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In Search of Gene Targets for Productive and Latent HSV-2 Infection of Cells of Neural Origin

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In Search of Gene Targets for Productive and Latent HSV-2

Infection of Cells of Neural Origin

A thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
University of Ottawa

In partial fulfillment of the requirement of the degree of
Masters in Science
Department of Microbiology and Immunology
Faculty of Medicine

by Irene Chan

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Abstract

Herpes Simplex Virus Type 2 (HSV-2) causes genital herpes and induces latency in nerve cells. Acyclovir is a guanosine analogue widely used for HSV-2 treatment. By investigating the effect of acyclovir and HSV-2 infection on gene expression, a number of genes that may be candidates for therapeutic targets of HSV-2 were identified.

HSV-2 was used to infect IMR-32 human neuroblastoma cells for 4 hours in the presence of acyclovir. RNA was extracted, and used as a template for both reverse transcriptase-polymerase chain reaction (RT-PCR)s using either $^{32}$P for filter microarray or fluorescent labels for glass slide microarray. Filter microarrays were spotted with 375 immune genes; glass slides were spotted with 19,000 human genes and expression sequence tags. Analysis of filter data using two statistical methods revealed up-regulation of eight genes: fibroblast growth factor receptor 2, interleukin-16, CXCR-5, interferon-alpha/beta receptor beta, ephrin-B1, endothelin-2, CD6, and interleukin-3 receptor alpha. Good correlation between gene modulation on both filter and glass slide microarrays was found.

IMR-32 cells were exposed to acyclovir for 4 hours, RT-PCR was performed with $^{32}$P, and the labeled product was hybridized with microarray filters spotted with 375 immune genes. Two methods of analysis revealed the up-regulation of cadherin-5, CXCR-5, and activated leukocyte adhesion molecule.

IMR-32 cells were infected with HSV-2 and incubated for 4 hours at either 37°C for productive infection or 40°C for latent infection. Two methods of analysis were performed on filter microarrays, revealing the up-regulation of macrophage-derived
chemokine, a chemokine involved in natural killer cell recruitment, in latently-infected cells.

HSV-2 challenge in the presence of acyclovir triggers up-regulation of many immune genes, whose effects include vasoconstriction and CD4+ T cell recruitment. The effects of acyclovir were investigated as a control for the HSV-2 experiments. Acyclovir furthermore was found to have effects on gene expression in neuroblastoma cells in addition to its well-known antiviral effect. As well, an increase in temperature, well known for its capability to induce latency, is associated with an up-regulation with a molecule involved in natural killer cell recruitment.
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Finally, thanks to my mother, Lydia Chan, for her patience and support, and my father, Francis Chan, for his guidance and counsel.
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<th>Description</th>
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<tbody>
<tr>
<td>ALCAM</td>
<td>Activated leukocyte adhesion molecule</td>
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<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>CAD</td>
<td>Cadherin</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediamine-N,N,N',N'-tetraacetic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>EST</td>
<td>Expression sequence tag</td>
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<tr>
<td>ET</td>
<td>Endothelin</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FGF R2</td>
<td>Fibroblast growth factor receptor 2</td>
</tr>
<tr>
<td>HFL</td>
<td>Human fetal lung</td>
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<tr>
<td>HHV</td>
<td>Human herpesvirus</td>
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<tr>
<td>HSV-2</td>
<td>Herpes simplex virus type 2</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s Modified Dulbecco’s Media</td>
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<tr>
<td>LAT</td>
<td>Latency-associated transcript</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>MDC</td>
<td>Macrophage-derived chemokine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PRR</td>
<td>Poliovirus receptor related protein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SRCRSF</td>
<td>Scavenger receptor cysteine-rich superfamily</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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Chapter 1: Introduction

The human body has evolved different methods for defending itself against microscopic foreign invasions: these include innate and adaptive immune systems. Innate immunity refers to non-specific defenses that protect against microorganisms, their toxic products, and inert microscopic particles. Examples of innate immunity include the skin, which forms a semi-waterproof boundary against the outside world; and mucus, which is a sticky secretion in which potential pathogens can become trapped and expelled by sneezing or coughing. As well, innate immunity includes the non-specific recognition and targeting of foreign particles by cells of the immune system. Phagocytes, for example, can engulf and kill common microorganisms. Furthermore, a class of lymphocytes known as natural killer (NK) cells can recognize and kill tumours and virus-infected cells, without the help of a specific antigen-specific receptor. Innate immunity decreases the number of organisms against which the adaptive immune system must fight.

Adaptive immunity refers to specific defenses mounted by the host against intracellular or extracellular microorganisms and their toxic products. These include antibodies against pathogen-derived antigens as well as cytotoxic T cells, which recognize and destroy cells that are infected with intracellular pathogens. Although innate immunity plays a significant role in the human immune system, adaptive immunity is pivotal in helping the host combat foreign organisms. The adaptive immune system consists of humoral and cell-mediated immunity.

The humoral response, characterized by the production of antibodies, typically helps defend against extracellular pathogens. The antibodies produced are tailored to
recognize specific epitopes present on the surface of the pathogen itself, or to recognize toxins produced by the pathogen.

By contrast, the cell-mediated response characterizes the recognition and destruction of host cells infected with intracellular pathogens. In these cases, the infected host cell exhibits a processed portion of the pathogen, which is recognized by specific lymphocytes. These lymphocytes kill the cell before the intracellular pathogen can replicate and infect other cells. Together, the adaptive and innate immune responses help protect the human body from infections.

1) Th1 and Th2 immunity

Two major types of lymphocytes exist: T and B. B lymphocytes differentiate into plasma cells, which secrete antibody; T lymphocytes play a number of different roles in immunity. Naive T and B cells circulate in the bloodstream and lymphatic system.

B cells can be stimulated to become plasma cells with the help of T cells, or independent of T cell help. T-cell independent B cell stimulation occurs when B cell receptors bind an antigen in such a way that allows cross-linking of the B cell receptors. This results in signal transduction via phospholipase C, protein kinase C, and tyrosine kinases. This cascade leads to the activation of transcription factors and production of proteins that lead to the differentiation of the B cell to a plasma cell [Middleton, 1998].

The first steps of T-cell dependent adaptive immunity take place when an antigen-presenting cell (APC) presents a foreign particle to a circulating T cell. APCs include dendritic cells, macrophages, and B cells, and APCs circulate in the lymph system. If an APC binds a T cell specific for the major histocompatibility complex (MHC)-antigen
complex, and co-stimulation occurs, the T cell becomes activated. Without co-stimulation, T cell anergy occurs and the T cell undergoes apoptosis [Paul, 1993].

Migration of lymphocytes to peripheral lymph tissues depends on cell adhesion molecules, which mediate cell-cell interactions. Cell adhesion molecules include selectins, vascular addressins, and integrins. These are expressed on cells of the immune system as well as by the endothelial cells, which line blood vessels [Butcher et al., 1999].

The conversion of naïve T cells to effector T cells requires the binding of an MHC molecule with an antigen. The MHC-antigen complex is then presented by one of the following APCs: a dendritic cell, macrophage, or B cell. There are two MHC classes: I and II. MHC I-antigen complexes are derived from pathogens that replicate within the host cell cytoplasm, while MHC II-antigen complexes are derived from pathogens or their products that are endocytosed by macrophages [Germain, 1994].

T lymphocytes may be further subdivided into two different functional groups and classified according to their surface molecule expression. The two major molecules represented on T cell surfaces are CD4 and CD8. During development, T lymphocytes usually express both CD4 and CD8, but most mature T cells express only one of these molecules. There is a small subset of T cells (5%) that does not express either of these molecules [Janeway et al., 1999].

MHC I complexes are presented to CD8+ T cells, which are stimulated to proliferate and differentiate into cytotoxic T cells. Cytotoxic CD8+ T cells recognize and destroy infected host cells. Naïve CD8+ T cells can then be stimulated to become cytotoxic T cells. Differentiation into cytotoxic T cells requires interleukin-2 (IL-2) in addition to antigen-ligand binding and CD28 co-stimulation. First, APCs can induce
differentiation of CD8+ T cells, which then produce IL-2 and drive their own proliferation. Second, a nearby, previously-activated CD4+ T cell can secrete IL-2 during the binding of CD8 to the MHC I-antigen complex; this can also activate the CD8+ T cell. Armed effector T cells do not require co-stimulation to function; this is advantageous for cytotoxic CD8+ T cells because often, infected cells do not express co-stimulatory molecules [Paul, 1993].

MHC II complexes are presented to CD4+ T cells, which are stimulated to proliferate and differentiate into two types of effector cells. The two Th categories are referred to as Th1 and Th2, and may be preceded by the cell type Th0. The activation of a naïve CD4+ T cell requires specific antigen-ligand binding as well as co-stimulation of B7 molecules by CD28. The subtypes of CD4+ T cells can be characterized by the release of different cytokines. Cytokines released by Th1 cells include IL-2, interferon gamma (IFN-γ), and lymphotoxin. Th2 cells release IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 [Chtanova & Mackay, 2001]. Th0 cells may produce both Th1 and Th2 cytokines [Del Prete, 1992].

Immunoglobulins (Igs) are antigen-specific proteins produced by plasma cells. There are various isotypes of Igs: IgA, IgD, IgE, IgG, and IgM. Isotypes are differentiated by structure and specificity. Th1 CD4+ T cells induce B cells to produce opsonizing IgG antibodies and induce macrophage microbicidal activity. Human IgG subtypes are numbered IgG1, IgG2, IgG3, and IgG4. Each of these subtypes has a specific role; for example, IgG1 and IgG3 are the subtypes most involved with complement activation. Th2 cells stimulate antigen-specific IgM production by naïve antigen-specific B cells, and may also induce the production of other antibody isotypes.
The antibodies induced by Th2 may have pathogen neutralizing activity, in contrast to the opsonizing antibodies induced by Th1 CD4+ T cells [Janeway et al., 1999].

2) Viruses and the immune system

The human immune system combats viruses with innate immunity as well as adaptive immunity. Innate immunity is the first line of defense against any foreign invader, and is especially important because adaptive immunity can require several days in order to function optimally. Viral infection can be stopped by physical barriers, such as skin. Enzymes such as lysozyme, present in tears, can degrade infectious particles. Sentinel cells release non-species-specific antiviral agents, such as interferons, which help cells to resist viral replication. As well, NK have no antigen-specific receptors, but can recognize and destroy virus-infected cells. [Scalzo, 2002].

Adaptive immunity, in the context of viral infection, refers to defenses that are specific to viral antigens, and helps defend the host from re-infection with a virus. It requires the clonal expansion of effector lymphocytes that are specific to the virus, and can result in life-long immunity against a pathogen. Adaptive immunity may include both the development of cytotoxic T cells and production of antibodies specific towards a foreign organism [Paul, 1993].

3) Herpes Simplex Virus (HSV)

The Herpes Simplex Viruses (HSV) are members of the Herpesviridae family. They are large, enveloped, double-stranded DNA viruses, which infect a large range of hosts throughout the animal kingdom. A representative virus particle is 150-250 nm in
diameter, and is comprised of two covalently-linked nucleic acid segments (long L and short S) and their associated proteins [Wadsworth et al., 1975], which include 100 nm diameter icosahedral capsid, tegument, and envelope. The envelope is derived from the host cell during the budding process, and therefore includes host cell proteins as well as viral glycoproteins.

At least eight herpesviruses are known to infect humans; these are known as human herpesviruses (HHV). Human herpesviruses include herpes simplex viruses (HSV) types 1 and 2 (HHV-1 and HHV-2), Varicella-Zoster virus (VZV, HHV-3), Epstein-Barr virus (EBV, HHV-4), cytomegalovirus (CMV, HHV-5), and also HHV-6, 7, and 8 (the latter being responsible for Kaposi’s sarcoma). [Mandell, 2000]

HSV-1 and HSV-2 share 50% identity at the DNA level and 70% identity at the protein level. Both are responsible for painful oral and genital ulcers, but typically, HSV-1 is associated with oral lesions while HSV-2 is associated with genital lesions. Both types induce acute infection, latency in neurons, and potential recurrence.

The HSV life cycle includes both acute infection as well as latency. In general, during an acute infection, the virus particle attaches to a permissive cell and interacts with host cellular molecules. A cascade of reactions ensues, which results in the fusion of the host plasma membrane with that of the virion. The nectins, also known as the poliovirus receptor-related proteins, have been described as being herpesvirus receptors [Geraghty et al., 1998]; hence, this family of proteins has also been named herpesvirus entry mediators (Hve). They have also been termed poliovirus receptor-related proteins (Prr). Specifically, HveB/Prr2 has been identified as a receptor responsible for mediating HSV-2 entry [Warner et al., 1998]. The virion then releases host shutoff factor (degrades
host RNA and shuts down host protein synthesis) as well as VP16 (a viral transcription factor), and then travels to the nucleus. Viral DNA enters the nucleus, where transcription and DNA replication occur. New capsids are also made in the nucleus. The virus matures and buds out of the nuclear membrane and exits the cytoplasm to infect other cells. One cycle of infectivity takes between 18-20 hours [Roizman & Knipe, 2001].

Three stages are necessary for entry of HSV into cells. First, the virus particle attaches to the cell. Then, the viral glycoprotein D (gD) interacts with a cell surface receptor. Finally, the viral envelope fuses with the cell membrane and the capsid-tegument enters the host cell [Taylor et al., 2002]. Each of these steps is discussed further below.

During the first step, viral glycoprotein C (gC), and to a lesser extent, glycoprotein B (gB), interact primarily with glycosaminoglycan substituents of the cell membrane [Shieh et al., 1992]. Although heparan sulfate is preferred for attachment, other moieties such as chondroitin sulfate can also be used at a lesser efficiency [Banfield et al., 1995]. The first interaction brings the virion into close proximity such that the second step can occur.

The second step involves the binding of gD with one of the following three types of receptors: tumour necrosis factor (TNF) family; immunoglobulin (Ig) family; and 3-O-sulfated heparan sulfate [Roizman & Knipe, 2001]. The latter of these receptors has been found to mediate HSV-1 entry exclusively [Shukla et al., 1999]. The other two groups have been found to mediate entry for both HSV-1 and HSV-2 [Terry-Allison et al., 2001; Whitbeck et al., 1997].
The TNF family of receptors for gD includes herpes virus entry modulator A (HveA). This receptor is present primarily on lymphoid cells, and therefore is unlikely to be the main mediator of entry into human cells, since herpesviruses are known to infect most types of cells and not predominantly cells of lymphoid origin [Roizman & Knipe, 2001].

Because they mediate intercellular adhesion at the adherens junction, “nectin” is another term used to describe the immunoglobulin family of receptors [Roizman & Knipe, 2001]. Four isoforms are known at present, though only nectins 1 and 2 have been implicated in HSV entry. Nectin-1 is also known as Prr1 and HveC; nectin-2 is also known as Prr2 and HveB [Geraghty et al., 1998]. Nectins 1 and 2 also play a role in cell-cell spread of wild-type HSV-1 [Cocchi et al., 2000].

