance can be restored by supplying the ICPO in *trans* (Härle, Carr, and Halford, unpublished observation). Therefore, the ICPO-encoded protein counteracts the antiviral effect of type I IFNs by an unknown mechanism. Collectively, HSV-1 has evolved a number of strategies as countermeasures to the innate and adaptive immune responses to viral infections, allowing a sufficient amount of time for the virus to establish a latent infection and escape immune detection within the neuronal bodies of the sensory ganglion.

Immune Surveillance and HSV-1 Reactivation

Following the acute ocular infection of the host by HSV-1, the virus traffics to the sensory ganglion (trigeminal ganglion, TG) where latency is established in the neurons. During latency, the virus is thought to be quiescent, with no viral protein or transcript expression with the exception of a family of transcripts referred to as latency associated transcripts or LATs. Although there is debate as to whether the LAT gene family encodes (a) functional protein(s) (105), LAT expression antagonizes apoptosis-enhancing neuronal survival (106), which may increase the pool of latently infected neurons and facilitate viral reactivation (107). Such results are consistent with a study that found a direct correlation between the number of latently infected neurons and the potential for reactivation (108). Therefore, it seems probable that LAT is a key gene to be targeted for further study in advancing our understanding of latency and HSV-1 reactivation.

In the murine host during HSV-1 latency, it is difficult to identify any viral transcripts or proteins. However, numerous laboratories have shown a persistent immune response in the form of infiltrating cells and cytokine and chemokine expression during latency (86, 87, 109, 110), a time, by definition, when no viral transcript or protein is expressed, with the exception of LAT. Taken together, these observations suggest that LAT may be involved in the chronic induction of the immune system during times of viral latency. However, mice latently infected with a LAT null mutant dLAT2903 (doesn't express LAT transcripts) still show an elevation in cytokine and anti-HSV-1 titers during latency, indicating that the persistent expression of cytokine mRNA in the TG of latently infected animals is not due to the expression of LAT (111). Additional studies have been conducted to address the persistent immune response in the TG of HSV-1 latently infected mice. In one study, the antiviral compound acyclovir (inhibits viral DNA polymerase and causes chain termination) was used to orally feed mice latently infected with HSV-1. If viral gene transcription was active, acylovir would antagonize this process and potentially reduce the stimulus for the local, persistent immune response. In fact, in mice latently infected with HSV-1 and chronically treated *per os* with acyclovir, the expression of the cytokines (IFN-γ and TNF-α), as well as antiviral antibody titers, significantly decreased longitudinally over 120 days postinfection (112). These results were interpreted to suggest that the virus spontaneously or incompletely reactivates infrequently at levels below detection, but enough to provide a antigenic stimulus for a continued immune response during latency. To further assess the chronic nature of cytokine expression in the TG, an acyclovir-resistant HSV-1 mutant (termed KG111) was used to infect mice and establish a latent infection. A dose-response study established a concentration of 0.3 mg/ml in the drinking water of mice infected with the parental virus (KOS) efficiently blocked HSV-1 replication in the TG during the acute infection. However, this dose antagonized only 50% of viral replication in the mice infected with the acyclovir-resistant HSV-1 mutant, KG111 (Fig. 4). Using this same dose of acyclovir to treat mice latently infected with parental or mutant HSV-1, results show that the mRNA expression of a prevalent pro-inflammatory cytokine IL-6 was significantly reduced in the TG of parental, but not KG111 latently infected mice (Fig. 5). In addition, anti-HSV-1 antibody titers were not different in the acyclovir-treated mice latently infected with the acyclovir-resistant mutant KG111 compared with the untreated controls (Fig. 6). However, such titers were reduced in the parental latently infected mice treated with acyclovir compared with nontreated controls (Fig. 7). Collectively, one interpretation of these results is that viral replication does continue during latency and acts as a stimulus for cytokine synthesis.

