Host Interferon: a Silent Partner in the Regulation of Herpes Simplex Virus Latency

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Abstract
Herpes simplex virus (HSV) establishes latent infections as a consequence of a non-cytolytic immune response that represses HSV replication, but fails to destroy neurons that harbour HSV’s genetic material. It has become increasingly evident that, in both mice and men, the host interferon system plays a critical role in tipping HSV’s latency-replication balance in favour of latency. HSV can resist interferon-induced repression provided that HSV’s two interferon antagonists, ICP0 and ICP34.5, are synthesized. Failure to synthesize either protein renders HSV interferon-sensitive and prone to establishing latent infections. Intriguingly, ICP0 and ICP34.5 are encoded within HSV’s latency-regulating R1 regions. We propose that differential synthesis of ICP0 and ICP34.5 may endow HSV with the capacity to ‘choose’ between latency and replication. HSV may choose to establish a latent infection by downregulating ICP0 or ICP34.5, and render itself sensitive to the interferon-induced antiviral state. Conversely, synthesis of ICP0 and ICP34.5 may ensure that HSV resists interferon-induced repression and completes another cycle of replication.

Prologue
Most virus families such as the picornaviruses are similar enough in genetic content to be meaningfully discussed as a group. This is not the case with the herpesviruses. The >130 known herpesviruses employ a conserved set of ~40 enzymes and structural proteins to synthesize virions that are indistinguishable from one another (Davison, 2002; McGeoch et al., 2006). All herpesviruses appear to establish life-long infections in animals that oscillate between relative states of latent versus productive infection. Beyond this, the similarities quickly disappear. For example, herpes simplex virus establishes life-long infections in neurons using a complement of ~75 genes, whereas cytomegalovirus relies on ~200 genes to establish life-long infections in monocytes (Murphy et al., 2003). This review will focus on herpes simplex virus such that the biology of a single herpesvirus may be considered in some depth. In particular, herpes simplex virus 1 (HSV-1) is one of the easiest human herpesviruses to study because it replicates to high titres, is amenable to genetic manipulation and recapitulates most aspects of human infection in animal models. Thus, HSV-1 provides a powerful system to explore the many gaps in knowledge that exist at the interface between virology and immunology.

In recent years, it has become apparent that host interferons play a more pivotal role in the biology of HSV infections than was previously recognized. The central tenet of the current review is that HSV exploits the host interferon system as a negative-feedback mechanism that controls the timing of viral exit from the productive cycle of replication. The intensity of interferon-inducible repression of HSV replication varies over several orders of magnitude (Halford et al., 2005a). Thus, when host interferon signalling exceeds a critical threshold, it is possible that HSV’s interferon antagonists (ICP0 and ICP34.5) may fail to accumulate such that HSV is rendered sensitive.
to repression by the interferon response. Such an interferon-dependent shutoff mechanism might endow HSV with the capacity to cease all viral protein synthesis just as CD8+ T-cells are preparing to destroy HSV antigen+ cells. Unlike cytolysis by T-cells, interferon-inducible repression may be reversible. Once the local immune response wanes, a subset of HSV-infected neurons may re-enter the productive cycle of HSV replication weeks to months later.

It is unlikely that the principles discussed herein are unique to HSV. Rather, the opposing forces provided by interferon-dependent repression and virus-encoded interferon antagonists may be exploited by many herpesviruses to regulate their oscillations between latent and reactivated infections. Likewise, most persistent viral infections may be described as a balance, or equilibrium, that is achieved between the host immune response and a virus that avoids clearance by this host response.

Students are generally familiar with the concept that a process that is static to the eye may, in fact, be controlled by two equal and opposing forces. Therefore, we wish to suggest that students might grasp the biology of persistent viral infections more readily if these processes were described in terms of an equilibrium between virus and host. Aside from the simplicity of the approach, it accurately reflects the weight of evidence that the host immune system plays an integral role in restricting the spread of most, if not all, persistent viral infections. Below, we describe the in vivo biology of HSV infections in such terms.

**Introduction**

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) are similar viruses that share a nearly identical set of ~75 co-linear genes. HSV-1 and HSV-2 produce a similar spectrum of disease. In this review, the terms HSV-1 and HSV-2 will be used to denote specific viruses when summarizing the results of a particular study. However, because the biology of HSV-1 and HSV-2 infections are similar in principle, the term ‘HSV’ will be used wherever possible to indicate that the principles under discussion are probably relevant to both HSV-1 and HSV-2.

In cultured cells infected with HSV, ~75 viral proteins are synthesized in a temporal cascade during productive replication (Honess and Roizman, 1974). Virion protein 16 (VP16) in the tegument of HSV virions forms a complex with the cellular transcription factor Oct 1 to initiate a cascade of viral gene expression (Triebenstern et al., 1988). Only five immediate-early (IE) genes are initially induced based on the presence of VP16-responsive elements in their promoters. Viral IE proteins such as infected cell protein 0 (ICP0) and 4 (ICP4) are thought to activate viral genes, which encode ~70 early (E) and late (L) viral proteins that form the machinery necessary for replicating and packaging HSV genomes into new virions. The IE → E → L model of HSV gene expression summarizes the series of events that lead to HSV virion synthesis (Honess and Roizman, 1974). However, this linear depiction of HSV gene expression does not address one fundamental question that has intrigued scientists for decades, ‘How does HSV ‘choose’ whether infection of a cell will be productive or latent?’

Most reviews of HSV latency tend to focus on the molecular biology of HSV gene expression, and often overlook the fact that latent HSV infections are the consequence of a host immune response to HSV. This is not to say that considerations of HSV gene regulation are irrelevant. Rather, it is important to appreciate that entry of HSV into a latent state occurs in at least two steps: (1) generation of a repressive stimulus that influences whether or not HSV replication will stall, and (2) silencing of HSV gene expression. The available evidence indicates that the host immune system is the primary source of the ‘repressive stimulus.’ In contrast, the effector mechanisms that silence HSV during latency remain unclear, and thus remain an ongoing area of research (Knipe and Cliffe, 2008; Bloom et al., 2010; Perng and Jones, 2010).

Latent HSV infections appear to exist as a dynamic equilibrium that is controlled by the opposing forces of (1) viral activators and (2) host immune repressors of HSV infection (Fig. 5.1A). HSV virology and HSV immunology have largely been studied in isolation from one another for the past 40 years. However, a holistic explanation of HSV latency will require the balanced...
Figure 5.1 An equilibrium model of the latency-replication balance of HSV. (A) Schematic of a human trigeminal ganglion. The ophthalmic and maxillary branches collect nerve fibres from the eye and face, respectively. The lower, mandibular branch collects nerve fibres from the mouth and is the predominant site of latent HSV-1 infection in humans. Neuronal cell bodies collect sensory inputs from the epithelium and relay signals to the brain. A single HSV latently infected neuron is illustrated as a large blue cell surrounded by smaller, red CD8+ T-cells. In the schematic to the right, it is proposed that HSV infections of neurons are controlled by the opposing forces of host interferons (red) and viral activator proteins (blue). Innate interferons (α/β) and CD8+ T-cell-secreted interferon-γ are proposed to favour the establishment of latent HSV infections. Viral activator proteins ICP0 and ICP34.5 are proposed to favour viral spread by dismantling two interferon-inducible blocks to HSV replication. Adapted from the Netter Anatomy Illustration Collection© (Netter, 1997) with permission from Elsevier Inc. All rights reserved. (B) Schematic cross-section of a trigeminal ganglion showing a single, HSV latently infected neuron (blue cell) surrounded by CD8+ T-cells (red cells) that have infiltrated the tissue as part of the host response to viral infection. Green lines in the nucleus of the HSV latently infected neuron represent the HSV latency-associated transcript (LAT) RNAs that may be detected in the HSV latently infected ganglia of experimental animals and humans. A colour version of this figure is located in the plate section at the back of the book.

consideration of both the viral activators and immunological repressors that control how HSV alternates between a silent and reactivated state in vivo (Fig. 5.1A).

Reviews of HSV latency that consider only the virus are akin to teaching someone to drive an automobile while focusing solely on the gas pedal (blue arrow in Fig. 5.1A). The accelerator is important because it explains how HSV-encoded activators allow the virus to undergo rapid amplification and spread in an infected animal. However, such virus-focused explanations imply
that withdrawal of pressure on HSV’s accelerator (i.e. lack of viral activators) is adequate to explain how the spread of HSV infection stops in vivo. This is analogous to suggesting that a commuter may safely stop a car during rush hour by simply removing their foot from the gas pedal. As with the commuter who attempts this method of deceleration, such explanations of HSV latency tend to crash upon scrutiny.

Immune-based explanations of HSV latency are akin to teaching someone to drive an automobile while focusing solely on the brake pedal (red arrow in Fig. 5.1A). The braking mechanism is important in explaining how HSV replication stops in vivo when T-cells infiltrate an HSV-infected tissue (Fig. 5.1B). If the mechanisms that controlled HSV latency simply involved the degree of pressure on the brake pedal (i.e. magnitude of the T-cell response), then latent HSV infections would never re-enter the productive cycle of replication. However, 2% of the human population is shedding HSV at any given time (Buddingh et al., 1953; Wald et al., 1997; Miller and Danaher, 2008).