The third step of virus entry is the fusion of the viral envelope with the host cell membrane. It is believed that glycoproteins D [Ligas & Johnson, 1988], B [Sarmiento et al., 1979], and the H-L heterodimer [Forrester et al., 1992] are needed for this step. Entry of HSV into the cell results in a cycle of acute infection that culminates with the replication of HSV virions and the egress of viral particles from the cell via budding. In this way, the viral particle derives its envelope from the host cell.

The infected cell can undergo structural changes such as alterations in chromatin and cell membranes, and the rearrangement of its cytoskeletal network. As well, the host cell can form intranuclear inclusion bodies. Apoptosis has been blocked by some HSV strains, and has also been induced by many HSV-1 mutants, though these mechanisms remain poorly understood. Finally, the virus can travel to the dorsal root ganglia of the human host and establish a lifelong latency [Taylor et al., 2002].
4) Latency

HSV typically establishes latency in the dorsal root ganglia. The virus reaches this area by travelling in a retrograde fashion along a neuron that innervates the infected area. During latency, there is no replication of infectious virus particles. The HSV-1 genome becomes circularized [Rock & Fraser, 1983] and some latency transcripts (LATs) are produced. Occasionally, HSV is reactivated due to an interaction between host, virus, and other environmental factors. During reactivation, the virus travels in an anterograde fashion to epithelial cells close to the site of primary infection [Roizman & Knipe, 2001]. The recurrence can be asymptomatic, or manifest as an acute lesion, whose severity varies considerably.

The latent phase of HSV adds to the challenge of diagnosis and treatment. The typical chronology involves a primary infection, characterized by vesicular lesions, followed by cycles of latency and recurrence. After primary infection, HSV enters local sensory neurons and travels via microtubule transport to the cell nucleus, whereupon its genome undergoes circularization [Rock & Fraser, 1983]. Following primary infection, HSV undergoes retrograde nerve transport and establishes latency in the dorsal root ganglia. The virus persists in the dorsal root ganglia during the latent period, but no acute viral infection is observed [Taylor et al., 2002].

Much about virus reactivation remains unknown, but certain triggers have been described. Exposure to ultraviolet light (UV) B, for example, has been shown to induce HSV reactivation [Rooney et al., 1992]; furthermore, use of sunscreen has been shown to decrease the likelihood of herpes recurrence [Rooney et al., 1991]. As well, the absence of several cytokines seems to play an important role. Mice that are IFN-γ deficient, and
those that are TNF-α deficient, have a higher likelihood of HSV reactivation after exposure to UV B light [Minami et al., 2002].

The manifestation of recurrence can fall anywhere between the range of painful vesicular lesions to asymptomatic, depending on many factors including the immune status of the infected individual [Mandell, 2000]. The extent of viral reactivation also depends on the severity of the primary infection. If a greater amount of tissue had been initially infected, the virus would have access to an increased number of sensory neurons in which it could establish latency. [Roizman & Knipe, 2001].

Establishment of latency is characterized by the production of LATs [Stevens et al., 1988]. These are derived from inverted repeat sequences in the HSV genome. Evidence suggests that LATs down-regulate expression of lytic genes: reduction of LAT transcription is associated with decreased virus reactivity [Hill et al., 1990]. The precise roles of LAT are not very clear, but may play a role in the following conditions: (1) inhibition of acute viral infection; (2) facilitation of viral reactivation in the presence of triggers; (3) maintenance of viral latency; and (4) prevention of neural apoptosis [Roizman & Knipe, 2001].

5) HSV and the immune response

Lesions resulting from HSV infection can fall into three categories. Firstly, the lesion could be a primary infection, which denotes the first ever exposure to any herpes simplex virus. The primary infection can spread extensively, unchecked by pre-existing antibodies. Typically, it consists of multiple vesicular lesions [Drew, 1994]. Secondly, the lesion could be a recurrence. Recurrences are typically less severe and are localized to
the area (or close to the area) of previous infection. Third, the lesion could be a first episode infection. A first episode infection occurs in an individual who has previously been exposed to HSV but not the same type as the current infection; e.g. the individual has anti-HSV-1 antibodies but is currently exhibiting lesions due to infection with HSV-2. The first episode infection is not as extensive as the primary infection because HSV-1 and HSV-2 share some homology. Furthermore, marked cross-reactions have been observed between HSV-1 and HSV-2 molecules [Lehtinen, 1985], including glycoprotein B and p45 nucleocapsid protein [Balachandran et al., 1987]. Hence, protection against one type of HSV offers some protection against the other type.

The host immune response to herpesvirus infection involves both Th1 and Th2 immunity. The Th1 response includes CD4+ T cell induction of HSV antibodies, induction of IFN-γ, activation of macrophages and production of IL-2, resulting in long-lasting immunological memory to viral antigens. The Th2 response includes CD4+ T cells stimulation of neutralizing IgM and IgG1 antibodies [Janeway et al., 1999].

The T cell response to HSV-2 infection includes responses mediated by CD4+ and CD8+ lymphocytes. In addition to the activation of B cells to produce antibody, CD4+ T lymphocytes have been reported to offer host resistance upon lethal genital challenge with virus [McDermott et al., 1989]. Memory CD4+ T cells have been shown to persist in mucosal membranes for at least 1.5 years after exposure, though this mucosal immune memory is not seen subsequent to administration of an HSV vaccine [Gallichan & Rosenthal, 1996].

The CD8+ T lymphocyte response ranges from the destruction of infected cells by armed T cells to the induction of memory cells. Armed cytotoxic T cells recognize and
kill virus-infected cells. The killing mechanism involves the release of membrane damaging compounds such as perforin [Trapani & Smyth, 2002]. Memory CD8+ T cells induce a faster and efficient immune response when the host is challenged with a known pathogen, and can even prevent systemic HSV-2 infection of IL-2 receptor deficient mice [Tsunobuchi et al., 2000].

Th1 immunity in response to HSV-2 challenge includes the release of IL-2 and IFN-γ [Carmack et al., 1996]. Treatment of IL-2 knockout mice with recombinant IL-2 decreases the ocular replication of HSV-1 [Ghiasi et al., 1999]. Although IFN-γ is not necessary for viral clearance from the genital tract, it may decrease the amount of HSV-2 present such that fewer particles reach the sensory ganglia for latency [Milligan & Bernstein, 1997].

Th2 response to HSV-2 infection includes the release of ILs 4, 5, and 10 [Carmack et al., 1996]. The secretion of IL-4 seems to favour virus replication: HSV-1 replication in IL-4 knockout mice was increased when the mice were exposed to this cytokine [Ghiasi et al., 1999]. IL-4 also seems to interfere with some aspects of Th1 immunity, such as the inhibition of TNF-α secretion [Paludan et al., 2000]. The role of IL-5 in HSV-2 infection is poorly described, but the decreased production of this cytokine is correlated with progression of HIV disease [Resino et al., 2001]. IL-10 production is associated with a decrease in pro-inflammatory response and subsequent decrease in extent of disease in the in vivo HSV-1 infected murine cornea model [Yan et al., 2001].

Upon HSV-2 infection, a rise in IL-10 and IFN-γ is accompanied by a fall in serum IL-4 concentrations in humans [Carmack et al., 1996]. There is also an increase in
IL-12 production, which synergizes with IFN-γ to produce a strong antiviral effect [Malmgaard et al., 2000]. This would lead to a decrease in pro-inflammatory response and less severe disease and enhancement of effects that would favour virus clearance.

B cells play a role in the host immune response to local HSV-2 infection. B cells differentiate into plasma cells, which produce antibodies specific to HSV-2 antigens; for this reason, recurrences of genital herpes tend to be localized and of shorter duration than the primary infection [Drew, 1994]. When infected with attenuated HSV-2, B-cell-deficient mice develop more extensive viremia and local inflammation [Harandi et al., 2001]. B cell mediated protection against HSV-2 seems most important in the 20 hours following re-infection, but other factors such as T cell immunity become more important during the 20-48 hours after infection [Parr & Parr, 2000].

HSV-2 is capable of infecting macrophages. Virus-infected macrophages secrete TNF-α, which results in the production of nitric oxide [Paludan et al., 2000]. The production of nitric oxide in the context of viral infection may have both beneficial and deleterious effects on the host. Beneficial effects include the inhibition of viral replication via interference with proteases and polymerases, while deleterious actions include the suppression of Th1 cells and oxidative injury [Torre et al., 2002].

HSV has evolved several mechanisms to evade the immune system, which increase its chance of survival. For example, CD4+ T cell proliferation and secretion of cytokines is inhibited upon infection with HSV in humans in an in vitro experiment [Barcy & Corey, 2001]. As well, HSV-1 inhibits CTL function in seropositive individuals, such that there is a significant decrease in the lysis of target cells [Posavad et al., 1994]. Furthermore, herpes simplex viruses induce Fc receptor expression on the
surfaces of infected cells, which enables interference with the host innate and adaptive immune system. The induction of gE, an IgG-binding glycoprotein, and gC, a complement-interacting glycoprotein, enable the virus to block antibody-dependent cellular cytotoxicity and complement formation [Lubinski et al., 2002].

6) Acyclovir

A number of treatments are available for patients infected with HSV-2. Acyclovir was the first deoxyguanosine analogue used to treat herpesvirus infection, and remains an effective agent against the virus. Typically it is administered orally or intravenously; in some cases topical acyclovir is used, but is much less effective if effective at all [Mandell, 2000]. This drug enters all cells, but interferes with the replication of HSV-2 in infected cells and usually does not affect growth of uninfected cells. In an HSV-infected cell, viral thymidine kinase and other enzymes phosphorylate acyclovir to yield acyclovir triphosphate, which is present at much higher concentrations in HSV-infected cells (40-100x) [Mandell, 2000]. The result is a gradient that enhances acyclovir concentration in HSV-2 infected cells.

Acyclovir triphosphate lacks the 3’ OH group that is required for DNA elongation. Incorporation of acyclovir into the growing nucleic acid strand can thereby result in the inactivation of viral DNA polymerase [Mandell, 2000].

Oral acyclovir exhibits relatively low bioavailability, at 15-21% [Mandell, 2000]. In one study, after 200-mg doses every four hours up to five times a day, serum levels of acyclovir peaked between 1.5 to 1.75 hours after oral administration. Levels rose as high as 4.0 micromolar (0.9 µg/mL) and an average high of 2.5 micromolar (0.6 µg/mL) [Van
Dyke et al., 1982]. Acyclovir can cross the blood-brain barrier, although levels are lower in cerebral spinal fluid (CSF) than in serum [Mandell, 2000]. Clearance of acyclovir is mediated by the kidney. Its half-life is approximately 2.5 hours, though this value is higher if the individual exhibits kidney dysfunction [Mandell, 2000].

Although newer treatments with increased oral bioavailability (such as valacyclovir and famciclovir) are currently available, these are more expensive. As such, acyclovir remains an effective treatment. Administration of 200-mg doses of acyclovir, five times daily for 5 days, before or during the outbreak of first lesions, is associated with reduced pain, itching, duration of viral shedding, time to lesion crusting and healing, and formation of new lesions [Nielsen et al., 1982]. Newer drugs, can also be used: 1 g of valacyclovir twice daily for 10 days, or 750 mg of famciclovir three times daily for 7-10 days is also effective for first episode treatment of herpes [Mandell, 2000].

7) Microarray analysis

Microarray analysis is a recently developed technique that was first described in 1995 [Schena et al., 1995]. Because it is capable of assessing such a large scope of genes in a relatively minimal amount of time, it is a very powerful tool. The microarray itself is a collection of cDNAs of genes of interest, covalently bound to nitrocellulose filters or spotted onto glass slides. The genes are usually spotted in duplicate such that artifacts and microarray abnormalities can be more easily detected.

There are two microarray techniques, one using a radioactive signal and the other using a fluorescent signal. Both methods involve extracting RNA from a sample, performing RT-PCR incorporating the labeled nucleotides, hybridizing the labeled
cDNAs to the microarray, and detection and analysis. Signals are detected by measuring radioactivity ($^{32}$P) or fluorescence (Cy3 and Cy5).

The radioactive microarray system uses two filters: one for hybridization with the control product and another for that of the experimental condition. Detection of radiolabeled cDNA is performed via autoradiography (phosphor screen) with a phosphorimager. Densitometry is then performed on the dots to reveal up- or down-regulation of genes of the sample filter, compared to the control.

Densitometry consists of computerized quantitation of pixels using a specialized program, such as ImageQuant (Amersham Biosciences, Baie d'Urfé, PQ, Canada). This quantitation can then be exported into a spreadsheet, where adjustments can be made (including the deletion of values which are due to foreign artifacts on the filter, and normalization of background by subtraction of negative control values). The gene expression of a certain sample at a specific time point can then be analyzed in a semi-quantitative manner.

A series of mathematical transformations must then be performed on the filter data before gene expression is quantitated, in order to normalize the background signal between the test sample and control filters. Some techniques, currently under development, involve the subtraction of background radioactive signals taken from the vicinity of gene signals. Older techniques involve the subtraction of values from negative control genes. Further possible calculations could include calculation of the median of duplicate spots and calculation of fold or times change between control and experimental conditions for any given gene.
The glass slide microarray system only requires one glass slide for both control and experimental conditions. Different fluorochromes are used to label the test sample and the control RT-PCR products. The signals from the two samples are detected by lasers, which operate at different wavelengths. Because the two fluorochromes compete for the same sites on the glass slide, the ratio between the signals can also be calculated. The labeled cDNA is detected by laser, and the varying intensities of the fluorochromes can then be statistically analyzed.

Glass slide analysis simply involves the interpretation of the ratio yielded by the fluorescent signals. Dividing the intensity of the signal from the Cy5 laser by that of the Cy3 laser yields a ratio that can be interpreted as gene up- or down-regulation. The ratios from each duplicate spot are also assessed for reproducibility.

8) Targets for HSV-2 prophylaxis and treatment

In addition to drugs that inhibit viral replication, other methods to combat HSV-2 are being investigated. These include the use of immunomodulators such as imiquimod [Harrison et al., 1994] and resiquimod [Bernstein et al., 2001], DNA vaccines [Baghian et al., 2002] and recombinant subunit protein vaccines, such as those against glycoproteins B and D [Straus et al., 1997; Corey et al., 1999; Kohl et al., 2000]. DNA vaccines have not yet been tested in humans, but several have received favourable testing results in mice and guinea pigs [Bernstein et al., 1999; Gebhard et al., 2000]. The vaccine against glycoproteins B and D has met with limited success, as antibody titres are not always adequate in individuals who are immunized with this vaccine [Kohl et al., 2000]. For unknown reasons, challenge with these glycoproteins, which are expressed on the viral
envelope, may not induce sufficient amounts of antibody [Kohl et al., 2000]. Furthermore, Corey et al. (1997) found that antibody titres alone may not be sufficient for protection against HSV-2. More recently, a vaccine using glycoprotein D has met with limited success [Stanberry et al., 2002]. The efficacy of this vaccine in HSV-seronegative women was 38-42%. Although antibodies were induced in female subjects who were seronegative for HSV-1 and HSV-2, antibodies were not induced in male subjects [Stanberry et al., 2002]. These results suggest that gender, susceptibility, frequency of viral acquisition, genetics, previous viral exposure, and their interactions play a significant role in determining the efficacy of these antiviral modalities.