The question remains as to how HSV-1 can reactivate in the sensory ganglion during a seemingly chronically active local immune response and reappear proximal to the original site of infection. For example, TNF-α and IL-6 produced by satellite cells (Schwann cells) and infiltrating immune cells are implicated in the control of acute HSV-1 infection (18, 113, 114) and are expressed during latency (115). Since these cytokines are expressed in the sensory ganglion during latency, it is predicted that HSV-1 may not successfully reactivate. In part, this conclusion is supported by data. Specifically, mice, unlike rabbits and humans, do not appear to spontaneously reactivate with the recovery of virus. Furthermore, there is evidence showing that expression of the pro-inflammatory molecules (IL-6 and TNF-α) in the sensory ganglion has been restricted to the murine host. Therefore, it is currently not known if a similar cytokine profile is exhibited in the TG of the human host latently infected with HSV-1.

In the murine model, the latent virus is induced to reactivate by environmental stressors such as temperature or ultraviolet light. One advantage of this model is that cellular or molecular events may be specifically correlated with the acute or latent infection, allowing investigators to identify and map potential mediators of reactivation. As an example, during the acute, but not latent infection, MHC class I

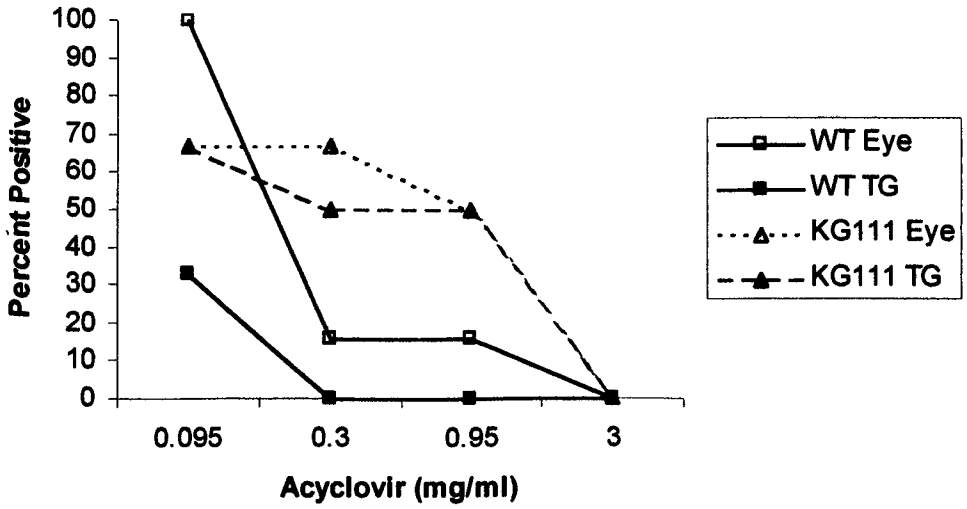

Figure 4. Acyclovir treatment reduces HSV-1 replication. Mice (n = 3–6/group) were infected with 1000 plaque forming units (PFUs)/eye of wild-type (WT) or mutant KG111 HSV-1 (KOS strain) following corneal scarification. At the time of infection, acyclovir was added to the drinking water at the indicated concentrations. Seven days postinfection, the mice were sacrificed and the eyes and trigeminal ganglia were removed and assayed for infectious virus by plaque assay.