Both an accelerator and braking mechanism are required to explain how HSV oscillates between latent and active infections over the lifetime of a human host. Against this background, it is our goal to review the literature that indicates (1) host interferons are key components in the braking mechanism, and (2) HSV-encoded interferon antagonists, ICP0 and ICP34.5, are key components in the accelerator that allows HSV to periodically overpower the immune system and re-enter the productive cycle of replication (Fig. 5.1).

The biology of latent HSV infections

HSV infections in humans and experimental animals

Human carriers of HSV-1 or HSV-2 frequently shed infectious virus in their mouth or genital region, and this commonly occurs in the absence of symptoms (Buddingh et al., 1953; Wald et al., 1997; Miller and Danaher, 2008). When transmitted to a naïve person, HSV replicates in the epithelium at the point of entry, which is typically the mouth or genitals (Fig. 5.1A). Over a 1- to 3-day period, HSV may undergo a 100- to 10,000-fold amplification in the epithelium, and spread to nerve endings. Upon HSV infection of nerve endings, nucleocapsids are carried by retrograde transport through axons to neuronal cell bodies several centimetres away in the innervating ganglia (Fig. 5.1A). Hence, HSV infection of the mouth is quickly followed by HSV infection of the trigeminal ganglia whose neurons innervate the mouth, eyes, and face. In contrast, dorsal root ganglia coming off the lower backbone house peripheral neurons that innervate the anogenital region. Hence, HSV-2 infection of the genital epithelium results in HSV-2 infection of dorsal root ganglia (Stanberry et al., 1982).

Within 3 weeks of infection, sites of productive HSV replication become exceedingly rare and the infection is said to be ‘latent.’ Empirically, a latent HSV infection simply means that HSV DNA persists in a tissue in the relative absence of infectious HSV virions. The phrase ‘relative absence’ is important to bear in mind, because the sensitivity of detecting infectious HSV in latently infected animals and humans is poor, and tends to underestimate the actual frequency of spontaneous HSV reactivation (Wald et al., 1997).

During a latent infection, the LAT gene is the only locus in the HSV genome that routinely produces high levels of latency-associated transcript (LAT) RNA (Fig. 5.2). LAT-specific riboprobes may be hybridized to nucleic acids present in trigeminal ganglia to reveal the presence of LAT+ neurons, which are a common feature in HSV latently infected ganglia harvested from experimental animals or human cadavers (Stevens et al., 1987, 1988; Fig. 5.1B). LAT RNAs are stable 1.5 and 2.0 kb RNA introns that are derived by differential splicing from a common primary transcript (Farrell et al., 1991). The LAT gene lies in the long-repeated (RL) regions of HSV’s genome, which appear to regulate the balance between HSV latency and replication (Fig. 5.2). The LAT introns that accumulate in HSV latently infected neurons share an ~750-bp antisense overlap with the ICP0 gene (Fig. 5.2) whose IE protein strongly promotes HSV reactivation (Halford and Schaffer, 2001; Halford et al., 2001). Thus, numerous hypotheses including LAT-derived microRNAs
have been offered to explain why HSV’s LAT gene should share >70 turns of a DNA double-helix with the reactivation-promoting ICP0 gene (Tang et al., 2008; Umbach et al., 2008). While non-coding LAT RNAs provide a useful marker for identifying HSV latently infected neurons (Fig. 5.1B), their function in the HSV life cycle has not been clearly established (Fraser et al., 1992; Bloom, 2004).

How often does HSV spontaneously establish latent infections in neurons?

Stevens and Cook (1971) established that neurons were the long-lived reservoir of latent HSV infections. During the acute phase of a primary HSV infection, homogenization of the innervating ganglia reveals the presence of large quantities of infectious virions. However, one month later, infectious virions are rarely detectable in HSV latently infected ganglia (Stevens and Cook, 1971). When HSV latently infected ganglia are removed from animals and placed in cell culture medium for one week, this physiological change causes latent HSV to reinitiate the de novo synthesis of new infectious virions (Stevens and Cook, 1971). The study of Stevens and Cook (1971) was groundbreaking in pinpointing the site of latent HSV infections, and in establishing an experimental system that provided the first means to study how HSV infections may alternate between a latent and reactivated state in bona fide latently infected neurons.

Because neurons serve as the reservoir of latent HSV infections, the unique biology of neurons is often suggested to hold the key to explaining how latent HSV infections are established (Roizman et al., 2005; Knipe and Cliffe, 2008). Several neuron-specific repressors including Oct-2 (Lillycrop et al., 1991; Latchman, 1996), Zhangfei (Akhova et al., 2005; Misra et al., 2005), and CoREST (Roizman et al., 2005) have been proposed to explain why HSV establishes latent infections in neurons. Such explanations are based upon the assumption that neurons are inherently refractory to HSV replication, and thus HSV establishes latent infections immediately upon entering neurons. This assumption is inconsistent with the available evidence, which is summarized as follows.

If epithelial cells and neurons intrinsically differed in their propensity to support productive HSV replication, then these differences should be reflected in the quantity of infectious HSV that is recovered from the epithelium versus the
innervating ganglia during a primary HSV infection (Fig. 5.1A). However, when animals are inoculated in the cornea with HSV-1, robust viral replication is first observed in the eyes on days 1 and 2 post inoculation (p.i.), and this is followed by equally robust HSV replication in trigeminal ganglion neurons between days 3 and 5 p.i. (Knotts et al., 1974; reviewed in Rawls, 1985).

HSV encodes its own DNA nucleotide biosynthetic enzymes, including thymidine kinase, which are strictly required for HSV to replicate in neurons (Coen et al., 1989). HSV thymidine kinase-null viruses replicate efficiently in cultured cells and in the epithelium of animals, which produce their own deoxynucleotide triphosphates (dNTPs). However, HSV thymidine kinase-null viruses fail to replicate efficiently in terminally differentiated neurons that do not synthesize their own dNTPs. Consequently, HSV thymidine kinase mutants are avirulent in animals because they fail to replicate in neurons, and fail to use peripheral nerve fibres as a conduit of spread (Coen et al., 1989; Valyi-Nagy et al., 1994b).

The relative efficiency with which HSV establishes latent infections in neurons upon its initial entry into the trigeminal ganglia may be determined by comparing the relative abundance of LAT+ neurons versus HSV antigen+ neurons during the acute phase of HSV infection. If HSV established latent infections immediately upon entry into the nuclei of neurons, then a predominance of LAT RNA+ neurons should be observed shortly after inoculation of the eye. Between 3 and 5 days p.i., some HSV LAT RNA+ neurons are indeed observed in the trigeminal ganglia (Table 5.1). However, HSV antigen+ neurons (i.e. sites of viral replication) are ~100-fold more abundant than HSV LAT RNA+ neurons in the trigeminal ganglia at early times after inoculation of mice (Halford et al., 2006; Table 5.1).

The unique biology of neurons is insufficient to explain how latent HSV infections are established in vivo. In contrast, the unique biology of neurons appears to be critically important in explaining (1) why LAT RNAs only accumulate in neurons (Stroop et al., 1984; Stevens et al., 1987), (2) why neurons are the only cell type in which latent HSV genomes are maintained over time (Sawtell, 1997), and (3) why neurons are the only cell type from which latent HSV infections are successfully reactivated (Halford et al., 1996a; Halford and Schaffer, 2001).

<table>
<thead>
<tr>
<th>Mice</th>
<th>LAT+ neurons</th>
<th>HSV Ag+ neurons</th>
<th>LAT+ neurons</th>
<th>HSV Ag+ neurons</th>
</tr>
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<tbody>
<tr>
<td>Wild-type</td>
<td>0.02–0.04% (12)</td>
<td>7–15% (4399)</td>
<td>0.1–0.2% (45)</td>
<td>9–18% (5300)</td>
</tr>
<tr>
<td>rag2−/−</td>
<td>0.02–0.04% (12)</td>
<td>10–20% (6122)</td>
<td>0.1–0.3% (82)</td>
<td>18–36% (10897)</td>
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Mice were inoculated with 10⁵ pfu per eye of wild-type HSV-1 strain KOS, and were sacrificed 72 hours (day 3) or 132 hours (day 5.5) after inoculation. Neurons in sections of HSV-infected trigeminal ganglia that hybridized with a latency-associated transcript (LAT)-specific riboprobe and which were HSV antigen-negative, as determined under a fluorescent microscope. Neurons in sections of KOS-infected trigeminal ganglia that were labelled by rabbit anti-HSV antibody and which were LAT-negative, as determined under a fluorescent microscope. The estimated per cent of neurons that were LAT+, or HSV antigen+, is based on the total number of neurons counted (number shown in parentheses) in a total of 144 TG sections derived from 6 independent TG (i.e. 24 sections per TG were analysed). It was estimated that this number of sections contained at least 30,000 neurons but not more than 60,000 neurons.