9) Rationale for experiments performed

The incidence and spread of HSV-2 have increased in the last two decades. One study found the incidence of HSV-2 to be 40% in five STD clinics [Gottlieb et al., 2002]. A comparison of genital herpes prevalence in two studies of American adults has shown that the incidence had risen 30% between 1980 and 1994 [Armstrong et al., 2001]. Despite the public health impact and disease burden of this disease, very little is known about the induction and maintenance of latency in infected individuals. Little is also known about the reason why infected neurons are not recognized by the immune system despite harboring the virus. Latent viruses require the expression of few viral genes for the maintenance of this state [Suzuki & Martin, 1989]. Shedding light into the nature and mechanisms of HSV-2 latency in infected persons could help identify strategies for therapeutic use. In order to identify gene targets for potential therapeutic intervention, we
designed an experimental approach in an *in vitro* model of HSV-2 acute infection and latency.

Infection and latency in sensorial neurons characterize human infections with this virus. This infection may result in activation and symptomatic recurrence of the virus despite the presence of an immune response characterized by antibodies and memory and cytotoxic cells that are specific to HSV-2. The ontogeny of both the immune and the nervous systems derives from the ectoderm; both systems serve a similar role [Chun, 2001]. They help with the interaction with the environment and by necessity they have functional similarities. Many biological molecules are produced in common by both systems [Chun, 2001]. Immune molecules released by neural cells or present in the cell surface are involved in the communication between these systems. By studying the global gene expression, but in particular, immune genes expressed in infected neurons, one might expect to discover key molecules that may suppress or induce communication between infected neurons and the immune system. This effect could be a viral strategy for survival. These potential therapeutic targets may be able to allow the viral infection to be seen by or become invisible to the immune system. Their identification could help design strategies to eradicate latency in neurons by allowing the immune system to identify and destroy infected cells. The results of these experiments could shed light on potential cellular strategies to induce or suppress stress and infection signals to the immune system.

The recurrent nature of human infections caused by HSV-2 and its cell tropism and latency in neurons is the rationale for the selection of a human neuroblastoma cell line as an *in vitro* model of HSV-2 infection. Although HSV-2 can infect a number of
different cell types, it is the persistence of the virus in neurons that is key to the virus' survival in the host. The rationale for using a human neuroblastoma cell line in this experimental approach is that this system has been used successfully in the past to induce and study HSV-2 latency [Yura et al., 1987]. In addition, this preliminary work could be the basis for further analysis in an in vivo model of HSV-2 infection.

Although the neuroblastoma cells are immortalized and their genetics are different to normal in vivo neurons, they are a good HSV-2 infection model because they represent cells of neural origin, akin to those that harbour the HSV-2 genome during latency. As well, it is easier to manipulate them than live animals, and it is logical to perform initial experiments with a cell line.

In order to determine the cellular genes that could be affected by the initial stages of infection with HSV-2, neuroblastoma cells were infected with the virus and after four hours the mRNA was isolated and converted to labeled cDNA. This population of cDNAs was examined by microarrays to determine the relative ratios of expression between infected and uninfected cells. Because HSV-2 can hijack the entire cell machinery if viral replication is not impeded [Taylor et al., 2002], a specific antiviral agent was used to limit the extent of the infection to the first stages of virus replication. Any attempt of the virus to replicate would be curtailed by the presence of acyclovir [Mandell, 2000]. Infection with HSV-2 in the presence of acyclovir does, however, trigger an immune response [Halford et al., 1997]. Therefore, at the four-hour timepoint, viral early genes and some cellular genes would be produced even in the presence of a nucleoside analogue. Identification of genes that are differentially expressed early in infection could help pinpoint candidates that may be likely to enhance the host immune
response; a subset of these genes could be investigated for antiviral therapy. The first experiment can thus be summarized as follows: human neuroblastoma cells, four hours, 100 μg/mL acyclovir, 2 MOI of HSV-2 vs. control.

After studying the acute HSV-2 infection, the ability of acyclovir to induce or suppress cellular genes was questioned. Thus, the second series of experiment investigated whether acyclovir alone had any effects on neural gene expression. It is important to confirm that acyclovir does not induce or suppress gene expression by itself, since the first experiment entailed the exposure of neuroblastoma cells to both HSV-2 and the drug. The results of this experiment would then be compared to the experiment that investigated the effect of HSV-2 infection in the presence of acyclovir. Therefore, the second series of experiments can be summarized as: human neuroblastoma cells, four hours, 100 μg/mL acyclovir vs. control.

Latent infection was investigated for comparison to acute infection. During a latent infection, the virus persists in neurons whilst being undetected by the immune system. It is well known that after an acute infection there is an intense immune response targeted to the site of virus replication. In contrast, during latency, the immune system is unable to recognize and eliminate infected cells. Comparison between latent and acute infection could pinpoint genes whose differential expression contributes to HSV-2 being able to survive in neurons without triggering an immune response.

There is a well-established HSV-2 latency model of infection in human neuroblastoma cells. HSV-2 infected cells undergo acute infection at 37°C while at 40°C the virus becomes latent [Yura et al., 1987]. By examining the genes that are differentially expressed during latency and acute infection, targets for forcing viral
latency or inhibition of the immune response may be identified. The induction of HSV-2 latency or the induction of an immune response that could recognize infected neurons could be important areas for therapeutic development. In brief, the Experiment 3 series can be summarized as follows: human neuroblastoma cells, four hours, 2 MOI of HSV-2, 37°C vs. 40°C.

10) Objectives

The goals of these investigations are to assess the differential gene expression of neural cells *in vitro* as they are subjected to infection with HSV-2 and acyclovir, using a neuroblastoma cell line as a model system. Furthermore, the differential gene expression of neural cells as they are infected with HSV-2 under conditions of acute infection (37°C) as compared with latent infection (40°C) will be investigated.

Specifically, the objectives for this study were the following:

1) To investigate the early effects of HSV-2 infection on neuroblastoma cells in the presence of acyclovir.

2) To investigate the effect of acyclovir exposure on neuroblastoma cells in the absence of HSV-2.

3) To investigate the differences between acute and latent HSV-2 infection of neuroblastoma cells.
Chapter 2: Materials and methods

1) Cell culture and culture media

Human neuroblastoma cells were obtained from the American Type Culture Collection (ATCC), catalog designation IMR-32. Cells were stored frozen in liquid nitrogen (frozen stock) or at −80°C (refrozen cells) when not in use. Growth media consisted of Iscove’s Modified Dulbecco’s Media (IMDM), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma), 1 x 10^2 IU/mL penicillin (Sigma, Mississauga, Canada), and 1 μg/μL gentamicin (Sigma, Mississauga, Canada) per mL of media. Cells were grown as a monolayer in T-75 flasks (Falcon) at 37°C (unless otherwise specified) with 5% CO₂.

For subculture, cells were washed twice with phosphate-buffered saline (PBS), washed once with a commercial solution containing 0.5 g/L porcine trypsin, 0.02% EDTA (Sigma, Mississauga, Canada) and incubated for one minute with 1 mL of 0.5 g/L porcine trypsin, 0.02% EDTA solution. Cells were detached by tapping of the flask three times. Trypsin-EDTA was quenched with 9 mL growth medium. Cells were centrifuged at 443xg (1600 rpm) for 5 minutes at room temperature (22°C), washed twice with PBS, resuspended in growth medium, and plated. Cells were allowed to attach for a minimum of 24 hours before media was changed.

Growth media was changed every 2-3 days, and cells were subcultured when a confluence of >60% was reached. Subculturing was not carried out past the 15th passage, which corresponds to the 51st passage (see below for cell line details).
2) Cell lines and virus strains

a) Human neuroblastoma, IMR-32

IMR-32 is an ATCC human neuroblastoma cell line isolated from the abdominal tumour of a 13-month-old Caucasian male during exploratory surgery in 1967. It was grown, characterized at different passages, and finally submitted to ATCC at passage 36, which represents a stable karyotype of 49. IMR-32 contains two extra, large marker chromosomes which are unlike any other human chromosome, contains an extra C group chromosome, and is missing one copy of chromosomes #1 and #16 [Tumilowicz et al., 1970].

This cell line consists of two morphologically distinct types of cells, one of which seems to differentiate from the other. The vast majority of cells (hereby to be referred to as Type A, consisting of >95% of culture) are fibroblastic or teardrop-shaped, neuroblast-like, and highly light-refractile. This type of cell often forms clusters, or focal accumulations. The remaining cells (Type B), which occurred in very small numbers, were much larger and poorly light-refractile. Type B cells were initially observed due to their decreased susceptibility to trypsin digestion in comparison to Type A cells. Species specificity tests performed on Type B cells confirmed that they were of human origin, and chromosomal analysis of these cells confirmed that all contained the two distinct marker chromosomes [Tumilowicz et al., 1970].

The characterization of IMR-32 cells has revealed that its karyotype is subject to change, depending on the passage of cells. At passage 10, cells with karyotypes 46 (normal) and 48 were present. The two extra chromosomes were morphologically
dissimilar to any other human chromosome; they were much larger and displayed a
submedian centromere. Some cells with karyotype 48 had only one chromosome #1,
while others retained two copies [Tumilowicz et al., 1970].

At the 24th passage, there were no more cells with the normal karyotype of 46. At
the 35th subcultivation, the absence of normal cells remained, but the majority of cells
represented a karyotype of 49. Most of these cells displayed the two marker
chromosomes, and a deleted copy of chromosome #1. Also, one copy of chromosome
#16 was observed, and there were two additional C group chromosomes (i.e. a
chromosome numbered 6-12 by the Denver convention) [Tumilowicz et al., 1970].

In the 56th subcultivation, the majority of cells remained at karyotype 49, but two
sublines of 48 and 50 chromosomes appeared. Cells of karyotype 50 were
chromosomally similar to those of karyotype 49, with the addition of another
chromosome to the C group [Tumilowicz et al., 1970].

Although IMR-32 has been subcultured to the 83rd passage, they were not
subcultured past the 51st passage for use in these experiments for fear that they would
undergo more chromosomal changes. The 56th passage has been observed to have yet
another extra C group chromosome [Tumilowicz et al., 1970].

There are established HSV-2 infection latency models using this cell line. By
increasing the temperature of culture from 37°C to 40°C, the HSV-2 infection becomes
latent [Kondo et al., 1990].
b) Human fetal lung cells

The human fetal lung (HFL) cells used are designated HF-62-L, and were developed in the 1970s by Mr. Bryce McCulloch of the Regional Virus Laboratory at the Children's Hospital of Eastern Ontario in Ottawa. They were derived from lung tissue from human embryos and are susceptible to HSV-2. Upon infection with HSV-2, HFL cells lose their characteristic fibroblastic shape and form blebs and syncytia. Because HFLs are HSV-2-susceptible and are of human origin, they were used to amplify the HSV-2 virus. Propagation of HFLs was not carried out past the 10th passage.

c) HSV-2, G strain

HSV-2 strain G, designated ATCC VR-734, was used. It is capable of infecting Vero, Hep-2, and other tissue cell lines. It was originally isolated from a human genital infection [ATCC, 2002]. The cytopathic effect (CPE) caused by HSV-2 strain G in susceptible cells consists of medium-sized rounded clumps interconnected by strings of single rounded cells, as first described by Ejercito [Ejercito et al., 1968].

3) Virus propagation

HFL cells were grown to 95% confluence in two B-150 Falcon flasks, in growth media. The growth media was replaced by 5 mL of maintenance media, which consisted of IMDM supplemented with 2% heat-inactivated FBS, $1 \times 10^2$ IU/mL penicillin, and 1 μg/μL gentamicin per mL of media. HSV-2 was added to one flask at multiplicity of infection (MOI) between 0.01-0.05. The other flask was not infected with the virus, but was used as a control. After one hour of virus adsorption, an additional 16 mL of
maintenance media was added. The virus was allowed to propagate for three days, or until CPE was evident in the entire infected flask. The flasks were then exposed to three freeze-thaw cycles at -80°C and 4°C (on ice) in order to liberate the maximum amount of virus particles from the cells. After the third thaw, the supernatant was removed and centrifuged at 443xg (1600 rpm) for 5 minutes at 4°C to remove HFL cell particles and debris. The supernatant was then titered and frozen at -80°C.

4) Virus titration assay

HFL cells were plated in a 24-well Falcon plate and allowed to grow to 100% confluence. All virus titrations were performed one day after cells had reached 100% confluence.

Virus was thawed on ice and serial dilutions were made using maintenance media as diluent. All solutions were vortexed to ensure complete mixing. Dilutions of 10^4 to 10^8 were used for virus titration, and a negative control (diluent only) was included. Samples were titrated in quadruplicate. Two hundred μL of virus suspension (or negative control) was applied to cells for 1 hour, and then 1 mL of 1:1 methyl cellulose: nutrient media overlay was applied for three days. During these three days, the cells were incubated at 37°C and 5% CO2. The plate was microscopically observed every day, plaques were counted on Day 2 and verified on Day 3 such that any new plaques would not be missed. On Day 3, virus/methyl cellulose was removed, the plate was stained with crystal violet/formalin solution, and the plaques were counted by microscopy. Crystal violet is a basic dye that permeates healthy cells. It is believed that either the dye binds to
nucleic acid, or to anionic components inside cells [Popescu & Doyle, 1996; Llewellyn, 1998].

Nutrient media

20% Eagle’s medium (Earle’s + glutamine 10X), 20% heat-inactivated FBS, and 6.67% NaHCO₃ (7.5%) in demineralized, double-distilled H₂O. 1 x 10² IU/mL penicillin, and 1 µg/µL gentamicin per mL of media (optional).

1% Methyl cellulose

1% methyl cellulose powder 4000 centipoise (w/v) in demineralized, double-distilled H₂O.

5% Crystal violet stock solution

5% Crystal violet powder (w/v) in 100% ethanol.

Working staining solution

20% of 5% Crystal violet stock solution and 5% of 40% formaldehyde added to 75% of 0.85% NaCl. [Hsiung, 1994]

5) Infection of neuroblastoma with acyclovir and HSV-2

Neuroblastoma cells were grown to 100% confluence in growth media.

Experiments were performed the following day to ensure that confluence had been reached.
Growth media was removed from the flasks and replaced with 5 mL maintenance media. Ten μL of 1 mg/mL acyclovir was added to each flask (final concentration 2 μg/mL). HSV-2 was thawed slowly on ice over at least 1 hour and added at an MOI of 2 in the experimental flask. The final volume of the media/acyclovir/(virus) solution was 5 mL. The neuroblastoma cells were allowed to incubate in this solution for 1 hour.

After the one-hour adsorption, 20 μL of 50 mg/mL acyclovir and 10 mL IMDM supplemented with 2% FBS, 1 x 10^2 IU/mL penicillin, and 1 μg/μL gentamicin per mL of media was added to each flask. The cells were allowed to incubate for an additional 3 hours.