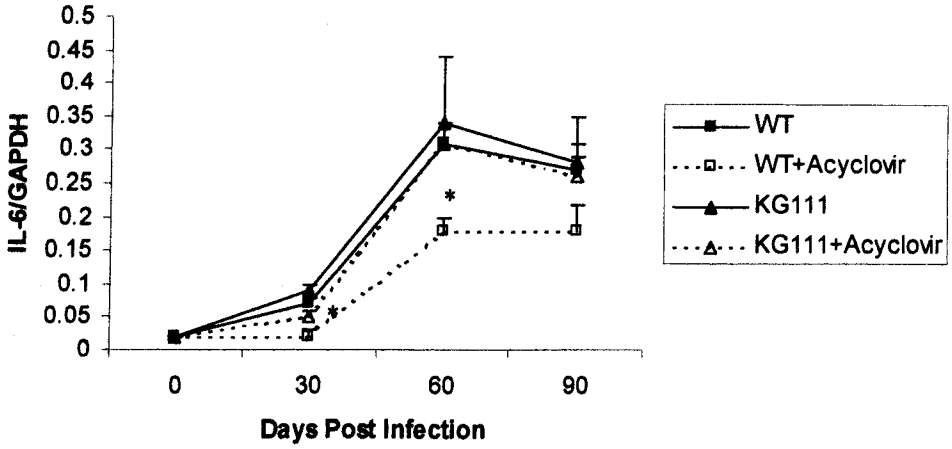

Figure 5. Acyclovir treatment reduces IL-6 expression in the trigeminal ganglion of latent HSV-1-infected mice. Mice (n = 3/group per time point) were infected with wild-type (WT) or acyclovir-resistant mutant (KG111) HSV-1 and treated with acyclovir (0.3 mg/ml) in the drinking water starting 14 days postinfection. Mice were sacrificed at the indicated time postinfection and the RNA from the trigeminal ganglia was processed and analyzed for IL-6 mRNA expression by reverse transcriptase-PCR. The data is presented as a ratio of IL-6 to the housekeeping gene, GAPDH. *$P < 0.05$ comparing the vehicle with acyclovir-treated WT group.

molecule expression is found on neurons and satellite cells (116, 117) facilitating the clearance of the virus by CD8 T cell recognition (83). Since MHC class I expression is reduced or absent during latency, such an occurrence may allow the virus to escape immune detection via $CD8^+$ T cells. Moreover, during reactivation along with TNF-α and IL-6 detection, IL-4 is also present correlating with a reduction in IL-2 and IFN-γ expression as the reactivation process evolves (115). IL-4 has previously been reported to exacerbate HSV-1 infection (118, 119), while IFN-γ has been associated with antagonizing reactivation (88). Taken together, these observations suggest that the expression of IL-4 during the reactivation cascade may reduce T_H1 cytokine production (including IL-2 and IFN-γ) and allow the virus to fully reactivate.

Other factors inevitably play a role in controlling HSV-1 reactivation, including $CD8^+$ T cells. For example, exposure of latently infected trigeminal ganglion cell cultures to an elevated temperature (43°C) for a brief period of time (10 min) eliminates the presence of $CD8^+$ T cells and induces viral reactivation in primary, latently infected mixed neuronal cell cultures (120). In a similar vain, anti-CD8 monoclonal antibody added to latently infected TG cultures has also been found to induce reactivation of quiescent virus (84). In support of these observations, adding lymphocytes to latently infected TG explant cultures at the initiation of culture results in a significant decrease in the percentage of cultures undergoing reactivation (121). By eliminating $CD8^+$ T cells from the mix, the antagonism is completely lost. Therefore, it would appear that $CD8^+$ T cells participate in blocking HSV-1 reactivation by an as yet unidentified mechanism. Since these studies have been conducted in a rodent model, it is presently unclear if such results can be applied to the human host.

As previously stated, IL-6 is a prevalent cytokine expressed during acute HSV-1 infection, latency, and reactivation. Within the central nervous system (CNS), astrocytes, but not microglia, are sensitive to HSV-1 infection as measured by plaque assay (Fig. 7), producing a significant amount of IL-6 compared with microglia following HSV-1 infection (Fig. 8). Consistent with previous reports (113, 114), the addition of neutralizing antibody to IL-6 enhances viral replication, suggesting that during an acute infection, IL-6 antagonizes HSV-1 infection (Fig. 9). However, the role of IL-6 during viral reactivation from latency is less clear.