This table was adapted from Halford, et al. (2006) in accordance with the open-access policy of BioMed Central.
T cells and latent HSV infections: inseparable partners
In animals that lack T-lymphocytes, wild-type HSV fails to exit the productive cycle of replication and HSV infections uniformly spread to the central nervous system, producing a fatal encephalitis (Oakes, 1975; Walz et al., 1976; Nagafuchi et al., 1979). Thus, the establishment of latent HSV infection is experimentally inseparable from the host immune response that represses productive HSV replication in the peripheral nervous system (Fig. 5.1B). This correlation does not prove that immunological repression is the mechanism by which HSV latency is established (Tenser et al., 1993; Gesser et al., 1994). However, the hypothesis that the host immune response is causally related to the establishment of latent HSV infections is based upon more than lethal outcomes of HSV infection in immunodeficient mice.

The strongest support for this hypothesis is derived from the pioneering studies of Simmons and Tscharke (1992) whose work focused attention to the possibility that CD8+ T-cells might be required to suppress HSV replication in neurons (Fig. 5.1B). Simmons and Tscharke asked a simple question that yielded powerful results: ‘Do CD8+ T-cells influence outcomes of HSV-1 infection in ganglionic neurons?’ In immunocompetent mice inoculated in the left flank, HSV-1 replication peaked in the innervating dorsal root ganglia by day 5 p.i., at which time 13% of neurons were HSV antigen+. On day 8 p.i. in immunocompetent mice, only rare dorsal root ganglion neurons could be found that were HSV antigen+. In contrast, HSV antigen+ neurons were ~50-fold more abundant on day 8 p.i. in the dorsal root ganglia of mice depleted of CD8+ T cells (Simmons and Tscharke, 1992).

The relative frequency of neurons that survived HSV infection was compared in dorsal root ganglia harvested on day 8 p.i. Relative to uninfected ganglia, immunocompetent mice were estimated to have lost no more than 0.5–3.5% of ganglionic neurons, despite the fact that 13% of neurons were HSV antigen+ on day 5 p.i. (Simmons and Tscharke, 1992). In contrast, on day 8 p.i., CD8-depleted mice were estimated to have lost 36% of neurons and gross pathology was observed in their dorsal root ganglia.

The findings of Simmons and Tscharke established the first direct link between CD8+ T-cells and the establishment of latent HSV infection in neurons. Specifically, CD8+ T-cell infiltration of ganglia appeared to be necessary to trigger the cessation of HSV replication in ganglionic neurons (Fig. 5.1B). Moreover, failure to recruit CD8+ T-cells into ganglia in a timely manner resulted in the rapid and widespread destruction of ganglionic neurons, as HSV infection spread unchecked through the peripheral nervous system. Subsequent studies corroborated that T-cell-dependent repression of HSV replication did not compromise the health of neurons, but rather precipitated an extraordinarily rapid clearance (turnover) of HSV antigens in neurons over a span of just 12–16 hours (Speck and Simmons, 1998).

Host response to HSV infections
Before discussing those aspects of the host immune response most critical to the establishment and maintenance of latent HSV infections, we consider the larger biology of the immune response to HSV infection. This is not a comprehensive treatment of the topic, but rather is meant to provide the non-immunologist a context for appreciating the types of molecules and cells that are called into action when the body responds to an HSV infection. For a more thorough consideration of the host immune response to HSV, we refer the reader to several excellent reviews on the topic (Carr and Tomanek, 2006; Sheridan et al., 2007; Cunningham et al., 2008; Koelle and Corey, 2008).

Innate immune response to HSV infections
HSV infects and destroys epithelial cells. Cell death disrupts the integrity of a tissue such as the epithelium, and this disturbance initiates a healing response. The first phase of healing is the scavenging of cellular debris by acute inflammatory cells such as polymorphonuclear leucocytes (PMNs) and long-lived professional antigen presenting cells (APCs). In the process of phagocytosing dead cells, PMNs and professional APCs secrete an array of chemokines and cytokines, which enhance the inflammatory
process causing capillaries to become leaky, and promote the movement of immune mediators out of the vasculature and into the site of tissue damage (Watanabe et al., 1999; Cook et al., 2004; Li et al., 2006). Professional APCs (MHC class II+ cells) circulate through such damaged tissue, gather antigen samples, return to draining lymph nodes, and there present these antigens to T- and B-lymphocytes (Allan et al., 2006; Heath and Carbone, 2009).

During this process of infection and repair, pro-inflammatory cytokines such as interleukin (IL)-1 and tumour necrosis factor (TNF)-α and chemokines such as CXCR-3, CXCL-10, and IL-8 are critical in drawing leucocytes out of the bloodstream to sites of viral replication and tissue damage (Miyazaki et al., 1998; Thapa and Carr, 2008; Wuest and Carr, 2008). IL-1 and TNF-α promote inflammation by causing local capillaries to become leaky and sticky for leucocytes. Leukocyte adhesion molecules upregulated on capillaries cause blood leucocytes to stick to the inner capillary walls, and diapedesis may occur, whereby leucocytes crawl between cells of the blood vessel wall and into the inflamed tissue. Chemokines are critical in establishing the gradient of molecules that leucocytes follow to their source, the site of viral infection and tissue damage.

Much has been claimed about the role of natural resistance and natural killer cells in host defence against HSV infections (Lopez, 1975; Biron et al., 1989). The evidence that supports these claims is equivocal, and is discussed elsewhere (Halford et al., 2004, 2005b). Scores of chemokines and cytokines elicited by the immune system are critical to leucocyte migration, differentiation, and the proliferation of lymphocytes that culminate in a robust adaptive immune response to viral infection (Salazar-Mather and Hokeness, 2003).

Aside from their role as inhibitors of viral replication, host interferons play an immunoregulatory role, which promotes the proliferation of CD4+ T-cells that promote cell-mediated immune responses. Moreover, the second immunoregulatory function of interferons lies in the potent upregulation (10- to 20-fold increase) of MHC class II molecules on macrophage, dendritic cells, and B-lymphocytes. This enhances the vigour of the anti-viral immune response, as MHC class II molecules are the vehicle by which 15–30 amino acid peptide antigens are presented to CD4+ T-helper lymphocytes. CD4+ T-cells act in turn to promote the proliferation and differentiation of effector CD8+ T-lymphocytes and B-lymphocytes that generate the adaptive immune responses that control HSV infection.

Adaptive immune response to HSV infections

B-lymphocytes

Each bone marrow-derived lymphocyte (B-cell) in a draining lymph node is developmentally committed to produce a particular class (or isotype) of antibody, and this antibody can only bind a single, unique antigenic epitope. In terms of protein antigens, the antigen-binding site of an antibody typically accommodates 5 or 6 amino acids derived from a single protein. These amino acids may be continuous in the polypeptide chain of the antigen, but are often discontinuous amino acids derived from 2 distinct polypeptide loops that converge at a single point on the surface of the protein antigen.

Temporally, naïve B-cells always produce IgM isotype antibody first because generation of other antibody isotypes requires (1) cytokine help from CD4+ T-lymphocytes (e.g. IL-4), and (2) the downstream event of genetic rearrangement of the B-cell receptor heavy chain gene. Antibody class switching involves deletion of a 5′ exon that encodes the IgM heavy chain, and this deletion event commits the B-cell to usage of an alternative 3′ exon that encodes the IgA, IgE, or IgG heavy chains. Thus, HSV-specific IgM antibody is measurable in serum 3–5 days into a primary HSV infection, whereas at least 7 days are required before HSV-specific IgG antibody begins to accumulate.

The IgM antibody is a 750kDa pentameric molecule, which is largely confined to the vascular compartment because of its large size. IgM antibody is critical in host defence against many bacteria, particularly bacteria capable of colonizing the bloodstream. HSV-specific IgM antibodies are a useful diagnostic indicator of primary HSV infections. HSV-specific IgM antibodies do not
play a major role in host defence against HSV infections, as IgM antibodies fail to diffuse into virus-infected tissues unless there is a severe loss of vascular integrity. Given the short serum half-life of IgM antibodies (~10 days), and the fact that B-cells undergo isotype class-switching as an obligate step in their differentiation into long-lived memory cells, IgM antibody rapidly declines in titre following the resolution of a primary HSV infection.

The IgG isotype antibody response follows but overlaps the IgM response (Kurtz, 1974). Owing to its lower molecular weight, 150 kDa, IgG antibody is found both intravascularly and extravascularly. Some of the extravascular IgG escapes through leaky capillary walls or is transported across cells into tissues by means of IgG-specific receptors. HSV-specific IgG reduces the severity and spread of HSV infections, but is insufficient to prevent recurrent herpetic infections. A subset of B-cells differentiate into short-lived, IgG antibody-secreting plasma cells, and secrete IgG antibodies that have a serum half-life of ~21 days. Asymptomatic carriers of HSV infection maintain high serum titres of HSV-specific IgG antibody over their lifetime. This raises questions about the source of HSV antigen that drives the continued proliferation and differentiation of HSV-specific B-cells into short-lived plasma cells years after a primary HSV infection, hence maintaining HSV-specific IgG titres over time.

**T-lymphocytes**

Thymus-derived lymphocytes (T-cells) are the antigen-specific cells of the cell-mediated immune system. Within this category of lymphocytes, there are two subpopulations that are easily identified by plasma membrane markers: Cluster of Differentiation molecule 4 (CD4) and Cluster of Differentiation molecule 8 (CD8). These lymphocytes circulate from blood to lymph and back, and are found in all lymphoid tissues.