After a total of four hours of incubation with virus (or control), the neuroblastoma cells were detached using trypsin-EDTA (method described above). Cells were washed twice with sterile PBS (pH 7.2, Gibco, Burlington, Canada) and then frozen at −80°C until microarray analysis was performed.

6) Electron microscopy

Uninfected and HSV-2 infected neuroblastoma cells were harvested after 24 hours of incubation at 37°C, 5% CO₂. Trypsin-EDTA was used to detach cells from the plate (method described above). Cells were washed twice with 5 mL PBS and centrifuged for five minutes at 443xg (1600 rpm). Cells were then resuspended and centrifuged in 1.5 mL of glutaraldehyde fixative (2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.3). Cells were kept at 4°C before electron microscopy was performed.
Electron microscopy was performed with a JEOL STM-10 transmission electron microscope. Micrographs were taken by Ian Robb of the Pathology department at the Children’s Hospital of Eastern Ontario.

7) RNA isolation

Total RNA was isolated using the Qiagen RNA Midi Extraction Kit (Mississauga, Canada). Two hundred µL RNase-free water was used to elute the column for the final step. The RNA was then quantitated using A260/A280 spectral absorption methods [Sambrook et al., 1989] and frozen at −80°C until needed. RNA samples were thawed on ice for an hour prior to the RT-PCR step. Microarray experiments always used first-elution RNA fractions.

8) Microarray analysis

a) Filter microarray

Panorama Human Cytokine Gene Arrays used in this research for radioactive microarray studies were obtained from Sigma-Genosys (Oakville, Canada). They are filters containing 375 cytokine-related genes each, as well as 9 housekeeping genes, and positive genomic DNA and negative controls. All genes were spotted on nitrocellulose in duplicate. These filters were chosen because they focused on immune-related genes. Gene clusters represented on these microarrays include the following: adhesion molecules; angiogenic factors; cell surface proteins; chemokines; chemokine receptors; cytokines; cytokine receptors; binding proteins, epidermal growth factors; ephrins; ephrin receptors; fibroblast growth factors; integrins; interleukins; interleukin receptors;
neurotrophic factors; nitric oxide metabolism proteins; proteases and related factors; TGF superfamily members, TNF superfamily members; and orphan receptors.

Two µg of RNA from each sample was used for each filter microarray.

i) RT-PCR

First, RT-PCR was used to generate $^{32}$P-labeled cDNA. Four µL of Human Cytokine cDNA Labeling Primers were added to 2 µg of total RNA, such that the volume totaled 15 µL. The mixture was heated to 90°C for 2 minutes and ramped to 42°C over 20 minutes. Once the mixture has reached 42°C, the following reagents were added to each sample, in the form of a mastermix, to a final volume is 30 µL: 20% of 5X Reverse Transcriptase Buffer; 3% of 10 mM dATP; 3% of 10 mM dGTP; 3% of 10 mM dTTP; 1% of 100 µM dCTP; 10% of 10 µCi/µL [α-$^{32}$P] dCTP; and 7% of 25 U/µL Reverse Transcriptase. Mixtures were carefully mixed by pipetting and incubated at 42°C for 2-3 hours.

Unincorporated [α-$^{32}$P] dCTP was removed by applying the mixture to a Sephadex G-25 gel filtration spin column. The column was resuspended by inversion and suspension buffer was drained by gravity. Excess buffer was removed by centrifuging the column in a 15-mL tube for 2 minutes at 1100xg, 20°C. The sample was then added to the column and centrifuged for 4 minutes at 1100xg. The eluate, consisting of $^{32}$P-labeled cDNA, was saved.
ii) Microarray and detection

Salmon testes DNA was added to Sigma-Genosys Hybridization Solution (HS), which was then pre-warmed to 65°C. The eluate was added to this solution, which was heated to 90°C for 10 minutes to denature the cDNA. HS consisted of the following reagents: 5X SSPE; 2% of SDS; 5X of Denhardt’s Reagent; and 100 μg/mL of sonicated, denatured salmon testes DNA as provided by the manufacturer.

Prior to hybridization, the microarray filters were rinsed at room temperature in 50 mL 2X SSPE for 5 minutes. Hybridization oven was pre-warmed to 65°C. Filters were pre-hybridized in 5 mL HS/salmon DNA for at least 1 hour at 6 rpm, with roller bottles. Pre-hybridization was performed in order to decrease non-specific binding, and therefore decrease background signal. Hybridization Solution was decanted and the 32P-labeled cDNA/HS mixture was added to the roller bottle with filter microarray, and allowed to hybridize for 12-18 hours at 65°C. Wash Solution I (WSI) consisted of the following reagents: 0.5X SSPE, and 1% SDS. Wash Solution II (WSII) consisted of the following: 0.1X SSPE, and 1% SDS.

Arrays were washed three times with 50 mL of room-temperature WSI. Bottles were inverted by hand and decanted. WSI was then pre-warmed to 65°C. Arrays were washed twice with 100 mL of WSI, which was added to the roller bottles and allowed to wash for 20 minutes in the hybridization oven (65°C) at 6 rpm. WSII was pre-warmed to 65°C. Arrays were washed with 100 mL of WSII at 65°C for 20 minutes in the hybridization oven.

The arrays were then removed from the roller bottles and air-dried for 1 minute. The filters were then wrapped in plastic wrap and 32P was detected using a STORM
phosphorimager and ImageQuant. Accurate densitometry was ensured by carefully
placing circles around the dot signals. The data was then imported into Microsoft Excel
97 and analyzed.
b) Glass slide microarray

The glass slides used for fluorescent microarray analysis contain 19,000 human genes and ESTs. The glass microarrays were spotted by the Toronto Microarray Consortium (Ontario Cancer Institute, University Health Network, Canada). Unlike the Panorama Human Cytokine Gene Array filters, the glass slide genes do not have any particular focus on immune genes; they were only used as a verification of the results yielded by the nitrocellulose filters. Each glass slide microarray set consists of two slides of 9600 genes each, plus positive Arabidopsis controls.

i) RT-PCR and probe purification

Ten μg of RNA from each sample was used for each glass slide microarray. A mastermix was made on ice, consisting of these reagents: 43% of 5X First Strand reaction buffer; 8% of AncT mRNA primer (5'-T20VN, 100 pmol/μL); 16% of 20 mM dNTP (-dCTP) (6.67 mM each of dATP, dGTP, dTTP); 5% of 2 mM dCTP; 22% of 0.1 M dithiothreitol (DTT); and 5% of Arabidopsis control RNA (1 ng/mL, use 0.5-1.0 ng).

Mastermix was added to samples of 10 μg of total RNA before adding to labeling reaction. Labeling reaction reagents are listed below. RNase-free water was added to the mixture to account for varied concentrations of RNA. Labeling reaction was produced as follows: Mastermix, 46%; 1 mM of Cy3 or Cy5 dCTP, 3%; and RNA sample, 10 μg. RNase-free water was added such that the total volume was 40 μL.
Labeling reaction was incubated for 5 minutes at 65°C, followed by 5 minutes of incubation at 42°C. Reverse transcriptase (2μL) and RNase inhibitor (1μL) were then added to each sample. The RT-PCR was allowed to proceed for 2-3 hours.

The labeling mixture was then briefly centrifuged and placed on ice to stop the reaction. Four μL of 50 mM EDTA and 2 μL of 10 N NaOH were added. The mixture was then incubated for 20 minutes at 65°C for RNA hydrolysis.

Purification of fluorochrome-labeled cDNA probes was performed using the Millipore Amicon/Microcon PCR Centrifugal Filter Device (Etobicoke, Canada). Four hundred μL Milli-Q water and the sample were put into the reservoir, and centrifuged for 15 minutes at 1000xg (3100 rpm), 20°C. Five μL Milli-Q water was then added to the reservoir, which was placed in a new tube. After 30 seconds at room temperature, the reservoir was inverted inside the tube and the entire apparatus was centrifuged for 2 minutes at 1000xg, 20°C. Eluate consisted of fluorochrome-labeled cDNA.

ii) Microarray and detection

For the hybridization step, 5 μL of yeast tRNA (10 mg/mL) and 5 μL of calf thymus (10 mg/mL) were added to 100 μL of DIG hybridization solution (Roche, Laval, Canada). Eighty μL of this solution was then added to the 5 μL of labeled cDNA. The hybridization mixture was mixed and incubated at 65°C for 2 minutes before being cooled to room temperature. Meanwhile, the hybridization chamber was prepared by adding a small amount of DIG hybridization solution to the bottom, to ensure humidity.
Glass slides were examined to ensure cleanliness from particles, which could cause bubbles during hybridization. Then, one slide was placed face down on the other slide, such that the sides spotted with genes were facing each other. The slides were slightly offset to provide an entry point for the hybridization solution. The hybridization solution, containing labelled cDNA, was pipetted slowly and carefully into the crack between the slides, avoiding any bubbles. The slides were then placed in the hybridization chamber, which was wrapped tightly with plastic wrap. The chamber was then incubated at 37°C for 8-18 hours.

Just before the slides were ready to be washed, fresh 1X SSC/0.1% SDS solution was prepared and pre-warmed to 50°C. The slides were put into 1X SSC and separated. They were then placed in a staining rack, which was placed into a staining dish containing fresh 1X SSC solution. Slides were washed three times for 10 minutes each at 50°C by placing staining rack into boxes containing the 1X SSC/0.1% SDS, with gentle shaking. Glass slides were rinsed in 1X SSC 4-6 times, and rinsed again in 0.1X SSC. Slides were spun dry for 5 minutes at 500 rpm. To minimize photobleaching of fluors, slides were kept in the dark at room temperature until read.

9) Statistical analysis

a) Filter microarrays

Statistical analysis for the filter microarrays was performed in two different manners, and comparisons between the two calculations were made. It was hypothesized that consensus genes identified between the two methods could help compensate for the weakness and bias that would exist if only one method was used. Specifically, it would
be unlikely that artificially high values would be calculated by both of the two separate methods. As well, the raw data (i.e. scan of filter or glass slide) would help to distinguish between artificially low or high values. Method A was developed by the Virology Research Laboratory at the Children’s Hospital of Eastern Ontario, while Method B was developed by the Department of Mathematics at the University of Ottawa.

The filters were first examined for major discrepancies between duplicate spots, which were discounted. Method A analysis was performed by subtracting the highest negative control from all values in order to account for non-specific binding. The arithmetic mean was then taken from the duplicate spots. The control and experimental values were then subjected to the following formula:

\[ s = \frac{(x-y)}{y}, \text{ where } x = \text{ the higher value, whether experimental or control; and } y = \text{ the lower value, whether experimental or control.} \]

If either \( x \) or \( y = 0 \), the non-0 value was divided by the average of the negative controls. Significance was accorded to genes whose \( s \) value was greater than 2 (i.e. a two-fold up- or down-regulation). Genes whose \( s \) values were greater than 2 on 2 or more runs were deemed significant.

Method B analysis was performed by taking the log of the raw data in order to normalize the wide range of values. The arithmetic mean of the duplicate spots, between all experimental runs, was then taken. The following formula was then applied to the control and experimental values:

\[ s = \text{round } [(\text{effect}/||\text{max effect}|) \times 10] \]

The “max effect” was the maximum value from any experimental run. In this case, it was represented by IL-16 in Experiment 2.
The s values for control and experimental values were plotted with the help of the computer program SAS (Statistical Analysis System) and clustered. All experimental runs were incorporated into the program at the same time. This yielded a number from -10 to +10, with +10 representing the highest up-regulation in any experimental situation and -10 representing the most significant down-regulation. Highest gene increases and decreases were noted, and a cutoff of 5 was given as a significant up-regulation.

b) Glass slide microarrays

For the glass slide microarrays, RNA was extracted from the same experiment as the filter experiments. Only the first experiment (HSV-2 vs. control) was verified by glass slide microarray.

After the fluorochrome signals from the microarray were detected by laser, the scan was examined for discrepancies and artifacts. These spots were discounted from the analysis. Accurate densitometry of the spots was ensured by carefully placing circles around each dot signal for measurement. The calculation of gene expression was performed by a straight ratio between the fluorochrome signals (experimental signal divided by control signal). Calculation of fluorochrome ratio was performed by QuantArray and imported into Microsoft Excel 97.

The transcription of genes that were represented on both filter and glass slide microarray systems was compared for accuracy between array types. The r and p values of the glass slide experiment repeats were calculated, and the extent of gene up- and down-regulations from Experiment 2 were assessed. The r value is a coefficient of linear correlation represented by a number between -1 and 1. A value of 0 implies no linear
correlation. A value of 1 implies perfect positive linear correlation, while a value of -1 implies perfect negative linear correlation [Mendenhall et al., 1994]. The p value represents the likelihood that the evidence is true. The smaller the p value, the less likely that data outside the hypothesis will be found; therefore, the smaller the p value, the more likely that the evidence is true [Texasoft, 2001].

10) Experiments performed

After HSV-2 propagation and titration, and electron microscopy, three experiments were performed in triplicate. These experiments were the following:

Experiment 1: human neuroblastoma cells, 37°C, 4 hours, acyclovir (100 µg/mL), control vs. HSV-2 (2 PFU/cell)

Experiment 2: human neuroblastoma cells, 37°C, 4 hours, control vs. acyclovir (100 µg/mL)

Experiment 3: human neuroblastoma cells, 4 hours, HSV-2 (2 PFU/cell) 37°C vs. 40°C
Chapter 3: Results

1) Preparation and determination of HSV-2 virus stocks

Quadruplicates of 10-fold HSV-2 stock dilutions were applied to HFL cells that had been grown to confluence (see Figure 1). Dilutions of $10^4$ to $10^8$, plus a negative control, were incubated for 3 days, followed by quantitation of plaques in the plates. At $10^4$, the HFL monolayer was completely destroyed, while at $10^8$, no plaques were seen. The concentration of HSV-2 was determined every time a stock virus solution was made. Concentrations usually reached $5 \times 10^7$ PFU/mL. Concentrations of less than $1 \times 10^7$ PFU/mL were not used for subsequent studies.

2) Electron microscopy

Electron microscopy was performed to confirm that the HSV-2 prepared in our laboratory was capable of infecting the human neuroblastoma cells. Human neuroblastoma cells were grown to confluence in two flasks. One flask was infected with one MOI of HSV-2, while the other flask remained uninfected as a control. Both flasks were incubated for 24 hours. Cell morphology was observed by electron microscopy.

Figure 2 shows the uninfected human neuroblastoma cells at 10 000X magnification. Intact cell and nuclear membranes are clearly seen.