Originally, a clinical trial using ciliary neurotrophic factor (CNTF) to treat patients with amyotrophic lateral sclerosis was found to frequently induce herpes labialis in patients receiving a high dose of the compound (122). Since

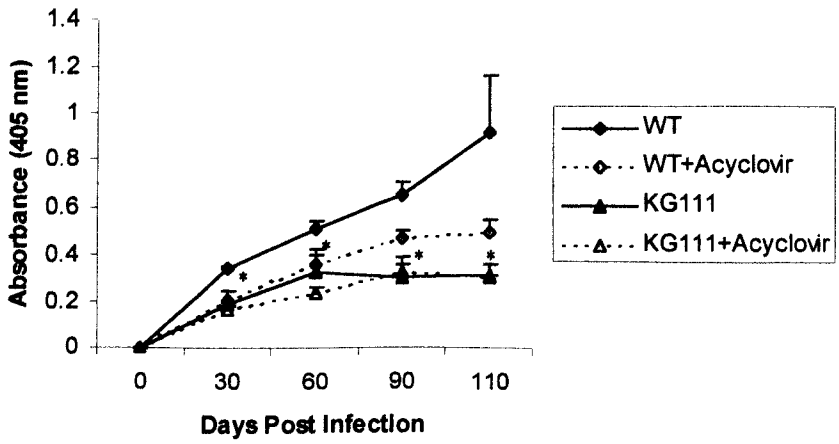

Figure 6. Acyclovir treatment reduces anti-HSV-1 antibody titer in wild type but not acyclovir-resistant mutant during latent infection. Mice (n = 3–7/group per time point) were infected with wild-type (WT) or acyclovir-resistant mutant (KG111) HSV-1 and treated with acyclovir (0.3 mg/ml) in the drinking water starting 14 days postinfection. Mice were sacrificed at the indicated time postinfection and serum samples were assessed for HSV-1 reactivity by ELISA. The results are expressed as absorbance. *$P <$ 0.05 comparing the vehicle with acyclovir-treated WT group.

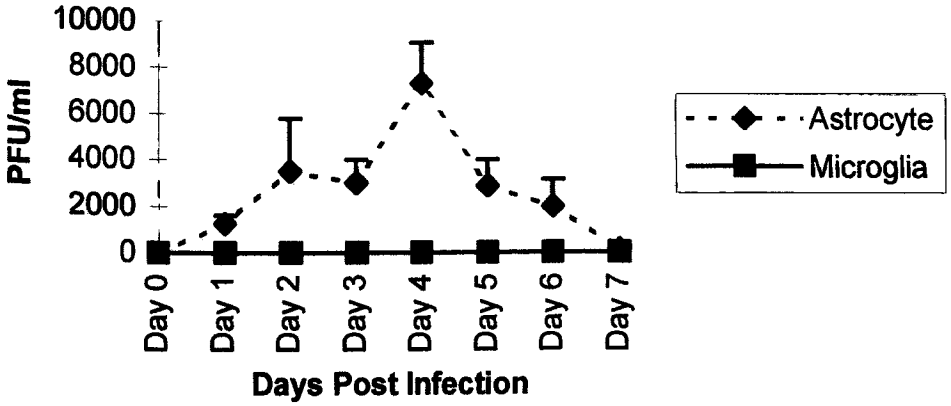

Figure 7. HSV-1 efficiently replicates in astrocytes, but not in microglia. Astrocytes (1×10^4 cells/culture) and microglia were infected with HSV-1 (McKrae strain, multiplicity of infection = 0.01) and the culture supernatants were assayed for infectious virus on the days indicated by plaque assay, using CV-1 cells as the indicator cell. The data are plotted as mean numbers of plaque forming units ± SEM. Values were derived from two experiments with triplicate cultures in each.