CD4+ T-cells have acquired the name ‘helper T-cells’ because they assist and promote the maturation of antibody responses and cellular immune responses to foreign antigens. During an active HSV infection, HSV-specific CD4+ T-cells promote antibody class-switching and the subsequent differentiation of HSV-specific B-cells into long-lived memory cells. Likewise, HSV-specific CD4+ T-cells promote the clonal expansion of HSV-specific CD8+ T-lymphocytes, and their differentiation into autonomous effector cells capable of (1) destroying HSV-infected cells or (2) secreting cytokines that interfere with HSV replication.

CD8+ T-cells are best known as ‘cytotoxic T-lymphocytes’ (CTLs) that kill virus-infected target cells. Cytolysis of virus-infected target cells is achieved when a CD8+ T-cell secretes granules containing perforin, granzymes, and fas ligand while directly apposed to a target cell. Internalization of fas ligand by the target cells leads to the initiation of a programmed cell death response, and consequently the virus-infected target cell is destroyed. Because CD8+ T-cells are capable of renewing their cytotoxic granules, a single CTL may kill scores of virus-infected target cells. CD8+ T-cells may also control viral infections via the secretion of interferon-γ, which interferes with viral replication without compromising the health of the host cell. Such non-cytolytic mechanisms are probably critical in explaining how CD8+ T-cells restrict the spread of HSV infection without destroying the neurons in which HSV persists (Fig. 5.1B).

**Secondary immune responses to HSV: memory response or chronic response?**

Lymphocytes are capable of remembering a prior encounter with antigen and responding with heightened vigour upon a second encounter with their cognate antigen. The primary immune response to acute respiratory viruses such as influenza virus leads to CTL-dependent destruction of virus-infected cells, viral clearance, and subsequent contraction of effector T-cells and IgG-producing plasma cells (Woodland et al., 2001; Hikono et al., 2006).

It is not apparent that the term ‘memory immune response’ applies to persistent viral infections in which the spread of viral infection is restricted, but the viral infection is never cleared. In such cases, it may be more accurate to think of the adaptive immune response as a chronic immune response to an ongoing infection.
Consistent with this viewpoint, experimental animals and humans maintain high and constant serum titres of HSV-specific IgG antibodies over their lifetimes (Halford et al., 1996b, 1997). Likewise, T-cells remain clustered around neurons in latently infected ganglia and at the tips of axons in the genital epithelium of human carriers (Derfuss et al., 2007; Verjans et al., 2007; Zhu et al., 2007).

T-lymphocytes are normally resident in blood, lymphatics, lymph nodes and spleen. It is uncommon to see T-cells in non-lymphoid tissues except when there is an active disease process such as in chronic gingivitis, measles encephalitis, and autoimmune conditions (Liebert, 1997; Yamazaki et al., 2003; Kunz and Ibrahim, 2009). What then can we conclude about the presence of T-cells in the trigeminal ganglia of HSV latently infected hosts months to years after the primary infection (Fig. 5.1B)? The experimental animals and humans in which these subclinical events are observed appear outwardly healthy and exhibit no evidence of an ongoing viral infection. If these T-cells were relics of the primary infection, then we would expect them to be immunologically silent. However, T-cells in HSV latently infected ganglia are not randomly scattered in the ganglionic parenchyma, but are clustered around neuronal cell bodies. Furthermore, these T-cells are often observed secreting a variety of cytokines including TNF-α, IL-2, and interferon-γ (Fig. 5.1B). T-cells not engaged in an immune response would not be secreting such potent mediators of host immunity. Rather, the T-cells that persist in HSV latently infected ganglia appear to be part of an ongoing immune response that restricts the spread of HSV infection, but which fails to clear the virus from the body (Khanna et al., 2004; Decman et al., 2005; Divito et al., 2006).

**Host interferons: critical suppressors of HSV replication**

**Host interferons**

Interferon (IFN)-α, IFN-β and IFN-γ are secreted proteins that play important roles in host resistance to viral infections. The >10 isotypes of IFN-α share 20–30% amino acid homology with IFN-β (Pestka, 2007). IFN-α is secreted by leucocytes as an innate response to viral infection, whereas IFN-β is secreted by virus-infected host structural cells. IFN-α and IFN-β bind to a common IFN-α/β receptor, which is expressed on nearly all cells in the body (Samuel, 1998). Activation of IFN-α/β receptors modifies the transcriptional and translational environment in cells inducing an ‘antiviral state’ (Pestka, 2007). IFN-γ does not share amino acid homology with IFN-α or IFN-β, and binds a distinct IFN-γ receptor that is expressed on most cells in the body. Production of IFN-γ is far more restricted than IFN-α/β, and results when T-cells, natural killer cells, or professional antigen-presenting cells are activated in response to a foreign antigen or activating stimulus (Farrar and Schreiber, 1993; Frucht et al., 2001; Stober et al., 2001; Schoenborn and Wilson, 2007).

**Cellular sources of interferon-γ**

All cells in the body may produce some IFN-α or IFN-β as an innate response to viral infection (Toth et al., 1990). IFN-α produced by leucocytes appears to be the predominant source of IFN-α/β that restricts HSV spread in vivo (Gary-Gouy et al., 2002; Vollstedt et al., 2003). In the case of IFN-γ, the situation is more complex. T-cell biology and the dynamics of the adaptive immune response must be considered to appreciate why the role of IFN-γ in host immunity to HSV changes over time.

A CD8+ T-cell is activated to secrete IFN-γ as a result of contact with a virus-infected cell. One of the primary activating stimuli is the engagement of the T-cell’s antigen receptors by MHC class I molecules bearing an 8–10 amino acid viral peptide. In a naïve individual experiencing a primary HSV infection, HSV-specific T-cells initially occur at a frequency of less than 1 per 100,000. Thus, HSV-specific CD8+ T-cells do not contribute to host control of a primary HSV infection until 5–8 days p.i. (McNally et al., 1999). As an infection resolves, antigen receptors on HSV-specific CD8+ T-cells continue to be engaged in draining lymph nodes, which drives their proliferation. HSV-specific CD8+ T-cells increase in number by 10- to 300-fold by the time a latent HSV infection is firmly established (Posavad et al., 1996). Hence, the capacity of HSV-specific CD8+ T-cells to secrete IFN-γ increases by several orders of
Interferon-α: a key mediator by which CD8+ T-cells suppress HSV reactivation

The findings of Simmons and Tscharke (1992) raised the possibility that CD8+ T-cells might control HSV replication in neurons. Several labs explored the validity of this inference, and confirmed that T-cells infiltrated HSV-infected ganglia at times that coincided with the shut off of productive HSV replication (Cantin et al., 1995; Shimeld et al., 1995; Liu et al., 1996). In each study, CD8+ T-cells were observed in direct contact with neurons, but neurons remained healthy in appearance suggesting that CD8+ T-cells relied on non-cytolytic mechanisms to control HSV replication in neurons. Surprisingly, activated CD8+ T-cells were retained in HSV-infected ganglia for months after the resolution of the acute infection (Cantin et al., 1995; Shimeld et al., 1995; Halford et al., 1996b; Liu et al., 1996). These findings suggested that HSV latency might be less 'latent' than originally envisioned. The persistence of IFN-γ-secreting CD8+ T-cells at sites of latent HSV infection suggested that either (1) low-level synthesis of HSV antigen was occurring, (2) HSV was spontaneously reactivating in neurons, or (3) both.

Several investigators questioned whether the IFN-γ that persisted at sites of latent HSV infection might play a role in suppressing HSV reactivation. Edouard Cantin's laboratory tested this possibility by comparing the frequency of stress-induced reactivation in HSV latently infected ganglia at times that coincided with the shut off of productive HSV replication (Cantin et al., 1995; Shimeld et al., 1995; Halford et al., 1996b; Liu et al., 1996). These findings suggested that HSV latency might be less 'latent' than originally envisioned. The persistence of IFN-γ-secreting CD8+ T-cells at sites of latent HSV infection suggested that either (1) low-level synthesis of HSV antigen was occurring, (2) HSV was spontaneously reactivating in neurons or (3) both.

How can CD8+ T-cells directly repress HSV reactivation in neurons?

In principle, HSV reactivation in neurons might be recognized by HSV-specific CD8+ T-cells patrolling the ganglia, IFN-γ secretion could result, and secreted IFN-γ could directly suppress HSV replication in neurons (Fig. 5.1B). The data of Cantin, et al. (1999a,b) and Liu, et al. (2000) were consistent with this possibility. However, the hypothesis that CD8+ T cells directly suppressed HSV replication in neurons seemed incongruous with other observations, considered as follows.

Do neurons express MHC class I?