Figure 3 shows the HSV-2 infected human neuroblastoma cells at 10 000X magnification. Acute infection can be seen, as well as degenerating plasma membranes. Infectious virus particles, as well as empty capsids, can be seen.
Fig. 1. Quantification of HSV-2 by virus titration assay. Ten-fold dilutions of HSV-2 stock were applied to confluent HFL cells for 3 days. From top to bottom: Row 1, $10^4$; Row 2, $10^5$; Row 3, $10^6$; Row 4, $10^7$; Row 5, $10^8$; and Row 6, negative control (no virus). Plaques of CPE were counted, and virus concentration was calculated.
Fig. 2. Electron micrograph of uninfected human neuroblastoma cells, 10 000X magnification. Uninfected human neuroblastoma cells. Intact cell membranes (arrowheads) and nuclear membranes (arrows) are clearly seen.
Fig. 3. Electron micrograph of HSV-2 infected human neuroblastoma cells, 10 000X magnification. HSV-2 infected human neuroblastoma cells, 1 PFU/cell. Acute infection can be seen, as well as degenerating plasma membranes (arrows). Infectious virus particles (arrowheads), as well as empty capsids (hollow arrowheads), can be seen.
3) Note on filter microarray results

All filter experiments were performed three times in order to increase accuracy and precision. Nevertheless, a wide range of variability is represented in some of the results for the three sets experiments performed on filter microarrays. It is thought that this large range of data sampling is due to the microarray technique itself and its inherent inconsistencies. Such variability includes differences between control and experimental incorporation of radiolabeled nucleotides. It was believed, however, that falsely elevated values would be discounted from the final list of significantly up- or down-regulated genes by comparing two different methods of statistical analysis. To minimize the inclusion of artifacts, the two methods incorporate different strategies: arithmetic analysis and logarithmic analysis.
4) Experiment 1: Effect of four-hour HSV-2 exposure on human neuroblastoma cells in the presence of acyclovir 100 μg/mL.

Neuroblastoma cells were grown to confluence, and then 100 μg/mL acyclovir was added to two flasks for four hours. The cells were then incubated at 37°C in the presence or absence of 2 PFU/cell of HSV-2, strain G. Two PFU/cell is considered a high MOI, but this concentration was used to ensure that changes in RNA expression would be more readily seen. A lower MOI would result in fewer cells being infected by virus particles, leading to fewer cells exhibiting the virus-induced changes. The cells were incubated with virus for four hours to examine the early genes whose expressions were induced by HSV-2 challenge. RNA was then extracted and subjected to both filter and glass slide microarray analysis.

a) Filter microarrays

Up-regulated genes following exposure of neuroblastoma cells to HSV-2, in the presence of acyclovir: Method A analysis

The genes found to be significantly up-regulated by Method A analysis are shown in Table 1. Significant up-regulation was defined as a fold change of equal to or more than 2. Except for IL-2 Rα, CD6, and BMP-7, all proteins were up-regulated on three filter experiments.

Up-regulated genes, Method B analysis

Many immune genes were found to be significantly up-regulated by Method B analysis. Genes that exhibited the highest levels of up-regulation included IL-16, CXCR-
5, endothelin-2, and IL-10 inducible chemokine (HCC-4) (see Table 2). Significant up-regulation was defined as an s value of equal to or more than +5.

No genes were found to be significantly down-regulated by either Method A or Method B analysis. For Method A, significant down-regulation was defined as a fold change of equal to or greater than −2. For Method B, a significant down-regulation was defined as an s value of equal to or greater than −5.

Genes commonly up-regulated, Methods A and B analyses

Table 3 shows the genes found to be up-regulated by both Methods A and B analyses. Of these genes, all but CD6 were up-regulated for all three filter experiments. The third experiment for CD6 was discounted because the negative control values were too high. No genes were found to be significantly down-regulated by both A and B methods of analysis.
Table 1: Experiment 1: Genes found to be significantly up-regulated by Method A analysis of filter microarray.

<table>
<thead>
<tr>
<th>Gene</th>
<th># of Experiment Runs</th>
<th>Median Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ephrin-B1</td>
<td>3</td>
<td>+8.8</td>
</tr>
<tr>
<td>Endothelin-2</td>
<td>3</td>
<td>+7.7</td>
</tr>
<tr>
<td>BMP-8</td>
<td>3</td>
<td>+7.1</td>
</tr>
<tr>
<td>IFN-α/β Rβ</td>
<td>3</td>
<td>+5.6</td>
</tr>
<tr>
<td>CXCR-5</td>
<td>3</td>
<td>+4.7</td>
</tr>
<tr>
<td>IL-16</td>
<td>3</td>
<td>+3.8</td>
</tr>
<tr>
<td>FGFR2</td>
<td>3</td>
<td>+3.6</td>
</tr>
<tr>
<td>IL-2 Rα</td>
<td>2</td>
<td>+3.1</td>
</tr>
<tr>
<td>GFRα3</td>
<td>3</td>
<td>+2.9</td>
</tr>
<tr>
<td>IL-3 Rα</td>
<td>3</td>
<td>+2.7</td>
</tr>
<tr>
<td>CD6</td>
<td>2</td>
<td>+2.3</td>
</tr>
<tr>
<td>BMP-7</td>
<td>2</td>
<td>+2.3</td>
</tr>
</tbody>
</table>

Human neuroblastoma cells were grown to 100% confluence. One hundred µg/mL acyclovir was added to both flasks; the experimental flask was infected with 2 PFU/cell of HSV-2, strain G. After four hours, RNA was extracted from harvested cells, 32P-cDNA was generated, and microarray analysis on filter arrays was performed. Negative controls were subtracted from all values to account for effects due to non-specific binding. Genes were deemed significant if the fold change for 2 or more experimental runs was greater than 2. If the control and experimental values were found to be 0, the entire experimental experimental run was discounted. Densitometry was measured with ImageQuant, while data was analyzed on Microsoft Excel 97.
Table 2. Experiment 1: Genes found to be significantly up-regulated by Method B analysis of filter microarray.

<table>
<thead>
<tr>
<th>Gene</th>
<th>s value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-16</td>
<td>+10</td>
</tr>
<tr>
<td>CXCR-5, Endothelin-2, HCC-4</td>
<td>+9</td>
</tr>
<tr>
<td>VEGF</td>
<td>+8</td>
</tr>
</tbody>
</table>

Human neuroblastoma cells were grown to 100% confluence. One hundred μg/mL acyclovir was added to both flasks; the experimental flask was infected with 2 PFU/cell of HSV-2, strain G. After four hours, RNA was extracted from harvested cells, ^32^P-labeled cDNA was generated, and microarray analysis on filter arrays was performed. Densitometry was measured with ImageQuant, while data was analyzed by the University of Ottawa Mathematics Department.
Table 3: Experiment 1: Genes found to be significantly up-regulated by both Methods A and B analyses of filter microarray.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Method A (Median Fold Change)</th>
<th>Method B (s value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ephrin-B1</td>
<td>+8.8</td>
<td>+6</td>
</tr>
<tr>
<td>Endothelin-2</td>
<td>+7.7</td>
<td>+9</td>
</tr>
<tr>
<td>IFN-α/β Rβ</td>
<td>+5.6</td>
<td>+6</td>
</tr>
<tr>
<td>CXCR-5</td>
<td>+4.7</td>
<td>+9</td>
</tr>
<tr>
<td>IL-16</td>
<td>+3.8</td>
<td>+10</td>
</tr>
<tr>
<td>FGF R2</td>
<td>+3.6</td>
<td>+7</td>
</tr>
<tr>
<td>IL-3 Rα</td>
<td>+2.7</td>
<td>+6</td>
</tr>
<tr>
<td>CD6</td>
<td>+2.3</td>
<td>+5</td>
</tr>
</tbody>
</table>

Human neuroblastoma cells were grown to 100% confluence. One hundred µg/mL acyclovir was added to both flasks; the experimental flask was infected with 2 PFU/cell of HSV-2, strain G. After four hours, RNA was extracted from harvested cells, 32P-labeled cDNA was generated, and microarray analysis on filter arrays was performed. Densitometry was measured with ImageQuant, while data was analyzed by Microsoft Excel 97 (Method A) and the University of Ottawa Department of Mathematics (Method B).
b) Glass slide microarrays

For Experiment 1, glass slide microarrays were also performed using the same total RNA as used for the filter microarray assay. Three glass microarrays were performed, and the data represented a ratio between the signals of the experimental and control dye samples.

The University of Ottawa Department of Mathematics calculated the \( r \) and \( p \) values for all three glass slide runs. The \( r \) value was calculated to be 0.74930, while the \( p \) value was found to be less than 0.0001. The \( r \) and \( p \) values show that the RNA expression for the genes represented on the glass slide was found to be similar between all repeats of the experiment.

Expression of genes that were represented on both the filter and glass slide microarrays was examined. The four genes that were common to both filter and glass slides were shown to be up-regulated (see Table 4). It can be seen that the increase in gene expression as analyzed by filter was greater than that found by glass slide ratio.
Table 4. Experiment 1: Genes found to be up-regulated by both Methods A and B analyses of microarray filters and by glass slide microarrays.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Filter (Method A)</th>
<th>Filter (Method B)</th>
<th>Glass slide (Ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ephrin B1</td>
<td>+8.8</td>
<td></td>
<td>3.02</td>
</tr>
<tr>
<td>IFN-α/β Rβ</td>
<td>+5.6</td>
<td>+6</td>
<td>2.89</td>
</tr>
<tr>
<td>IL-16</td>
<td>+3.8</td>
<td>+10</td>
<td>2.41</td>
</tr>
<tr>
<td>FGF R2</td>
<td>+3.6</td>
<td>+7</td>
<td>2.89</td>
</tr>
</tbody>
</table>

Human neuroblastoma cells were grown to 100% confluence. One hundred mg/mL acyclovir was added to both flasks; the experimental flask was infected with 2 PFU/cell of HSV-2, strain G. After four hours, RNA was extracted from harvested cells, 32P-labeled cDNA was generated, and microarray analysis on filter arrays and glass slides was performed. Densitometry was measured with ImageQuant, while data was analyzed by Microsoft Excel 97 (Method A), the University of Ottawa Mathematics Department (Method B), and QuantArray (glass slides).
5) Experiment 2: Effect of four-hour exposure of 100 μg/mL acyclovir on RNA expression of human neuroblastoma cells (filter microarray).

Neuroblastoma cells were grown to confluence and incubated for four hours either in the presence or absence of 100 μg/mL acyclovir. RNA was then extracted and subjected to filter microarray analysis.

**Up-regulated genes, Method A analysis**

Table 5 shows the genes found to be significantly up-regulated by Method A analysis. Significant up-regulation was defined as a fold change of equal to or more than 2, compared to the negative control values. CXCR-5 and ALCAM were up-regulated for all three filter runs. The other genes were only up-regulated on two filter experimental runs; since for one run the negative control was too high.

**Up-regulated genes, Method B analysis**

Table 6 shows the genes that were found to be significantly up-regulated by Method B analysis. Significant up-regulation was defined as an s value of equal to or more than +5.
Down-regulated genes, Method A analysis

Only one gene was found to be significantly down-regulated by Method A analysis (see Table 7). Significant down-regulation was defined as a fold change of equal to or more than $-2$.

No genes were found to be significantly down-regulated by Method B analysis. Significant down-regulation was defined as an $s$ value of equal to or more than $-5$.

Genes commonly up-regulated, Methods A and B analyses

Genes found to be significantly up-regulated by both A and B statistical methods are shown in Table 8. From Method A, CAD-5 was up-regulated on 2 filter experiments, while the other two genes were up-regulated on all 3 filters.

No genes were found to be significantly down-regulated by both A and B methods of analysis.
Table 5. Experiment 2: Genes found to be significantly up-regulated by Method A analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th># of Experiment Runs</th>
<th>Median Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAD-5</td>
<td>2</td>
<td>+15.5</td>
</tr>
<tr>
<td>E-Cadherin</td>
<td>2</td>
<td>+3.6</td>
</tr>
<tr>
<td>Integrin-β7</td>
<td>2</td>
<td>+3.4</td>
</tr>
<tr>
<td>ALCAM</td>
<td>3</td>
<td>+2.9</td>
</tr>
<tr>
<td>CXCR-5</td>
<td>3</td>
<td>+2.8</td>
</tr>
</tbody>
</table>

Human neuroblastoma cells were grown to 100% confluence and the experimental flask was exposed to 100 μg/mL acyclovir for four hours. RNA was extracted from harvested cells, 32P-labeled cDNA was generated, and microarray analysis on filter arrays was performed. Negative controls were subtracted from all values to account for effects due to non-specific binding. Genes were deemed significant if the fold change for 2 or more experimental runs was greater than 2. If the control and experimental values were found to be 0, the entire experimental run was discounted. Densitometry was measured with ImageQuant, while data was analyzed on Microsoft Excel 97.
Table 6. Experiment 2: Genes found to be significantly up-regulated by Method B analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>s value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TARC</td>
<td>+9</td>
</tr>
<tr>
<td>TECK</td>
<td>+8</td>
</tr>
<tr>
<td>TRAIL R2</td>
<td>+6</td>
</tr>
<tr>
<td>ALCAM</td>
<td>+5</td>
</tr>
<tr>
<td>BCAM</td>
<td>+5</td>
</tr>
<tr>
<td>CAD-5</td>
<td>+5</td>
</tr>
<tr>
<td>CXCR-4</td>
<td>+5</td>
</tr>
<tr>
<td>CXCR-5</td>
<td>+5</td>
</tr>
<tr>
<td>Ephrin-A3</td>
<td>+5</td>
</tr>
<tr>
<td>Ephrin-A4</td>
<td>+5</td>
</tr>
<tr>
<td>IFN-γ R1</td>
<td>+5</td>
</tr>
<tr>
<td>IGF-Binding Protein 5</td>
<td>+5</td>
</tr>
<tr>
<td>Integrin-αV</td>
<td>+5</td>
</tr>
<tr>
<td>Integrin-β1</td>
<td>+5</td>
</tr>
<tr>
<td>NGF R</td>
<td>+5</td>
</tr>
<tr>
<td>TRAIL R3</td>
<td>+5</td>
</tr>
</tbody>
</table>

Human neuroblastoma cells were grown to 100% confluence and the experimental flask was exposed to 100 μg/mL acyclovir for four hours. RNA was extracted from harvested cells, 32P-labeled cDNA was generated, and microarray analysis on filter arrays was performed. Densitometry was measured with ImageQuant, while data was analyzed by the University of Ottawa Mathematics Department. All three experimental runs were incorporated prior to mathematical transformation.
Table 7. Experiment 2: Gene found to be significantly down-regulated by Method A analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th># of Experiment Runs</th>
<th>Median Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γR2</td>
<td>3</td>
<td>-5.0</td>
</tr>
</tbody>
</table>

Human neuroblastoma cells were grown to 100% confluence and the experimental flask was exposed to 100 μg/mL acyclovir for four hours. RNA was extracted from harvested cells, and microarray analysis on filter arrays was performed. Negative controls were subtracted from all values to account for effects due to non-specific binding. Genes were deemed significant if the fold change for 2 or more experimental runs was greater than 2. If the control and experimental values were found to be 0, the entire experimental run was discounted. Densitometry was measured with ImageQuant, while data was analyzed on Microsoft Excel 97.