CNTF shares a common signaling cascade (gp130) with IL-6 following binding to its cell surface receptor, it was proposed that IL-6 might also elicit HSV-1 reactivation. To test this hypothesis, mice latently infected with HSV-1 and treated with neutralizing antibody to IL-6 were induced to reactivate using an ultraviolet light source. Mice treated with the anti-IL-6 antibody showed a significantly reduced level of ocular reactivation compared with control antiserum-treated mice (123). Upon further inspection, these investigators found a potential explanation for such an observation at the molecular level. Specifically, within the HSV-1 genome, consensus recognition sequences for cellular transcription factors associated with IL-6 receptor activation (namely, signal transducer and activator of transcription, STAT-3, and nuclear factor-IL-6, NF-IL-6) were identified within the inverted repeat regions associated with viral reactivation (124). Therefore, the virus was potentially capable of utilizing endogenous IL-6 levels produced by satellite cells of the peripheral nervous system or infiltrating leukocytes of the immune system to facilitate reactivation. Since the time of these initial observations, supporting and conflicting data have surfaced. In one study, antagonizing stress-induced elevations in TG IL-6 blocked HSV-1 reactivation (125). In another study, latently infected TG explant cultures treated with anti-IL-6 antibody showed a reduction in the frequency of reactivation (121). However, latently infected IL-6 knockout mice showed no difference in the frequency of viral reactivation in comparison with wild-type controls following ultraviolet light stimulation (114). One explanation for this discrepancy may reside in the redundancy of function within the IL-6 family of molecules, including CNTF, IL-11, and leukemia inhibitory factor that compensate for the absence of IL-6 in the knockout mice. Although it is interesting to note the unique and potential association between IL-6 and HSV-1, the current data only supports the maxim: *Guilt by Association.* Additional studies are warranted to more closely address the complex relationship between IL-6 and HSV-1 reactivation.

There is little doubt that during latency HSV-1 provides a stimulus for continued low level cytokine and chemokine production within the sensory ganglion harboring latent virus. The potential pathological manifestations that are a consequence of chronic cytokine production within the central or peripheral nervous system as a result of HSV-1 latency have not been addressed, but should be considered (126). In addition, given the recent data to suggest human herpes virus 6 contributes to the development of multiple sclerosis (127), it is tempting to speculate that HSV-1 may also contribute to neurological pathogenesis. To this end, a recent observation has shown cross-reactivity between HSV-1 antigens and the α-chain of the acetylcholine receptor (128).

Gene Therapy with Naked DNA Plasmid Constructs in the Eye: An Approach to Resolve HSV Infection?

Inherited and acquired diseases represent primary targets for this therapeutic approach and about 200 Recombi-

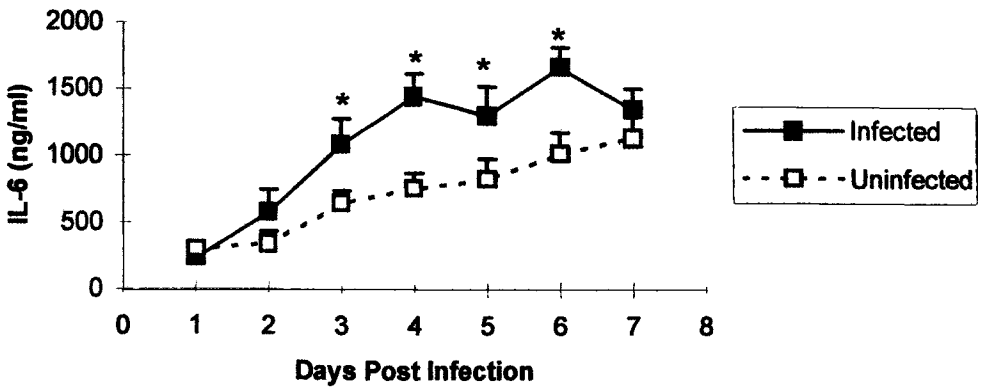

Figure 8. HSV-1-infected astrocytes secrete elevated levels of IL-6 compared to uninfected cells. Astrocytes (1×10^4 cells/culture) were infected with HSV-1 (multiplicity of infection = 0.01). Uninfected cells served as controls. The culture supernatants were collected daily and assayed for IL-6 levels by ELISA. The data are expressed as means ± SEM and were derived from three experiments with triplicate cultures for each time point. An asterisk indicates $P < 0.05$ comparing infected with uninfected cultures.