In the early 1990s, it was widely held that neurons do not express MHC class I, and thus were incapable of presenting intracellular peptides to CD8+ T-cells (Weinstein et al., 1990; Joly and Oldstone, 1992; Bailey et al., 1994). When more sensitive methods were employed, Tony
Simmons’ laboratory found that (1) neurons expressed low levels of MHC class I molecules on their cell surface, and (2) MHC class I density increased on neurons during HSV infection of ganglia (Pereira et al., 1994). This upregulation of MHC I molecules was most likely a result of HSV-induced IFN-α/β and/or IFN-γ secretion, as both cytokines induce MHC gene expression (Lampson and George, 1986; Njenga et al., 1997). Numerous studies have since corroborated the finding that neurons express a low density of MHC class I molecules on their cell surface (Redwine et al., 2001; Neumann et al., 2002).

How does interferon-γ inhibit HSV reactivation?

The possibility that IFN-γ secreted by CD8+ T-cells might control HSV reactivation in neurons (Cantin et al., 1999a; Liu et al., 2000) paralleled the findings of Chisari and colleagues, who had previously demonstrated that hepatitis B virus infections were controlled in a non-cytolytic manner that was dependent on CD8+ T-cells and IFN-γ (Guidotti et al., 1994; Guidotti et al., 1996). While Cantin and Hendricks’ data were impeccable, it was difficult to reconcile how IFN-γ could suppress HSV reactivation in neurons when IFN-γ had only negligible effects on the replication of wild-type HSV in cultured cells (Harle et al., 2002).

The resolution to this paradox lay in considering the in vivo context in which T-cells act to control HSV infections. T-cells never arrive at sites of HSV infection, and secrete IFN-γ into environments that are devoid of other cytokines. At a minimum, secretion of IFN-α/β, proinflammatory cytokines (e.g. TNF-α), and chemoattractant cytokines (e.g. IL-8) precedes the recruitment of T-cells into a site of viral infection. Therefore, a more accurate cell culture test of IFN-γ’s capacity to inhibit HSV replication would require treatment of cultured cell with a combination of IFN-γ and at least one other cytokine of the innate immune response.

Testing of this hypothesis revealed that IFN-γ acted in concert with IFNα/β to potently inhibit HSV-1 replication in cultured cells. For example, treatment of Vero cells with IFN-β or IFN-γ inhibited HSV-1 replication by less than 20-fold, whereas combinations of IFN-β and IFN-γ inhibited HSV-1 replication by nearly 1000-fold (Sainz and Halford, 2002). These results suggested that treatment of cells with ligands of IFN-α/β receptors or IFN-γ receptors rendered cells only partly resistant to HSV replication, whereas co-activation of both IFN-α/β receptors and IFN-γ receptors rendered cells fully resistant to HSV replication. Hence, IFN-γ secreted by CD8+ T-cells might act in concert with IFN-α/β secreted by virus-infected cells to render neurons fully refractory to HSV replication.

Co-activation of interferon-α/β receptors and interferon-γ receptors is sufficient to repress HSV replication in cultured cells

IFN-α/β- and IFN-γ-signalling pathways are often described in terms that suggest that the receptors function independently of one another. However, Takaoka and colleagues demonstrated that IFN-α/β and IFN-γ receptors co-immunoprecipitate in a 1:1 ratio (Takaoka et al., 2000). If the innate and adaptive interferon responses to viral infections are quite literally linked to one another, then ligand binding to IFN-γ receptors may directly enhance the antiviral effects of IFN-α/β. For example, IFN-γ ligand binding to its receptor might multiply the antiviral effect induced by IFN-β ligand binding to its receptor.

To test this hypothesis, Halford, et al. (2005) compared HSV DNA yields at 30 hours p.i. in Vero cells that were inoculated with 0.1 pfu per cell of HSV-1, and which had been pre-treated with 64 unique dose combinations of IFN-β and IFN-γ (Fig. 5.3A). The amount of HSV DNA synthesized in each culture was measured by dot blot analysis, where the blackness of each dot provided a direct measure of HSV DNA abundance. Inhibition of HSV DNA synthesis increased as the dosage of IFN-β was increased, and the dose–response relationship was described by a hyperbolic tangent function that produced an S-shaped curve (blue box in Fig. 5.3A; front edge of graph in Fig. 5.3B). Addition of IFN-γ had no effect on the shape of the IFN-β dose–response curve, but rather IFN-γ acted as a multiplier that increased the amplitude of the curve (red box in
Mathematical analysis demonstrated that IFN-α acted as a multiplier of the potency with which innate IFNs restrict the replication of HSV in otherwise permissive cells (Halford et al., 2005a).

The capacity of IFN-β and IFN-γ to inhibit viral replication synergistically is not unique to HSV-1, but has been observed with cytomegalovirus, SARS virus, and hepatitis C virus replicons (Larkin et al., 2003; Sainz et al., 2004, 2005). The mechanism by which IFN-α/β and IFN-γ synergistically modify the host cell remains obscure (Peng et al., 2008). What is clear is that IFN-α/β and IFN-γ do not synergistically inhibit HSV-1 replication via (1) a cytotoxic or apoptotic effect, (2) a block to HSV entry or (3) an overt suppression of IE and E mRNA or protein synthesis (Pierce et al., 2005). Rather, IFN-α/β and IFN-γ’s first, unambiguous synergistic effect on HSV replication is manifest at the level of HSV DNA synthesis, which in turn prevents the synthesis of new HSV virions (Pierce et al., 2005).

Mice that lack IFN-α/β receptors and IFN-γ receptors fail to control HSV infections

Cell culture studies raised the possibility that IFN-γ might act in concert with IFN-α/β to directly inhibit HSV-1 replication in animals. Luker et al. (2003) put this hypothesis to the test by comparing the spread of a ‘glowing’ HSV-luciferase virus following rear footpad inoculation of different IFN receptor-knockout mice (Fig. 5.4). In wild type mice, the spread of HSV-luciferase remained largely restricted to the site of inoculation between 1 and 4 days p.i., and all mice survived the infection (top panel in Fig. 5.4; Luker et al., 2003). In IFN-α/β receptor knockout mice, the HSV-luciferase virus transiently spread from rear footpads to the liver on day 3 p.i., but all mice survived the infection (middle panel in Fig. 5.4). In IFN-γ receptor-knockout mice, the spread of HSV-luciferase was indistinguishable from wild-type mice, and all mice survived the infection (Luker et al., 2003).
Mice that lacked both IFN-α/β receptors and IFN-γ receptors exhibited a very different pattern of HSV infection (Luker et al., 2003). HSV-luciferase spread in a rapid and uncontrolled manner from the rear footpads to the livers of double-IFN-receptor-knockout mice, and none survived beyond 4 days p.i. (bottom panel in Fig. 5.4). Although animal hosts are intimately dependent on host IFNs to restrict the spread of HSV infection, this fact was overlooked in prior studies that focused on the partial phenotypes that result when either IFN-α/β receptors or IFN-γ receptors are eliminated. In contrast, Luker, et al. (2003) revealed the full phenotype of uncontrolled HSV replication and spread that follows loss of both IFN-α/β and IFN-γ signalling pathways. Hence, IFN signalling is critical to the process by which animals normally restrict the spread of HSV infection to the initial site of infection, and the nerve fibres that innervate this portion of the epithelium (Fig. 5.1A).

Of mice and men
Owing to the wealth of mouse-specific reagents, most advances in HSV immunology have first been made in mice and later tested in humans. Such mouse studies have led us to conclude that T-lymphocytes and the combined activities of the IFN-α/β- and IFN-γ-signalling pathways are required for the process by which wild-type HSV is driven into a latent state in animals. But, do these findings translate to humans? As summarized...
below, T-cells and host IFN-signalling pathways appear to be highly relevant to the natural process by which latent HSV infections are established and maintained in humans.

CD8+ T cells persist in HSV latently infected ganglia of humans

It was documented in the mid-1990s that CD8+ T-cells persisted at sites of latent HSV infection in mice (Cantin et al., 1995; Shimeld et al., 1995; Halford et al., 1996b; Liu et al., 1996). The relevance of these findings was unclear at the time for two reasons.

First, it was not known if the CD8+ T-cells found in HSV latently infected ganglia represented sentinels that were keeping latent HSV infections in check, or rather were bystanders drawn to the scene of a crime. Bob Hendricks’ laboratory addressed this question by determining the fraction of CD8+ T-cells in ganglia that bore antigen receptors specific for HSV peptides. Almost two-thirds of the CD8+ T-cells in HSV latently infected ganglia were specific for amino acids 498–505 of the HSV-1 glycoprotein B, and another 4% were specific for amino acids 822–829 of the HSV-1 ribonucleotide reductase large subunit (Khanna et al., 2003). Therefore, the CD8+ T-cells retained in latently infected ganglia are HSV-specific (Fig. 5.1B), and appear to be actively engaged in the process of responding to subclinical HSV infections (Khanna et al., 2003, 2004; Divito et al., 2006; Knickelbein et al., 2008).