Table 8. Experiment 2: Genes found to be significantly up-regulated by both Methods A and B analyses.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Method A (Mean Fold Change)</th>
<th>Method B (s value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAD-5</td>
<td>+15.5</td>
<td>+5</td>
</tr>
<tr>
<td>ALCAM</td>
<td>+2.9</td>
<td>+5</td>
</tr>
<tr>
<td>CXCR-5</td>
<td>+2.8</td>
<td>+5</td>
</tr>
</tbody>
</table>

Human neuroblastoma cells were grown to 100% confluence and the experimental flask was exposed to 100 μg/mL for four hours. RNA was extracted from harvested cells, and microarray analysis was performed. Negative controls were subtracted from all values to account for effects due to non-specific binding. Genes were deemed significant if the fold change for 2 or more experimental runs was greater than 2. If the control and experimental values were found to be 0, the entire experimental run was discounted. Densitometry was measured with ImageQuant, while data was analyzed on Microsoft Excel 97.
6) Experiment 3: Comparison of gene expression between four hours of acute or latent HSV-2 infection of human neuroblastoma cells (filter microarray).

Neuroblastoma cells were grown to confluence and incubated for four hours in the presence of 2 MOI of HSV-2, at either 37°C or 40°C. RNA was then extracted and subjected to filter microarray analysis. This experiment examines the differential gene expression during an acute infection (37°C) in comparison to a latent infection (40°C). A higher MOI than usual latent models [Kondo et al., 1990] was used to infect the cells because changes in RNA expression would be more readily seen. A lower MOI would result in fewer cells being infected by virus particles, leading to fewer cells exhibiting the virus-induced changes. The cells were incubated for four hours to differentiate between early genes that were induced by acute vs. latent HSV-2 infection.

**Up-regulated genes, Method A analysis**

The only gene found to be up-regulated by Method A is shown in Table 9. Significant up-regulation was defined as a fold change of equal to or more than +2.

**Up-regulated genes, Method B analysis**

Table 10 shows the genes found to be up-regulated by Method B analysis. Significant up-regulation was defined as an s value of equal to or more than +5.
Down-regulated genes, Method A analysis

No genes were found to be significantly down-regulated by Method A analysis. Significant down-regulation was defined as a fold change of equal to or more than −2.

Down-regulated genes, Method B analysis

Genes found to be significantly down-regulated by Method B analysis are shown in Table 11. Significant down-regulation was defined as an s value of at least −5.

Genes commonly up- or down-regulated, Methods A and B analyses

The only gene found to be significantly up-regulated by both statistical methods is MDC, shown in Table 12.

No genes were found to be significantly down-regulated by both statistical methods for the third experiment.
Table 9. Experiment 3: Gene found to be significantly up-regulated by Method A analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th># of Experiment Runs</th>
<th>Median Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDC</td>
<td>2</td>
<td>+7.6</td>
</tr>
</tbody>
</table>

Human neuroblastoma cells were grown to 100% confluence, and infected with 2 PFU/cell of HSV-2, strain G. Cells were then incubated at either 37°C (control) or 40°C (experimental) for four hours. RNA was extracted, fluorochrome-labeled cDNA was generated, and microarray analysis of filter microarrays was performed. Negative controls were subtracted from all values to account for effects due to non-specific binding. Genes were deemed significant if the fold change for 2 or more experimental runs was greater than 2. If the control and experimental values were found to be 0, the entire experimental run was discounted. Densitometry was measured with ImageQuant, while data was analyzed on Microsoft Excel 97.
Table 10. Experiment 3: Genes found to be significantly up-regulated by Method B analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>s value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDC</td>
<td>+8</td>
</tr>
<tr>
<td>BMP RIIA</td>
<td>+6</td>
</tr>
<tr>
<td>IL-5</td>
<td>+5</td>
</tr>
<tr>
<td>PREF-1</td>
<td>+5</td>
</tr>
</tbody>
</table>

Human neuroblastoma cells were grown to 100% confluence, and infected with 2PFU/cell of HSV-2, strain G. Cells were then incubated at either 37°C (control) or 40°C (experimental) for four hours. RNA was extracted, fluorochrome-labeled cDNA was generated, and microarray analysis of filter arrays was performed. Densitometry was measured with ImageQuant, while data was analyzed by the University of Ottawa Mathematics Department.
Table 11. Experiment 3: Genes found to be significantly down-regulated by Method B analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>s value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAD-5, CD27L</td>
<td>-8</td>
</tr>
<tr>
<td>FGF basic, ICAM-2, Midkine</td>
<td>-7</td>
</tr>
<tr>
<td>CAD-8, CCR-5, CD30L, HB-EGF, IFN-γ R1, L-Selectin, P-Cadherin, R-Cadherin, TIMP-2</td>
<td>-6</td>
</tr>
</tbody>
</table>

Human neuroblastoma cells were grown to 100% confluence, and infected with HSV-2, strain G. Cells were then incubated at either 37°C (control) or 40°C (experimental) for four hours. RNA was extracted, fluorochrome-labeled cDNA was generated, and microarray analysis of filter arrays was performed. Densitometry was measured with ImageQuant, while data was analyzed by the University of Ottawa Mathematics Department.
Table 12. Experiment 3: Genes found to be significantly up-regulated by both Methods A and B analyses.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Method A (Median Fold Change)</th>
<th>Method B (s value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDC</td>
<td>+7.6</td>
<td>+8</td>
</tr>
</tbody>
</table>

Human neuroblastoma cells were grown to 100% confluence, and infected with 2 PFU/cell of HSV-2, strain G. Cells were then incubated at either 37°C (control) or 40°C (experimental) for four hours. RNA was extracted, fluorochrome-labeled cDNA was generated, and microarray analysis was performed. Densitometry was measured with ImageQuant, while data was analyzed by Microsoft Excel 97 (Method A) and the University of Ottawa Department of Mathematics (Method B).
Chapter 4: Discussion

HSV-2 reactivation from latency in nerve roots is the result of communication or imbalance between the immune and neuronal systems. Despite the importance of the interactions between these two systems, global gene expression studies have not been performed. The differential gene expression of neural cells as they are subjected to challenge with acyclovir and HSV-2 could help pinpoint candidates that could be suitable for further study for antiviral therapy. Furthermore, the differential gene expression of neural cells as they are infected with HSV-2 under conditions of acute infection as compared with latent infection could help identify candidates that could be subjected to further investigation for the induction of latency in already-infected individuals.

Although microarray is a powerful technique that enables the simultaneous assessment of gene expression, it has limitations. Many of the molecules that may be important for communication may not have been identified, and therefore are not represented on a microarray. This is only one of the caveats that must be considered before interpreting the data.

The microarray assay itself is prone to variability. Reasons for the wide range of results includes the following: (1) The process of RNA extraction. Discrepancies in RNA extraction would affect the amount of RNA available for RT-PCR, which would affect the signal detected. (2) The incorporation of labeled nucleotides into the cDNA. Differences in incorporation of labeled nucleotide would result in variable signals, even if the hybridization of cDNAs to the microarray were identical. (3) The hybridization of labeled product to the microarray; signal variability would result from uneven
hybridization of the cDNAs to the microarray. These variabilities, along with the sheer volume of data, have spawned techniques on how to distinguish noise from independently reproducible signals [Mills & Gordon, 2001].

Another limitation of these microarray techniques is that it only can examine the gene expression at the RNA level. For this reason, genes that are expressed at different levels in response to a stimulus must be verified at a protein level. Enzyme-linked immunosorbent assay (ELISA) could be used to verify the expression of secreted proteins; flow cytometry could be used to verify expression of cell surface proteins; and Western blot could be used to verify protein expression of cellular genes.

As well, the information yielded by these experiments is only from a single cell line of neural origin. These cells are chromosomally different from neurons found in vivo, as they have been immortalized. Because of this difference, future experiments using other neuronal cell lines, neurons, and in vivo experiments are needed in order to verify the results in more representative models.

The timepoint used for all these experiments is four hours. This only yields a small snapshot of what genes are being expressed at one time. For a clearer picture of the up- and down-regulation of immune genes and their interactions, a greater number of timepoints is needed. The immediate-early and early genes are transcribed approximately 5 hours after induction [Roizman & Knipe, 2001], while it takes up to 9 hours for latent HSV to reactivate [Sawtell et al., 1999]. Four-hour incubation would examine the immediate-early and early gene expression, but further experiments are needed for a complete picture of the events that unfold after HSV-2 infection.
Finally, the data yielded represent only the genes that are differentially expressed at an RNA level, which is not necessarily reflected by a difference in protein synthesis. For this reason, experiments that quantify the protein expression of the genes that are significantly up- or down-regulated by microarray analysis is needed. These experiments could include flow cytometry and immunoblot for surface proteins as well as ELISA for secreted proteins.

Regardless of these limitations, the genes found to be differentially expressed in these experiments likely represent a true difference in expression because it was found by both methods of analysis. Nevertheless, the performance of more experimental runs would likely increase the accuracy of the results.

a) Experiment 1: Effect of four-hour HSV-2 exposure on human neuroblastoma cells in the presence of acyclovir.

The infection of cells with virus triggers a wide range of changes in gene expression. Genes previously found to be differentially expressed in response to HSV-2 infection include IFN-γ, IL-10, and IL-4 [Carmack et al., 1996]. In experiment 1, genes that were up-regulated in response to challenge with HSV-2 in the presence of acyclovir include ephrin-B1, endothelin-2, IFN-α/β Rβ, CXCR-5, IL-16, FGF R2, IL-3 Rα, and CD6. No genes were found to be down-regulated in response to HSV-2 infection.
Ephrin-B1

Ephrin-B1 was up-regulated in response to HSV-2 challenge in the presence of acyclovir. Method A analysis found a median 9-fold up-regulation, while Method B yielded an s value of +6.

Ephrins are expressed by the majority of tissues during growth and development [Mellitzer et al, 2000]. Ephrins are notable for playing roles in fetal neural development as well as in the neurons of adults [Murai & Pasquale, 2001]. They have also been implicated in angiogenesis and vascular remodeling; up-regulation of this family has been associated with tumour growth and metastasis [Cheng et al, 2002].

The ephrin family consists of 8 ephrin proteins as well as 14 ephrin receptors [Mellitzer et al, 2000]. They are the largest subfamily of tyrosine kinase receptors. The fourteen known receptors, nine of which exist in vertebrates, play roles in germ layer development and cell migration during embryogenesis [Coulthard et al., 2002]. Ephrins and their receptors are each divided into two groups, based on extracellular domain amino acid sequence homology. Ephrin ligands consist of ephrin-A and ephrin-B subclasses, while ephrin receptors are divided into EphA and EphB subclasses [Orioli & Klein, 1997]. Generally, ephrin-A ligands bind EphA receptors, while ephrin-B ligands bind EphB receptors, but there have been exceptions [Flanagan & Vanderhaeghen, 1998].

Most ephrins and their receptors are expressed during neural development [Orioli & Klein, 1997]. Evidence suggests that ephrins and their receptors can direct the patterning of axon growth. Specifically, the theory of contact repulsion in neuronal development is paralleled by the expression of the ephrin-B and EphB subclasses.

Ephrin-B1 (ephrin ligand B1, also known as Lerk2) is expressed in neurons as well as in endothelial cells in arteries and veins. It has been implicated in contact repulsion in cerebral neurons [Flanagan & Vanderhaeghen, 1998], and has also been shown to induce angiogenesis in endothelial cells [Mellitzer et al., 2000]. Up-regulation of ephrin-B1 in response to HSV-2 infection in neurons is therefore suggestive of increased neuronal growth and development.

There have been no previous publications concerning ephrins and viruses. Further experiments are therefore needed to elucidate the role that ephrins have in the herpetic infection of neurons. An increase in ephrin-B1 could result in the generation of new blood vessels. As well, it could signify the organization or remodeling of neural migration. It is therefore possible that HSV-2 infection modulates the geography of neuronal development as well as the growth of blood vessels. If HSV-2 does influence both neural and endothelial growth, infective spread could increase. Further infection would be made possible by more extensive neural access; survival of the new neurons could be ensured by new blood vessels.

**Endothelin-2**

Endothelin-2 (ET-2) was up-regulated in response to HSV-2 challenge in the presence of acyclovir. Method A analysis found a median 8-fold up-regulation, while Method B yielded an s value of +9.
ETs are cell surface proteins that mediate adhesion on endothelial cells. They play important roles in angiogenesis, cell proliferation, and neural crest development. They also induce proliferation of various cell types and are involved with the expression of proto-oncogenes c-myc and c-fos [Vural et al., 2001]. Three isoforms have been isolated (ET-1, ET-2, ET-3), each composed of 21 amino acids [Gianessi et al., 2001]. The role of ETs has been best characterized in the vascular system, but there have also been reports of ETs being expressed in the nervous system.

In the vascular system, ETs are one of the most potent vasoconstrictors known. Vasopressor hormones, platelet-derived factors, and some cytokines are known to induce ET production, while nitric oxide and prostacyclin are known to decrease ET production. In addition to their vasoconstrictive effects, ETs have effects on myocardial tissue; they increase heart rate and strength of heart contraction [Bhatt, 1997]. Investigations of ETs and their receptors as therapy targets for hypertension and other cardiovascular dysfunctions are currently underway [van der Walle & Barlow, 1998].

Many neural cells have also been shown to express ETs. Evidence suggests that these proteins play important roles in post-ganglionic development and modulation. ETs enhance the activity of nerve growth factor and also prolong survival and promote differentiation of neural cells [Damon, 1999]. Astrocytes devoid of ET-1 expression exhibit increased vulnerability to ischemia [Ho et al., 2001]. ETs also ensure proper development of the enteric nervous system [Gershon, 1999].

Cells of the immune system also express ETs. Mast cells produce ETs and ET receptors, and it is thought that all of these proteins play a role in modulation of Th1/Th2 cytokine production of mast cells [Coulombe et al., 2002]. As well, the ET-1 isoform can
induce IL-6 production in primary osteoblasts [Perkins et al., 1997] and umbilical endothelial cells [Stankova et al., 1996].

Of the three isoforms, ET-1 is the most widely expressed. At least 16 organs produce ET-1 [Uchide et al., 2000], which is secreted mostly by vascular endothelial cells [Agapitov & Haynes, 2002]. ET-2 is less widely expressed than ET-1 [Uchide et al., 2000]. ET-2 is secreted by a variety of cells, including keratinocytes [Murakami et al., 2001], uterus, and intestine [Uchide et al., 1999]. ET-3 is expressed by a number of cells, including melanocytes [McGallion & Chakravarti, 2001] and kidney cells [Deng et al., 2000]. In contrast to ET-1 [Sventek et al., 1996], ET-2 expression has not been found to induce hypertension [Liefeldt et al., 1995].

The up-regulation of ET-2 in response to HSV-2 infection could result in increased vascularity of the infected area without hypertension. As well, enhanced cell survival could result of the enhancement of nerve growth factor activity. Both these effects would increase the chance of HSV-2 survival. Because ET-2 was not significantly up-regulated in Experiment 2, it is less likely that the differential expression of this gene is due to acyclovir only. The up-regulation of ET-2 could therefore be seen as a viral effect to enhance viability of infected cells and therefore increase chances of virus propagation and latency.