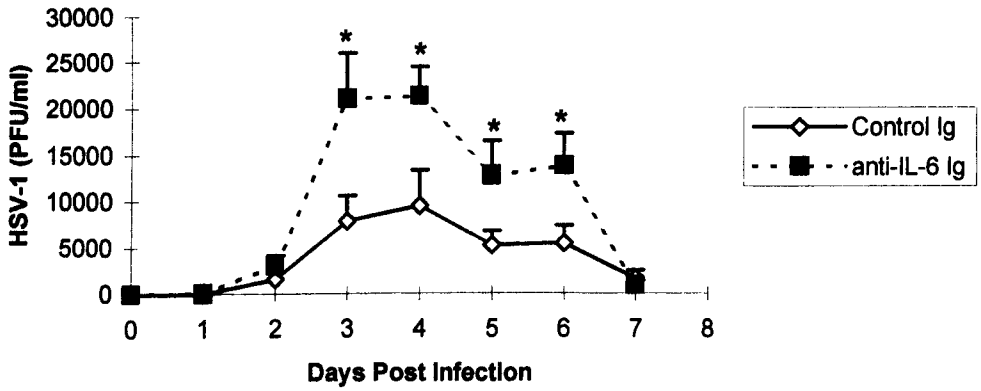

Figure 9. The addition of anti-IL-6 antibody augments HSV-1 replication in astrocyte cultures. Astrocytes (1×10^4 cells/culture) were infected with HSV-1 (multiplicity of infection = 0.01) and treated with anti-IL-6 or isotypic control immunoglobulin (Ig) (10 μg/culture). Supernatants were collected daily and assayed for infectious HSV-1 by plaque assay using CV-1 cells as the indicator cell line. The data are expressed as the means ± SEM of two experiments with four cultures at each time point. An asterisk indicates $P < 0.05$, comparing anti-IL-6-treated with Ig-treated astrocyte cultures.

nant DNA Advisory Committee- (RAC) approved clinical trials relating to gene therapy are under investigation. Protocols focus on the repair or replacement of mutated genes, the regulation of gene expression and signal transduction, the targeting of malignant cells for destruction, and the manipulation of the immune system with the development of new vaccination techniques. Viral vectors, including herpes viruses, adenoviruses, adeno-associated viruses, and retroviruses, are among the most commonly used gene delivery systems (129). However, cells are capable of taking up naked DNA and expressing their genes without the aid of transfer vectors. Technical questions regarding the appropriate vector or the choice of the most efficacious regulatory protein for each targeted disease still remain (130, 131). This implies that successful gene therapy requires not only the search for the best delivery system, but also requires a deeper understanding of the pathophysiology of the targeted disease.

Gene Therapy with Naked DNA Plasmid Constructs. Gene delivery with naked DNA is an accessible and cost-effective approach to delivering genes into somatic cells. The gene of interest is cloned into a plasmid vector that contains additional prokaryotic, eukaryotic, and viral sequences. The prokaryotic sequences are necessary for bacterial propagation, while the eukaryotic and viral sequences encode for promotors (e.g., cytomegalovirus and *Rous sarcoma* virus) and enhancers of transgene expression (e.g., SV40- and bovine growth hormone-polyadenylation signal, Kozak sequence) (129, 132). Prior to transgene expression, naked plasmid constructs are internalized by cells. This step occurs with low efficiency, but can be enhanced by chemical (e.g. $CaPO_4$-DNA precipitation, complex formation with negatively charged organic polymers, and others) and physical means (gene gun, electroporation, and microinjection of DNA directly into cell nuclei). Many enhancers of transfection efficiency induce a cellular response themselves, indicating appropriate controls are required to specifically identify transgene effects (133).