Second, it was possible that the persistence of T-cells in HSV infected ganglia was a bizarre artefact of the mouse model of HSV infection. Using HSV-1 seropositive organ donors as a source of human trigeminal ganglia, Diethilde Theil’s laboratory tested this possibility using a combination of in situ hybridization for HSV-1 LAT RNA and immunohistochemical staining for T-cells (Theil et al., 2003; Derfuss et al., 2007). Theil found that human ganglia from HSV seropositive donors contained single neurons that were frequently encircled by T-cells (Fig. 5.5A). These neurons were surrounded by T-cells expressing CXCL-3 receptors and other definitive markers of T-cell activation (Derfuss et al., 2007). Yet, neurons remained healthy in appearance, suggesting that T-cells did not unleash their cytotoxic potential on the neurons they encircled (Fig. 5.5A). In situ hybridization for HSV-1 LAT RNA confirmed the presence of frequent LAT+ neurons in human trigeminal ganglia that were infiltrated by T-cells (Fig. 5.5B and C). Although T-cell infiltrates were occasionally noted adjacent to HSV LAT+ neurons (Fig. 5.5B), more often T-cells were noted clustered around neurons that were LAT-negative.

Figure 5.5 T-lymphocytes persist at sites of latent HSV infection in humans. Representative photomicrographs of a tissue section from a human trigeminal ganglion latently infected with HSV-1 stained with anti-CD3 antibodies, which recognize the cell surface CD3 molecule that is associated with the T-cell receptor of both CD4+ T-cells and CD8+ T-cells. (A) A single, large neuronal cell body encircled by an infiltrate of small, brown-stained CD3+ T-cells (denoted by black arrow). Reprinted from Am. J. Pathol. 2003, 163: 2179–2184 with permission from the American Society for Investigative Pathology. (B) In situ hybridization for HSV-1’s LAT RNA introns highlights the nucleus of a single, human neuron that is latently infected with HSV-1 (denoted by white arrow), and immunohistochemical staining reveals the presence of small, brown-stained CD3+ T-cells in an adjacent mononuclear cell infiltrate. Adapted from Derfuss et al., 2007, Brain Pathol. 17: 389–398 with permission from John Wiley and Sons. (C) An infiltrate of CD3+ T-cells (denoted by black arrow) drawn to a site several neuronal cell bodies away from an HSV-1 LAT+ neuron (denoted by white arrow). Original images were generously provided by Diethilde Theil. A colour version of this figure is located in the plate section at the back of the book.
and which were well separated from HSV LAT+ neurons (Derfuss et al., 2007; Fig. 5.5C).

Theil’s findings were corroborated in an independent study performed on a large cohort of trigeminal ganglia collected from human cadavers (Verjans et al., 2007). Larry Corey and colleagues extended these findings to human carriers of latent HSV-2 infections (Zhu et al., 2007). Biopsies of genital epithelium were taken from the labia of female patients 3 weeks after a recurrence of genital herpes. HSV-specific CD8+ T-cells were found in direct contact with nerve endings at sites of recurrent herpes lesions, but were absent in control biopsies taken from sites where herpes outbreaks did not occur (Zhu et al., 2007).

Deficits in interferon signalling predispose humans to severe HSV infections

Deficits in T-cell-mediated immunity predispose humans to severe herpesviral infections caused by HSV, cytomegalovirus, or varicella-zoster virus. For example, unusually severe recurrences of HSV disease are often noted in patients with AIDS (Posavad et al., 1997), leukaemia (Angarone and Ison, 2008), organ transplants (Miller and Dummer, 2007), severe burns (D’Avignon et al., 2010) or primary immunodeficiencies that prevent T-cell development (Berthet et al., 1994).

Humans may also be born with severe defects in IFN signalling, which predispose these individuals to severe herpesviral infections (Jouanguy et al., 2007). For example, a homozygous defect in the interferon-activated Stat 1 transcription factor has been identified in three children, who each died before 18 months of age (Dupuis et al., 2003; Chapgier et al., 2006). One Stat 1-deficient child died of herpes simplex encephalitis (Dupuis et al., 2003), the second died shortly after a fulminant Epstein–Barr virus infection (Chapgier et al., 2006) and the third child died at home of an undiagnosed, viral-like illness (Dupuis et al., 2003). Likewise, genetic defects in the intracellular UNC-93B protein have been found to compromise the induction of an IFN-induced antiviral state, and have been associated with HSV encephalitis in two small children (Casrouge et al., 2006).

Infiltration of CD8+ T cells in herpes lesions coincides with the cessation of HSV shedding

In humans, the timing of arrival of CD8+ T-cells in recurrent genital lesions correlates strongly with the cessation of HSV-2 replication (Koelle et al., 1998; Posavad et al., 1998). In contrast, immunosuppressed people with deficits in T-cell function present with slowly progressing herpes infections that often fail to resolve without clinical intervention and antiviral drugs (Stanberry et al., 1994; Stewart et al., 1995; Kang et al., 2006; Yudin and Kaul, 2008).

The latency-regulating R_L regions of HSV

Switching gears: from the brakes to the accelerator

The braking mechanism that causes HSV replication to stop in vivo is a product of the host immune response that restricts HSV replication, but which fails to clear the virus. While the host immune response explains how the cessation of HSV replication is brought about, it does not address two critical questions: (1) How are latent HSV infections maintained in the human nervous system? (2) How do latent HSV infections periodically re-enter the productive cycle of viral replication? We lack detailed answers to these questions, but possess many clues that constrain the number of possibilities. For example, regulation of HSV latency appears to be controlled from a pair of 10 kb long-repeated (R_L) regions in the HSV genome (Fig. 5.2). Synthesis of two R_L-encoded proteins, ICP0 and ICP34.5, promotes HSV replication in neurons, whereas failure to synthesize either protein predisposes HSV to establish a latent infection (Fig. 5.1A). Before discussing the R_L regulatory proteins, we put this discussion of the HSV latency-replication balance into perspective by considering the natural frequency with which HSV infections oscillate between a latent and reactivated state.

How latent is ‘latent’?

Approximately 2% of the human population are shedding detectable levels of infectious HSV-1
in their oral cavity on any given day (Miller and Danaher, 2008). Thus, HSV infections are not ‘latent’ in the absolute sense of the word. Rather, the term ‘latent’ is relevant in differentiating low-grade HSV infections from viral infections that are truly chronic in nature. For example, humans chronically infected with hepatitis B virus or human immunodeficiency virus may produce millions of new infectious virions per day. In contrast, humans who are latently infected with HSV produce only vanishingly small quantities of infectious virus.

The study of Wald, et al. (1997) highlighted how the sensitivity of testing for HSV-2 shedding strongly influences our perception of the nature of a latent HSV-2 infection. Over 270 consecutive days, three different measures of HSV-2 reactivation were compared in women with recurrent genital herpes: (1) symptoms of herpetic disease, (2) infectious virus recovered from genital swabs or (3) HSV-2 DNA detected by PCR (Wald et al., 1997). On 3% of days, these women were aware of prodrome (nerve tingling) or active herpes lesions. On 8% of days, infectious HSV-2 was recovered from genital swabs. On 28% of days, genital swabs contained HSV-2 DNA that was detected by PCR, and 60% of HSV-2 DNA shedding events were too brief in duration to produce symptoms (Wald et al., 1997). Thus, our estimates of the frequency of HSV-2 reactivation increase by an order of magnitude when a more sensitive assay is used to detect subclinical HSV-2 reactivation events.

Latent HSV infections are unlikely to be static in nature (Buddingh et al., 1953). Rather, latent HSV infections appear to consist of a population of 1000–10,000 HSV latently infected neurons where, once a week, a single HSV-infected neuron exits the pool of latently infected neurons and re-enters the productive cycle of HSV replication. Hence, HSV virions are exported from the ganglia to the epithelium of human carriers on a regular basis (Buddingh et al., 1953; Wald et al., 1997). Consequently, an immune response persists in human ganglia that harbour these low-grade, persistent HSV infections (Theil et al., 2003; Verjans et al., 2007).

The $R_L$ regions of the HSV genome
The regulation of HSV latency appears to be controlled from a pair of 10 kb, inverted $R_L$ regions in the HSV genome (Fig. 5.2). There are four well-established genes in the $R_L$ regions: LAT, L/ST, ICP0 and ICP34.5. The LAT and L/ST genes share extensive antisense overlap with the ICP0 and ICP34.5 genes and produce what appear to be non-coding RNAs (Fig. 5.2). It remains unclear what purpose is served by the 1.5 and 2.0 kb LAT RNA-derived introns that accumulate in HSV latently infected neurons (Fig. 5.1B). Likewise, the function of the long/short spanning transcript (L/ST) RNA is unclear. Thus, LAT and L/ST RNAs are enigmatic and do not yet clarify how the HSV $R_L$ regions influence the outcome of an HSV infection.

In contrast, the ICP0 and ICP34.5 genes produce two proteins, ICP0 and ICP34.5, which regulate the probability of HSV replication in vivo. Importantly, HSV ICP34.5 null viruses replicate like wild type virus in cultured cells, and HSV ICP0 null viruses replicate like wild type virus at high multiplicities of infection. Thus, ICP0 and ICP34.5 are not essential cogs in the machinery of viral replication, but rather are regulatory proteins that allow HSV to ‘choose’ whether its replication machinery will start (ICP0) or keep functioning (ICP34.5) for long enough to produce new infectious virions. Synthesis of ICP0 and ICP34.5 strongly promote HSV replication in vivo. It has been postulated that the antisense LAT and L/ST genes may contribute to the establishment and/or maintenance of latent HSV infections by antagonizing ICP0 and ICP34.5 gene expression (Tang et al., 2008; Umbach et al., 2008).