IFN-α/β Rβ

IFN-α/β Rβ was up-regulated in response to HSV-2 challenge in the presence of acyclovir. Method A analysis found a median 6-fold up-regulation, while Method B yielded an s value of +6.
IFNs are non-specific antiviral agents that are primarily released by NK and T cells, as well as by macrophages [Middleton, 1998]. They exert broad antiviral and immunomodulatory effects. Two types of interferons exist: I (α, β, τ, ω) and II (γ). Type I interferons can protect cells from viral infection, inhibit viral replication inside infected cells, and up-regulate MHC I expression on cell surfaces [Middleton, 1998]. At the protein level, types I and II interferons bear little resemblance to each other [Bach, 1997].

IFNs α and β are produced soon after infection and increase up-regulation of MHC I molecules as well as β₂-microglobulin [Janeway et al., 1999]. This is one manner in which type I IFNs enhance the host anti-viral response. IFNs also influence cells to process viral proteins and present them to the surface more efficiently, which enhances T cell activation. As well, type I interferons enhance resistance to viral replication and activate NK cells [Janeway et al., 1999].

The IFN-α/β (IFN type I) receptor is composed of an alpha (α) subunit and a beta (β) subunit. The β subunit has three forms: soluble, long (βL), and short (βs). Signaling of the IFN type I receptor requires the α and βL subunits; the role of βs remains to be clarified. The βL subunit is required for signal transduction [Prejean & Colamonici, 2000].

The anti-viral effects of interferons have been well documented. IFN-α can inhibit HSV-1 transmission and viral spread in axons and epidermal cells [Mikloska & Cunningham, 2001]. Type I interferons have also been known to exert anti-proliferative effects, which have been used in the treatment of some cancers [Prejean & Colamonici, 2000]. As well, IFN-α has been shown to maintain neuronal growth [Plioplys &
Massimini, 1995]. An increase in IFN-α signaling could therefore prolong the survival of a healthy neuron as well as a latently-infected neuron. This could be an example of how a virus uses host defense mechanisms to its own advantage.

The up-regulation of IFN-α/β Rβ in response to HSV-2 infection could, however, also increase the host antiviral immune response. An increase in type I interferon signaling would increase MHC I presentation, which would enhance Th1 immunity. As well, NK cells would be more efficiently activated as a result.

CXCR-5

CXCR-5 was up-regulated in response to HSV-2 challenge in the presence of acyclovir. Method A analysis found a median 5-fold up-regulation, while Method B yielded an s value of +9.

Chemokines are small proteins produced by virtually all cells of the immune system. More than 30 have been identified, and all range in size from 8-12 kDa. Their most important role is in the induction of cell migration, but they have been shown to play roles in immunoregulation, cell activation, and control of angiogenesis and hematopoiesis [Middleton, 1998]. Chemokines are also referred to as immediate early genes, because their mRNA is often expressed within 2-3 hours of cell stimulation [Schall et al., 1992].

Chemokines are divided into three categories based on the pattern of cysteine amino acids in their N-terminal structure: CXC, CC, C, and CX3C. C denotes the amino acid cysteine, while X denotes a non-cysteine residue [Graves et al., 1999]. The majority of chemokines are secreted by leukocytes and platelets [Middleton, 1998].
Within their class, CXC chemokines share a 50% homology [Middleton, 1998]. They also share structural similarities with the CC chemokines, which echoes the functional overlap between these molecules. Typically, CXC chemokines are responsible for neutrophilic chemotaxis, whereas CC chemokines are involved in monocyte migration [Middleton, 1998]. Evidence has also shown that chemokines can play roles in viral immunity. For example, the CC chemokines MIP-1α (monocyte chemotactic protein 1), MIP-1β and RANTES (regulated upon activation, normal T cell expressed and secreted) have been shown to reduce HIV-1 replication [Cocchi et al., 1995].

CXCR-5 is a lymphocyte-associated chemokine receptor required for B cell homing. Its expression has not been described in neuronal cells. Its ligand is B lymphocyte chemoattractant (BLC) [Ansel et al., 1999]. CXCR-5 is expressed by a number of immune cells, including naive B cells [Bowman, 2000] and memory T cells [Campbell et al., 2001]. There exist no previous reports of this chemokine receptor being produced in cells of neural origin.

When conditions are favorable to CD4+ T cell migration, they up-regulate CXCR-5, which results in enhanced exposure to B cells [Ansel et al., 1999]. It is therefore possible that after acyclovir treatment, neurons also up-regulate CXCR-5 to signal migration of B-lymphocytes to their site. An increase in migration of B cells to the site of infection could enhance humoral immunity to the virus.

The up-regulation of CXCR-5 by neurons following challenge with HSV-2 could therefore enhance the immune response. The increase in CXCR-5 expression could result in more B cells being recruited to the area of infection, which could enable more rapid Th2 immunity. B cells are primarily involved with the production of opsonizing and
neutralizing antibodies [Janeway et al., 1999]. These antibodies could help decrease the amount of infectious virus by binding to specific viral antigens and thereby preventing the viral particles from infecting nearby cells.

CXCR-5 is up-regulated in response to acyclovir stimulation in the absence of HSV-2, but is also up-regulated in response to the virus in the presence of acyclovir. More experiments should be performed to elucidate the nature of this up-regulation, and clarify whether it is more closely related to the drug or to the virus.

IL-16

IL-16 was up-regulated in response to HSV-2 challenge in the presence of acyclovir. Method A analysis found a median 4-fold up-regulation in the three experimental runs, while Method B yielded an s value of +10, the highest given to any gene.

Cytokines are secreted proteins used for communication with cells of the immune system. ILs are cytokines produced by T cells; to date there are 27 ILs known [Ibelgauft, 2002]. Initially described as the first T cell chemoattractant, IL-16 is now found to be a chemoattractant for many CD4+ cells. It is produced by a number of cells, including both immune (dendritic cells, eosinophils, primary mast cells) and non-immune cells (fibroblasts, epithelial cells) [Cruikshank et al., 2000].

A variant of IL-16 is also expressed by cells of the neural system [Kurschner & Yuzaki, 1999]. Neuronal IL-16 (NIL-16) mRNA is expressed in the cerebellum and hippocampus throughout development and adulthood. The C-terminus of NIL-16 seems to be identical to that of IL-16, but the N-terminus is not the same.
Evidence suggests that IL-16 has at least two functions: chemoattraction and progression through the cell cycle. Pro-IL-16 is cleaved by caspase-3 into two functional fragments [Zhang et al., 2001]. The C-terminal product can be secreted and is processed to form mature IL-16. The N-terminal product can localize to the nucleus and can affect cell entry into the cell cycle [Zhang et al., 2001].

Roles of IL-16 include the induction of chemotaxis of immune cells. IL-16 triggers cell migration among CD4+ cells such as T cells, eosinophils, and dendritic cells [Cruikshank et al., 2000]. The potency of this chemoattraction seems to depend on the surface expression of CD4 [Ogasawara et al., 1999].

As well, IL-16 can stimulate CD4+ T cells to progress through the cell cycle, though not to proliferate. Yet, some transformed cells, such as lymphoma cells, are capable of proliferating in response to CD4 stimulation [Cruikshank et al., 2000]. T cell anergy can also be induced by IL-16 [Ogasawara et al., 1999]. These results suggest that IL-16 plays a larger role than just the recruitment of immune cells to the site of an infection. IL-16 seems capable of influencing cell differentiation and modulating cellular responses.

In the context of viral infection, IL-16 inhibits the replication of some viruses and also suppresses activation of lymphocytes. When peripheral blood mononuclear cells (PBMCs) from HIV-infected individuals are exposed to IL-16 under specific conditions, a significantly lesser number of cells undergo activation-induced cell death [Idziorek et al., 1998]. AICD susceptibility has been correlated with high viral load and progression of disease [Idziorek et al., 1998]. IL-16 seems capable of inhibiting transcription of HIV in vitro at the mRNA level [Zhou et al., 1997]; as well as the DNA level [Qi et al., 2002].
Furthermore, IL-16 interferes with HIV entry into macrophages and dendritic cells and inhibits viral replication within these cells [Truong et al., 1999].

Neuroblastoma cells demonstrated up-regulation of IL-16 in response to HSV-2 challenge in the presence of acyclovir. This is a novel finding; however, the notion that neurons produce a cytokine that acts as an immune cell migration signal after being infected by a virus is not surprising, since the cells had been invaded by a foreign organism. The recruitment of CD4+ cells to the site of infection would enhance host response to foreign invasion.

**FGF R2**

FGF R2 was up-regulated in response to HSV-2 challenge in the presence of acyclovir. Method A analysis found a median 4-fold up-regulation, while Method B yielded an s value of +7.

Fibroblast growth factors (FGFs) play important roles in many aspects of growth and development, such as germ layer formation, angiogenesis, neurogenesis, and wound healing [Nurcombe et al., 2000]. Not surprisingly, their receptors (FGF Rs) are ubiquitous. FGFs are divided into two categories: acidic (FGF1) and basic (FGF2). There are over 15 FGF Rs that have been identified, each with different affinities to their ligands [Stauber et al., 2000]. FGF R2 is best characterized for its role in bone development, dysregulation in FGF signaling has been linked to bone disorders characterized by early union of incorrect bones [Ingersoll et al., 2001]. FGF R2 has also been pinpointed as essential for proper development of lung epithelium and mesenchyme.
[Korfhagen & Whitsett, 1997]. Additionally, mutations in FGF R2 have been associated with gastric cancers [Jang et al., 2001].

FGFs are responsible for the timely differentiation and maturation of neurons [Goddard et al., 2001]. As well, many immortalized cell lines express receptors for these FGFs [Beitz, 1992]. FGF R2 expression has been observed in cells of neuronal origin, but its role has not been further examined [Reimers et al., 2001].

It has been hypothesized that FGFs have protective effects on the progression of viral disease in neurons. For example, FGF-induced receptor down-regulation is correlated with HIV seropositivity [Sanders, 2000]. Furthermore, the upregulation of FGF R2 in response to challenge with HSV-2 could reflect a viral effect on angiogenesis, since FGFs have been shown to play a role in vessel formation [Nurcombe et al., 2000]. There are no previous reports concerning the effects of HSV-2 on angiogenesis. Because FGF R2 was not significantly increased in Experiment 2, it is less likely that the upregulation of this gene is due to the virus and not acyclovir. Enhanced blood vessel formation could improve the accessibility of the infected area to immune cells.

Alternatively, the increased production of FGF R2 could also result in increased cell proliferation and therefore in enhanced chance of survival of HSV-2. Furthermore, FGF R2 mutations have been implicated in gastric cancers [Jang et al., 2001]; this molecule may therefore play a role in other tumours, such as neuroblastoma. Because the role of FGF R2 in acute HSV-2 infection is unclear, further experiments should be performed.
IL-3 Rα

IL-3 Rα was up-regulated in response to HSV-2 challenge in the presence of acyclovir. Method A analysis found a median 3-fold up-regulation, while Method B yielded an s value of +6.

IL-3 is a growth factor and cytokine secreted by T cells and mast cells. Roles of IL-3 include the production and activation of several types of mature blood cells, such as eosinophils, basophils, and monocytes [Korpelainen et al., 1996]. IL-3 plays important roles in hematopoiesis. Exposure to IL-3 results in the proliferation and differentiation of a wide range of immune cells [Frendl, 1992].

The IL-3 receptor consists of two chains, alpha (α) and beta (β). The α chain binds IL-3 with low affinity. The β chain alone does not bind to IL-3, but if the β chain is associated with the α chain, both chains bind IL-3 with high affinity. The α chain alone is not capable of signal transduction. The β chain is also common to the GM-CSF and IL-5 receptors. [Korpelainen et al., 1996].

IL-3 receptors are expressed by differentiated neurons, and nerve growth is stimulated by IL-3 [Kannan et al., 2000]. Expression of IL-3 Rα is also associated with a neuroprotective effect. Neurons that express IL-3 Rα are less vulnerable to ischemia and undergo apoptosis less rapidly [Wen et al., 1998].

Up-regulation of the IL-3 receptor alpha chain, if accompanied by expression of the β chain, could result in enhanced growth and activation of varied cells involved in the inflammatory response. This could result in an enhanced inflammatory response to HSV-
2 infection in neurons. An increase in monocytes could also increase Th2 immunity
directed towards extracellular virus particles.

Increased expression of IL-3 Rα could also result in delayed apoptosis and
enhanced neural proliferation. These two effects could benefit the virus by increasing its
survival time in the neuron. As well, neural proliferation could increase the spread of
HSV-2 and worsen the extent of future recurrent lesions.

CD6

CD6 was up-regulated in response to HSV-2 challenge in the presence of
acyclovir. Method A analysis found a median 2-fold up-regulation, while Method B
yielded an s value of +5.

CD6 is a co-stimulatory glycoprotein and cell surface receptor molecule whose
receptor is activated leukocyte adhesion molecule (ALCAM). CD6 is expressed on
immune cells such as T cells and some B cells, a subset of neural cells, and thymocytes
[Starling et al., 1996]. Expression of CD6 on neurons has not been described. This
molecule is capable of regulating T cell activation and proliferation signals, but the
mechanism remains poorly understood [Rogers et al., 2002].

CD6 is a type I membrane protein and a member of the scavenger receptor
cysteine-rich superfamily (SRCRSF) [Aruffo et al., 1997]. Its role in T cell activation has
been suggested by antibody cross-linking studies [Whitney et al., 1995]. The presence of
ALCAM and CD6 in T and B cells as well as activated leukocytes suggests that these two
molecules play roles in leukocyte activation as well as cross-talk between the nervous
and immune systems [Bowen et al., 1995].
The activation and proliferation of T cells in the context of HSV-2 infected neurons could result in antiviral effects, such as the release of interferons and the killing of virus-infected cells [Janeway et al., 1999]. The enhancement of T cell immunity would therefore be an adaptive response directed against the virus. Further studies are needed to investigate the nature and the extent of the immune system enhancement, and to elucidate the role of CD6-ALCAM interaction in T cell activation and proliferation.

b) Experiment 2: Effect of 4-hour exposure of acyclovir on human neuroblastoma cells.

The effects of acyclovir on neuroblastoma cells *in vitro* in the absence of HSV-2 are poorly studied. The accepted anti-viral mechanism of action for acyclovir, the phosphorylation of acyclovir to form acyclovir triphosphate, occurs at 40-100x higher levels in HSV-infected cells than uninfected cells [Mandell, 2000]. This is because the first phosphorylation is performed by viral thymidine kinase, which is not present in uninfected cells. Cellular enzymes subsequently phosphorylate acyclovir monophosphate to form acyclovir triphosphate [Elion, 1983]. Acyclovir triphosphate lacks the 3’ OH group that is required for DNA elongation, and incorporation of acyclovir into the growing nucleic acid strand can thereby result in the inactivation of viral DNA polymerase [Mandell, 2000].