A crucial consideration in naked DNA delivery is the route by which the plasmid construct is administered. Considering the treatment of a local disease, e.g., ocular HSV infection and the low transfection efficiency of naked DNA, the administration of the transgene at the site of infection is warranted to obtain maximum effect. However, studies have shown the local administration of plasmid DNA is expressed systemically (134). Locally administered transgenes are taken up by scavenger receptor-mediated pathways (135, 136) or polyanion-defined receptors (137) on targeted cells, preventing DNA degradation. In contrast, intravenous injection of naked DNA does not exhibit a systemic transgene expression pattern due to dilution of the transgene and rapid clearance from the blood stream by the reticuloendothelial system (129). Another efficient technique of administering plasmid DNA is via intramuscular injection, which tends to reduce local inflammation (138, 139) and has been proven effective against cytomegalovirus (CMV) (140).

Treatment of Ocular HSV-1 Infection with Naked DNA Vectors Encoding for Cytokines. The use of gene therapy has been studied in numerous diseases including cancer therapy, metabolic disorders, and various infectious diseases (3), including HSV-1. Antiviral agents including acyclovir, gancyclovir, penicyclovir, cidovir and foscarnet are effective in controlling the acute infection and continuous antiviral medication has been shown to significantly reduce the rate of recurrent stromal keratitis (141). Unfortunately, after ending the prophylaxis the recurrence rate raises to levels of placebo-treated patients. In addition to these observations, reports of resistance to the above mentioned antiviral drugs are accumulating (142, 143). These entities led to the recent attention of gene therapy for this disease.

One of the first studies evaluating cytokine gene therapy against ocular HSV-1 infection and pathology applied naked DNA encoding IL-10 into mouse eyes showing HSV-1 induced lesions (144). A one-time administration of IL-10 DNA resulted in reduced ocular pathology. The rationale for this therapeutic approach was the observation that tissue destruction was linked to T_H1 cytokines IL-2 and IFN-γ (53) and animals with spontaneous resolution of HSV-1 lesions revealed elevated T_H 2 cytokines, e.g., IL-10

(145). IL-10 is known to inhibit the activation and cytokine production of $CD4^+$ lymphocytes (T_H1-subtypes) and PMNs resulting in decreased immunopathology in the cornea. However, the reduction of type 1 cytokines does not reduce viral titers and does not inhibit the establishment of latency.

Therefore, a second approach was undertaken to inhibit viral replication and with this prevent the establishment of latency. To this end, previous results had identified (directly or indirectly) type I IFNs (i.e., IFN-α and IFN-β) to be potent inhibitors of viral replication during ocular HSV-1 infection (17, 146).

Based on these observations, a plasmid construct encoding for murine IFN-α1 (pCMVβ-IFN-α1) was constructed and administered onto mouse corneas prior to ocular HSV-1 infection. Transgene expression markedly reduced HSV-1 load and viral gene expression in the eye and TG, which correlated with a reduction in immune cell infiltration in cornea and iris (147, 148). In addition, pCMVβ-IFN-α1-transfected eyes induced a 5-fold increase in MHC class I mRNA expression over vector-treated controls, implying the local expression of the transgene product (148). The depletion of $CD4^+$ or $CD8^+$ T-lymphocytes completely abrogated resistance to ocular HSV-1 infection induced by the IFN-α transgene. These results imply that not only innate, but also adaptive immune defense mechanisms play a role in the efficacy of the IFN-α transgene. However, only the local application of the IFN-α transgene at the ocular site conferred protection against HSV-1 mortality, whereas administration at other mucosal sites, including intranasal or intravaginal, did not (148). Similar results were also noted for treatment of ocular herpes with a murine IFN-β transgene (149). Unfortunately, type I IFN naked DNA therapy only has been found to be effective if the plasmid is administered 24 hr prior to or after ocular infection. Due to the limitations of the present therapy, further studies are underway to identify additional type I IFN constructs that are effective against ocular HSV-1, as well as to characterize gene delivery systems to optimize transgene expression.

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