ICP0 functions as an activator/antirepressor of HSV replication
ICP0 was identified as a protein that transformed the HSV major transcriptional regulator, ICP4, from a weak transcriptional activator to a potent activator of mRNA synthesis; specifically, combinations of ICP0 and ICP4 are 20-fold more potent at driving mRNA synthesis than either ICP0 or ICP4 alone (Everett, 1984; Gelman and Silverstein, 1986). Functionally, synthesis of ICP0 causes the equilibrium of HSV to abruptly tip
towards productive replication, whereas absence of ICP0 produces the opposite effect. Synthesis of ICP0 is sufficient to trigger HSV reactivation in trigeminal ganglion neurons and other models of latent HSV infection (Halford et al., 2001; Terry-Allison et al., 2007). While HSV ICP0-deficient viruses replicate to nearly wild type levels at high multiplicities of infection, at low multiplicities of infection the same ICP0-deficient viruses establish quiescent infections in 99% of the cells they infect (Sacks and Schaffer, 1987; Everett, 1989). Such observations suggest that ICP0 antagonizes a repressor of HSV replication, whose repressive capacity can be saturated with high numbers of HSV genomes (Everett, 1989).

Because ICP0 is an E3 ubiquitin ligase (Boutell et al., 2002), it has been suggested that a repressor may silence HSV mRNA synthesis in the absence of ICP0, whereas accumulation of ICP0 may neutralize this repressor and allow HSV mRNA synthesis to proceed unhindered (Everett, 2000; Preston, 2000). The details remain an area of debate, but the big picture is clear; accumulation of ICP0 promotes productive HSV replication, whereas absence of ICP0 strongly favours the repression of HSV gene expression (Liu et al., 2010).

ICP34.5: a regulator of the progression of HSV replication in neurons

In the late 1980s, Rick Thompson’s laboratory identified a neurovirulence factor encoded from the $R_l$ gene, which came to be known as the ICP34.5 gene (Fig. 5.2). Deletion of the $R_l$ gene had no effect on the capacity of HSV to replicate in cultured cells, but prevented HSV from completing its replication cycle in neurons in vivo (Javier et al., 1987; Thompson et al., 1989; Bolovan et al., 1994). Chou, et al. (1995) discovered that the $R_l$ gene product, ICP34.5, interacted with cellular protein phosphatase 1 and stimulated the dephosphorylation of eIF-2α (Chou et al., 1995). What is most relevant to initially recognize about ICP34.5 is that absence of this protein strongly predisposes HSV to establish latent infections in neurons (Thompson et al., 1989). In fact, HSV ICP34.5-deficient viruses are so predisposed to establish latent infections in neurons, that high doses of HSV ICP34.5-deficient viruses may be injected into brain tumours of human patients. Owing to the failure of HSV ICP34.5-deficient viruses to replicate in healthy human neurons, these oncolytic viruses can selectively destroy cancer cells in the brain without harming healthy brain tissue in glioma patients (Markert et al., 2000; Todo, 2008).

The $R_l$ regions regulate the sensitivity of HSV to interferon

Hypothesis: host interferons versus viral interferon antagonists

Latent HSV infections appear to exist in a dynamic equilibrium that oscillates between a state of latency versus frequent reactivation in single HSV latently infected neurons (Fig. 5.1A). Host immune cells and host IFNs appear to be the source of the repressive stimuli that push the equilibrium of HSV towards a latent state. The $R_l$-encoded viral activators, ICP0 and ICP34.5, appear to push this equilibrium in the opposite direction towards HSV replication. These statements are consistent with the evidence, but beg the question, ‘How do ICP0 and ICP34.5 allow HSV to override constant repression by the host IFN response?’

ICP0 and ICP34.5 function as viral IFN antagonists. When synthesized, ICP0 and ICP34.5 endow HSV with the capacity to sustain replication in the face of the antiviral state induced by IFN-α/β (Leib et al., 1999; Leib et al., 2000). Specifically, the resistance of HSV to IFN-α/β-inducible repression increases by ~100-fold when both ICP0 and ICP34.5 are expressed. In contrast, failure to synthesize either ICP0 or ICP34.5 ensures that HSV will succumb to the IFN-α/β-induced antiviral state and will predominantly establish silent infections (Mossman et al., 2000; Harle et al., 2002; Mossman and Smiley, 2002).

The HSV $R_l$ regions function as an IFN resistance locus (Fig. 5.2). When this fact is integrated into an equilibrium model of HSV latency (Fig. 5.1A), three insights emerge. First, synthesis of ICP0 and ICP34.5 is necessary for the ‘IFN-resistant’ phenotype that allows HSV to sustain replication in vivo. Second, failure to express ICP0 or ICP34.5 renders HSV ‘IFN-sensitive’ and prone
to establish latent infections. Finally, the collective gene activity in the HSV R₅ regions appears to imbue HSV with the capacity to 'choose' whether viral replication will proceed, or halt, at one of two IFN-inducible restrictions to HSV gene expression (Mossman and Smiley, 2002; Halford et al., 2006). This hypothesis is attractive because it assigns a tangible function to the latency-regulating R₅ regions of HSV (Fig. 5.2). Moreover, this hypothesis implies that failure to synthesize ICP0 or ICP34.5 should promote the establishment of a latent HSV infection if, and only if, the host IFN response is intact and available to restrict the progression of HSV replication. We discuss below the evidence that the balance between latency and replication is tipped in opposite directions by ICP0 and ICP34.5 versus the host IFN-α/β response (Fig. 5.1A).

HSV must synthesize ICP0 and ICP34.5 to resist interferon-induced repression \textit{in vitro}

Mossman and colleagues found that the resistance of HSV to IFN-α/β was an active process, and was dependent on the synthesis of both ICP0 and ICP34.5 (Mossman et al., 2000; Mossman and Smiley, 2002). Wild-type HSV replicates ~3-fold less efficiently in IFN-α/β-activated cells relative to untreated cells. In contrast, HSV ICP0 null viruses replicate ~300-fold less efficiently in IFN-α/β-activated cells relative to untreated cells (Harle et al., 2002; Halford et al., 2006). Likewise, HSV ICP34.5 null viruses exhibit a similar IFN-sensitive phenotype (Mossman and Smiley, 2002). Importantly, the IFN-sensitive phenotype is not a non-specific phenotype that results when any of the ~30 accessory genes of HSV are mutated; mutations in the VP16, ICP22, UL13 or vhs genes do not affect the resistance of HSV to IFN-α/β-inducible repression in cultured cells (Mossman et al., 2000). Therefore, the ‘IFN-sensitive’ phenotype of HSV may be defined as an ~100-fold increase in sensitivity to IFN-α/β-induced repression \textit{in vitro} that results from failure to synthesize a single HSV protein. To date, ICP0 and ICP34.5 are the only HSV proteins that satisfy this stringent, functional definition of an interferon antagonist.

How do ICP0 and ICP34.5 antagonize interferon-induced repression of HSV?

ICP34.5

IFN-α/β often inhibits the replication of viruses via a shutoff of viral protein translation that is based on the IFN-inducible, dsRNA-activated protein kinase (PKR) and its substrate eIF-2α. When PKR phosphorylates eIF-2α, the eIF2 translation factor is unable to assemble 80S ribosome complexes on mRNA, and protein translation is inhibited (Gebauer and Hentze, 2004). ICP34.5 was first identified as a neurovirulence factor essential for HSV replication in neurons (Thompson et al., 1989). It was later discovered that ICP34.5 stimulated dephosphorylation of eIF-2α by binding protein phosphatase 1 (Chou et al., 1995; Mohr and Gluzman, 1996). Thus, ICP34.5 appeared to be a PKR antagonist, and the replication defect of HSV ICP34.5 null viruses in neurons was potentially the result of an IFN-inducible block to HSV protein synthesis.

Leib, et al. (2000) reasoned that if ICP34.5 antagonized an IFN-α/β-inducible, PKR-dependent block to HSV replication, then HSV ICP34.5 null viruses should re-acquire their virulence in knockout mice deficient in IFN-α/β receptors or PKR. Both predictions proved to be correct. High doses of HSV ICP34.5 null viruses produced little disease when injected into the brains of normal mice. However, the same HSV ICP34.5 null viruses were uniformly lethal when injected into the brains of PKR-knockout mice or IFN-α/β receptor-knockout mice. Hence, the failure of HSV ICP34.5 null viruses to replicate in neurons was the result of an active host response that was dependent on PKR and IFN-α/β receptors (Leib et al., 2000).

ICP0

Unlike ICP34.5, we lack a crisp, biochemical explanation for the capacity of ICP0 to overcome IFN-inducible repression of HSV. When ICP0 is synthesized, presence or absence of IFN-α/β has negligible effects on the efficiency of HSV IE and E mRNA synthesis (Mossman et al., 2000). In contrast, when ICP0 is not synthesized, pre-treatment of cells with IFN-α/β prevents the accumulation
of HSV IE and E mRNAs (Mossman et al., 2000). Thus, in IFN-α/β-activated cells, synthesis of ICP0 is required if HSV IE and E proteins are to accumulate to levels that support HSV replication and virion synthesis (Mossman et al., 2000; Harle et al., 2002).

HSV must synthesize ICP0 and ICP34.5 to resist innate immune repression in rag2−/− mice

The HSV latency-regulating R₇ regions (Fig. 5.2) may control the balance between latency and replication by allowing HSV to alternate between relative states of IFN-sensitivity (latency-prone) versus IFN-resistance (replication-prone). For example, down-regulation of ICP0 or ICP34.5 synthesis may predispose HSV to be repressed by the IFN-α/β-induced antiviral state, hence favouring the establishment of a latent infection. Conversely, efficient synthesis of ICP0 and ICP34.5 may render HSV resistant to repression by the IFN-α/β-induced antiviral state.

Severe-combined immunodeficient (SCID) mice and rag2−/− mice lack mature B and T-lymphocytes, and represent a powerful system to test the putative role of ICP0 and ICP34.5 in rendering HSV resistant to repressive effects of the innate immune system. In the absence of T-lymphocytes, wild type HSV-1 infection of SCID mice or rag2−/− mice is invariably fatal and culminates in encephalitis. Of course, the arrangement of genes in the R₇ regions favours the synthesis of ICP0 and ICP34.5 in the absence of T-lymphocytes, and thus wild-type HSV is genetically predisposed to resist repression by IFN-α/β (Fig. 5.2). But, what happens if this predisposition is eliminated by mutations that prevent the synthesis of ICP0 or ICP34.5?

HSV-1 ICP0 null viruses and HSV ICP34.5 null viruses are avirulent in SCID mice and rag2−/− mice (Valyi-Nagy et al., 1994a; Halford et al., 2006). HSV-1 ICP0 null viruses replicate efficiently in the eyes of wild-type mice and rag2−/− mice for the first 48 hours p.i. (Fig. 5.6A), and then appear to be driven into a latent state based on the relative absence of viral shedding and disease (Fig. 5.6A,B). When rag2−/− mice are sacrificed 60 days p.i., infectious virus is undetectable in homogenates of the eyes or trigeminal ganglia but HSV-1 ICP0 null viral DNA is readily detected in the trigeminal ganglia by PCR (Halford and Schaffer, 2000; Halford et al., 2006). Likewise, HSV-1 ICP34.5 null viruses are avirulent in

![Figure 5.6](image-url)

**Figure 5.6** HSV ICP0 null viruses are repressed in rag2−/− mice in a manner that is dependent on IFN-α/β receptors and Stat 1. (A) HSV-1 shedding in eyes of strain 129 mice, rag2−/− mice, stat1−/− mice, or rag2−/− stat1−/− mice inoculated with 2 × 10⁵ pfu per eye of the ICP0 virus, HSV-1 0-GFP (n = 4 mice per group). The dashed line indicates the lower limit of detection of the plaque assay. (B) Results of an independent experiment comparing duration of survival of strain 129 mice, rag2−/− mice, rag2−/− ifnar−/− mice, or rag2−/− stat1−/− mice that were inoculated with 2 × 10⁵ pfu per eye of the ICP0− virus, HSV-1 0-GFP (n = 8 mice per group). Adapted from Halford, et al. (2006) in accordance with the open-access policy of BioMed Central.
lymphocyte-deficient SCID mice (Valyi-Nagy et al., 1994a), and similar analyses suggest that HSV-1 ICP34.5 null viruses efficiently establish latent infections in rag2\(^{-/-}\) mice (W.P. Halford, unpublished).

Repression of HSV ICP0- or ICP34.5-null viruses in rag2\(^{-/-}\) mice is IFN-\(\alpha/\beta\) dependent

The fact that HSV ICP0 null or ICP34.5 null viruses are avirulent in rag2\(^{-/-}\) mice does not prove that these viruses are driven into a latent state. Thus, it is critical to distinguish between two equally viable explanations for these observations. Like all of the essential proteins of HSV (e.g. ICP4, ICP27, ICP8), synthesis of ICP0 and ICP34.5 may be required for HSV to sustain productive replication in any experimental animal including a rag2\(^{-/-}\) mouse. Alternatively, the IFN-sensitive phenotype of HSV ICP0 null or ICP34.5 null viruses may render these viruses prone to establish latent infections in rag2\(^{-/-}\) mice that possess an intact IFN-\(\alpha/\beta\) response.

To differentiate between these possibilities, replication of an HSV-1 ICP0 null virus was compared in lymphocyte-deficient rag2\(^{-/-}\) mice versus rag2\(^{-/-}\) mice that possessed secondary defects in their IFN-\(\alpha/\beta\) receptors (rag2\(^{-/-}\) ifnar\(^{-/-}\)) or the downstream Stat 1 transcription factor (rag2\(^{-/-}\) stat1\(^{-/-}\)). On day 3 p.i., replication of an HSV-1 ICP0 null virus was low to undetectable in the eyes of rag2\(^{-/-}\) mice (Fig. 5.6A). In contrast, an HSV-1 ICP0 null virus replicated to \(\sim\)1000-fold higher levels in rag2\(^{-/-}\) ifnar\(^{-/-}\) mice (Fig. 5.6A) or rag2\(^{-/-}\) stat1\(^{-/-}\) mice (Table 3 of Halford et al., 2006). While wild-type mice and rag2\(^{-/-}\) mice remained disease-free for 60 days p.i. (Fig. 5.6B), the same HSV-1 ICP0 null virus produced lethal disease within 11\(\pm\)1 days p.i. of rag2\(^{-/-}\) ifnar\(^{-/-}\) mice and rag2\(^{-/-}\) stat1\(^{-/-}\) mice (Halford et al., 2006; Fig. 5.6B). Similar comparisons of replication and pathogenesis revealed that HSV-1 ICP34.5 null viruses rapidly produce lethal infections in rag2\(^{-/-}\) stat1\(^{-/-}\) mice, despite their avirulent phenotype in rag2\(^{-/-}\) mice (W.P. Halford, unpublished).

Based on these considerations, neither ICP0 nor ICP34.5 is strictly required for HSV-1 to replicate in animals (Halford and Schaffer, 2000; Leib et al., 2000; Halford et al., 2006). Rather, failure to synthesize ICP0 or ICP34.5 strongly predisposes HSV to establish what appear to be latent infections in animals that retain a functional IFN-\(\alpha/\beta\) signalling pathway.

**Conclusion**

Knowledge of HSV latency has grown tremendously in the past 30 years. With the advent of recombinant DNA technology, HSV virology exploded as a science in the 1980s, and yielded new insights into the genetic code of HSV, LAT RNAs, and IE regulatory proteins. In the 1990s, it became clear that T-cells provided the stimuli that triggered the establishment of latent HSV infections in neurons. In the 2000s, host IFN-signalling pathways emerged as a critical connection that linked the repressive stimuli provided by leucocytes to the intracellular restriction of HSV replication in host cells including neurons.

The phenomenon of HSV latency continues to be studied and discussed in predominantly virological terms. Such discussions tend to be confusing because the ‘repressive stimulus’ that drives HSV into a latent state emanates from a source that lies outside the traditional scope of virology. Recent data illuminate the path out of this quandary: it is time to broaden our scope! Latent HSV infections are the result of an equilibrium between a virus and the host immune response to that virus (Khanna et al., 2004; Decman et al., 2005; Divito et al., 2006; Fig. 5.1). In light of this new principle, understanding of HSV latency is poised to advance to a more mechanistic level as the details emerge regarding how (1) the host IFN response drives HSV into a latent state, and how (2) differential regulation of the IFN antagonists of HSV is achieved.

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Figure 1 - Halford and Gebhardt

A

[Diagram showing CD8+ T cells and HSV latency and replication pathways]

B

[Diagram showing HSV DNA, HSV latently infected neuron, LAT RNA, and IFN-γ activation]

HSV latency
HSV replication

viral activators

host interferons

CD8+ T cells

innate IFN-α/β

IFN-γ
Figure 2 - Halford and Gebhardt
Figure 3 - Halford and Gebhardt

A

B

Fold inhibition

IFN-β (U/ml)

IFN-γ (U/ml)

IFN-β only

IFN-β + 100 U/ml IFN-γ

IFN-β (U/ml)

IFN-γ (U/ml)

1000 64 100 164 46 21 10 0

100 200 300 400

1000 100 10 0

0 215 100 10 0

0 46 21 10 0

0 464 215 100 10 0

0 1000 64 100 164 46 21 10 0

0 1000 64 100 164 46 21 10 0

IFN-β only

IFN-β + 100 U/ml IFN-γ
Figure 4 - Halford and Gebhardt

<table>
<thead>
<tr>
<th>Days after inoculation</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>10</th>
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<td><strong>wild-type mice</strong></td>
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<td><strong>IFN-α/β R⁻/⁻ mice</strong></td>
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<td><strong>IFN-γ R⁻/⁻ mice</strong></td>
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- HSV-luciferase

R.I.P.
Figure 6 - Halford and Gebhardt

A  HSV-1 ICP0− deficient virus

B  HSV-1 ICP0− deficient virus