The phosphorylation of acyclovir to produce acyclovir monophosphate does not occur to a significant degree in uninfected cells [Elion, 1983]. Acyclovir must therefore exert its effects, if any, on healthy cells via a different mechanism. It is possible that an
increased nucleoside concentration within the cell could affect enzyme function and protein production. Although acyclovir is a medication that is well-tolerated by most individuals, there are few people who are not able to tolerate this medication [Mandell, 2000]. It is possible that unknown cellular interactions may exist to explain these undesirable effects of nucleoside analogues.

Because experiment 2 was performed to acquire a baseline for the experiment 1 control condition, it is important to compare the results of these two experiments. The experimental condition for experiment 2 is the same as the control condition for experiment 1: neuroblastoma cells exposed to 100 μg/mL acyclovir for 4 hours. Both experiments 1 and 2 revealed the up-regulation of CXCR-5, suggesting that the up-regulation of this gene in the experimental condition of experiment 2 may be due to the effects of acyclovir, as opposed to solely due to HSV-2 infection. This phenomenon is discussed in more detail under the CXCR-5 subheading in this section.

CAD-5

Cadherin-5 (CAD-5) was up-regulated in response to acyclovir treatment. Method A found a median 16-fold up-regulation, while Method B yielded an s value of +5.

The cadherins are a group of calcium-dependent cellular adhesion molecules, involved in cell-cell interactions. Cadherins play roles in cellular proliferation, morphogenesis, and regulation of membrane permeability [Ferber et al., 2002]. During embryogenesis, cadherins are crucial for normal development of the vasculature [Dejana et al., 2000]. They are present on various tumours of stromal vessel endothelium,
irrespective of cell type growth pattern, or tumour grade [Markovic-Lipkovski et al., 2001]. The expression of CAD-5 has not been described in neurons.

CAD-5 is also known as vascular-endothelial cadherin (VE-cadherin). Its expression has not been described in neural cells. In the endothelium, however, two principal cadherins are expressed: CAD-5 and N-cadherin. CAD-5 plays a role in normal differentiation of endothelial barrier function during angiogenesis [DeFouw & DeFouw, 2000]. CAD-5 is expressed in bone cells, endothelial cells, and cells of hematopoietic origin. Its differential expression also plays a role in the modulation of angiogenesis in matrix-bound fibroblasts [Martin et al., 2001]. Increased levels of CAD-5 are correlated with development of restrictive endothelial barrier during normal angiogenesis [Cruz & DeFouw, 1999]. Loss of CAD-5-mediated adhesion leads to increased permeability of bone marrow endothelium monolayers [van Buul et al., 2002]. Cell-cell adhesion also controls the speed of endothelial expansion. CAD-5 seems to be associated with cell-cell junctional control of cell proliferation and migration [Underwood et al., 2002]. Up-regulation of CAD-5 could therefore lead to the proliferation of some blood vessels, and could affect the networking and direction of angiogenesis.

**ALCAM**

ALCAM was up-regulated in response to acyclovir treatment. Method A found a median 3-fold up-regulation, while Method B yielded an s value of +5 (see Table 4).

Cell adhesion molecules play many important roles in cell-cell interactions and selective cell recruitment. More than 35 of these molecules have been identified on
human cells [Middleton, 1998]. Cell adhesion molecules include the following families: selectins, integrins, and immunoglobulin superfamily [Janeway et al., 1999].

ALCAM is a 100-kDa member of the immunoglobulin (Ig) superfamily [van Kempen, 2001]. The Ig superfamily consists of more than 12 molecules [Middleton, 1998]. It is expressed on various cells, including spleen, lymph node, neurons, and various epithelial cells [Aruffo et al., 1997]. Also known as CD166, ALCAM is expressed mostly on various subsets of cells involved with growth and migration [van Kempen et al., 2001]. Although the expression of this molecule has been observed in neurons and glia in the brain, the importance of this interaction has not been further elucidated [Aruffo et al., 1997].

ALCAM expression has also been observed in cells of the immune system and those undergoing inflammatory response. Activated B and T cells, as well as synovial tissues affected by rheumatoid arthritis, express ALCAM [Joo et al., 2000]. Various primitive hematopoietic cells have also expressed ALCAM, notably those in adult and fetal bone marrow and fetal liver [Cortes et al., 1999]. The production of ALCAM on monocytes and macrophages is enhanced by M-CSF [Levesque et al., 1998]. On activated T cells, it is only transiently activated from 24-48 hours after activation and disappears 8 days post-activation [Aruffo et al., 1997].

ALCAM is capable of undergoing heterophilic (ALCAM-CD6) and homophilic (ALCAM-ALCAM) interactions [van Kempen et al., 2001], but the nature of these homophilic interactions has not been well explored.

As mentioned above, the ligand for ALCAM, known as CD6, is a member of the SRCRSF [Aruffo et al., 1997]. It is a co-stimulatory molecule for the activation of T cells
CD6 is expressed on many immune cells as well as sympathetic and parasympathetic spinal nerves [Konno et al., 2001]. CD6 is expressed by thymocytes, mature T cells, and neurons in the basal ganglia and cerebral cortex [Aruffo et al., 1997]. On T cells, CD6 stimulation can activate regulation signals [Rogers et al., 2002].

The up-regulation of ALCAM in response to acyclovir could result in a heightened immune and inflammatory response. Increased ALCAM production could contribute to enhanced activation and regulation of T cells and B cells. As well, activation of T cells to their effector function could result in various antiviral effects, such as the killing of HSV-2 infected cells.

CXCR-5

CXCR-5 was up-regulated in neuroblastoma cells in response to acyclovir treatment. Method A analysis found a median 3-fold up-regulation, while Method B yielded an s value of +5 (see Table 6).

CXCR-5, as described above, is a CXC chemokine receptor whose roles include the induction of B-cell migration [Ansel et al., 1999] and may include immunoregulation, cell activation, and control of angiogenesis and hematopoiesis [Middleton, 1998]. This chemokine is expressed on many immune cells such as naïve B cells [Bowman, 2000] and memory T cells [Campbell et al., 2001]. T cells up-regulate CXCR-5 when their conditions are suitable for migration, this results in increased B cell exposure [Ansel et al., 1999].

As previously discussed, in the experiments with acyclovir alone, CXCR-5 is significantly up-regulated. The up-regulation of CXCR-5 in response to challenge with
HSV-2 was higher than its up-regulation in response to acyclovir only. The effect of the acyclovir could also be increased due to the viral thymidine kinase increasing the cellular concentration of the drug in HSV-2 infected cells. Regardless, it seems that although acyclovir alone may be able to enhance the host immune system, acyclovir in the presence of HSV-2 infection may also be able to increases the immune response.

c) Experiment 3: Effect of temperature on four-hour incubation of human neuroblastoma cells in the presence of HSV-2.

The induction of latency by temperature increase is a well-established model for HSV [Harris & Preston, 1991; Kondo et al., 1990; Yura et al., 1987]. The differential gene expression between acute and latent infection has not been well described. Yet, these differences in gene expression could yield important information concerning genes that should be targeted for the induction of latency in HSV-2 seropositive patients.

The only gene found to be differentially expressed in a latent HSV model was macrophage-derived chemokine (MDC). The fact that only one gene was identified suggests that in this case, the parameters for significant gene expression were too stringent.

Macrophage-derived chemokine (MDC)

MDC was up-regulated in response to temperature increase (37°C to 40°C) in the presence of HSV-2. Although the temperature increase is necessary for latent infection [Yura et al., 1987], it remains possible that the differences in gene expression are due to
the increase in temperature only, and not to the induction of latency. More studies should be undertaken, including the investigation of uninfected and infected neuroblastoma cells at 40°C.

Method A analysis found a 7-fold up-regulation of MDC, while Method B yielded an s value of +8 (see Table 8). Because reproducible evidence of up-regulation was found by both methods of analysis, it is likely that MDC was up-regulated. Again, an increased number of experimental runs should be performed to increase accuracy and precision.

MDC is a chemoattractant for many immune cells, including NK cells. One study has shown its expression in neural cells in rats [Meucci et al., 1998]. MDC is a ligand for CCR4, which is expressed on many CD4+ T cell subsets [Kunkel et al., 2002] as well as platelets [Clemetson et al., 2000]. The upregulation of MDC in neuroblastoma cells that are latently infected with HSV-2 may enhance migration of immune cells to the site of neural infection, and may result in enhanced clearance of the virus and virus-infected cells. It is probable, however, that the secretion of MDC does not imply destruction of the latently-infected cells.

The effects of MDC on viruses have been contradictory and controversial. A truncated form of MDC had been shown to inhibit replication of HIV-1 [Pal et al., 1997] and HIV-2 [Struyf et al., 1998]. This effect of MDC on HIV replication has not been found by other investigators [Cota et al., 2000]. The inhibition of HIV-1 replication by MDC has been shown in some macrophages, however [Cota et al., 2000]. The inhibition of HIV-1 replication by MDC has not, however, been observed in similar experiments performed by at least one other researcher, and the originating laboratory has since admitted that effects could have been due to a contaminant [Comment, 1998].
Furthermore, low levels of MDC have been shown to enhance one strain of HIV [Greco et al., 1999].

Effects of MDC on HSV-2 have not been reported, but it is possible that similar effects could inhibit the replication of HSV-2 in neuroblastoma cells but not destroy them. Instead, it is possible that virus replication is inhibited only and that HSV-2 persists within the intact cell. It is therefore possible that an increase in temperature would upregulate MDC in HSV-2 infected cells and enhance latency by (a) increasing migration of immune cells to the site of latent infection, and (b) inhibiting replication, but not the destruction of, the virus in infected cells.
d) Possible Future Studies

Current work only concerns the infection of neuroblastoma cells with HSV-2 in the presence of acyclovir. For a better representation of what genes are transcribed during HSV-2 infection, experiments with HSV-2 infected neuroblastoma cells in the absence of acyclovir should be performed.

Because gene transcription begins soon after infection and continues for several hours afterwards [Roizman & Knipe, 2001; Sawtell et al., 1999], it would be worthwhile to investigate the transcription of genes at different time points after infection with acyclovir as well as with HSV-2. The earliest genes are induced within minutes of stimulation, while later genes are only induced many hours afterwards. Therefore, a more complete picture of gene transcription would be attained with time points ranging from 30 minutes to 12 hours.

The latency model for HSV-2 and neuroblastoma cells should also be further investigated by microarray. In this manner, genes that are expressed differently during latency and acute infection can be identified. This may help pinpoint target genes for immunotherapy for patients who suffer from recurrent lesions. If the expression or exogenous introduction of certain proteins can help induce latency in an HSV-2 seropositive patient, the patient may be able to live more comfortably.

Although the DNA microarray is a powerful tool that enables researchers to examine the expression of thousands of genes simultaneously, it has weaknesses. One of the most glaring limitations is the variability of the assay. The nature of the microarray is such that any number of steps could result in a different signal, even if the profile of RNA
expression were exactly the same in each experiment. Factors contributing to the variability include the RNA extraction, incorporation of labeled nucleotide into cDNA, and the hybridization of the cDNA to the microarray.

As well, because microarray technology is a quickly-expanding field in terms of facilities and programs available for information analysis, these new developments should be applied to the experiments described in this thesis as well as future experiments. These techniques include improvements in data analysis, such as the individual subtraction of background values due to non-specific binding, which may differ according to the location of gene spots on the microarray filter or slide. Individualization of the background normalization would increase the accuracy of results because the extent of non-specific binding could differ throughout the same slide.

Though the neuroblastoma is a good model for preliminary examination of HSV-2 infection, it does not offer a perfect reflection of the in vivo situation. This is due to many factors, including the fact that the neuroblastoma cells have a 49n karyotype, and that the nutrient media does not include all the constituents present in a living organism. Thus, microarray analysis of a mouse model of HSV-2 infection of the trigeminal nerve should be performed [Feldman et al., 2002]. Additional investigations of murine vaginal HSV-2 infections could also be performed [Parr & Parr, 1997].

Mouse knockouts of the genes found to be expressed differently would also be important. After the protein expression of these genes is verified via ELISA, flow cytometry, and Western blot, mouse knockouts would provide an in vivo model to ascertain the importance of these genes to the immune system during challenge with HSV-2.
New technologies are emerging regarding the microarray of proteins. The technology is still in its infancy, but if a viable method for investigating a wide range of protein expression is developed, it would be worthwhile to perform all the neuroblastoma and mouse experiments. Comparisons between the DNA and protein microarrays would also shed light onto the strengths and limitations of each of these techniques.

Finally, these microarray and mouse knockout experiments would yield differentially expressed genes which could be targeted for HSV-2 vaccines and immunotherapy. Targeting genes for induction of longer periods of latency in previously-infected HSV-2 seropositive patients could greatly improve their quality of life.
Chapter 5: Summary and Conclusions

Microarray technology has quickly blossomed in the past five years as a powerful research tool. It has enabled simultaneous assessment of a large number of genes. Because of its ability to measure the differential gene expression at one particular timepoint, possible interactions between genes can be identified for further investigation. Because of the previously-discussed limitations of microarray, a number of steps were taken to ensure that “real” changes in gene expression were differentiated from artifacts. The first step involved examining the scans of filter arrays and glass slides to determine whether the signal from all genes is uniform. If extreme irregularities, such as a dark signal that is not in the shape of a DNA spot, are seen, that spot is discounted from the data set.

The next step involved repeating the experiments three times. Increasing the number of runs of an experiment and comparing the results of each run will increase the significance of measured values. Naturally, it would be ideal for the experiments to be repeated as many times as possible, but this is not always practical.

Another step involved using the consensus of two methods for assessing significance. One method for calculating differential gene expression was developed in the Virology Research Laboratory at the Children’s Hospital of Eastern Ontario, while the other was developed by the Department of Mathematics at the University of Ottawa. By accepting only the genes that were deemed significant by both methods of analysis, the likelihood of pinpointing a gene that is not truly differentially expressed is diminished.
Despite the importance of interactions between immune and neuronal systems in viral reactivation from latency in nerve roots, these interactions have not been extensively examined. The study of neural cell gene expression as it is subjected to HSV-2 challenge could help pinpoint candidates suitable for further investigation for antiviral therapy. To distinguish between effects due to acyclovir and those due to the virus, the effects of acyclovir alone were measured. Finally, the differential gene expression of nerve cells infected with HSV-2 under conditions of acute and latent infection could help identify candidates that enable the virus to persist in the host whilst eluding the immune system.

In response to HSV-2 stimulation, FGF R2, IL-16, CXCR-5, IFN-α/β Rβ, ephrin-B1, endothelin-2, CD6, and IL-3 Rα were up-regulated. FGF R2 has been identified on cells of neural origin, but its role has not been further clarified [Reimers et al., 2001]. Because the roles of FGF R2 in other cells involve angiogenesis and tumorigenesis, it is possible that its up-regulation is a viral strategy for survival.

Dual functions for IL-16 exist: chemoattraction and progression through the cell cycle. IL-16 also exists in a neuronal form [Kurschner & Yuzaki, 1999]. Its roles include the enhancement of CD4+ cells and cell differentiation. In virus-infected cells, IL-16 can inhibit viral replication. Its up-regulation could therefore result in many antiviral effects.

There have also been no reports of CXCR-5 expression in neurons. In B and T cells, however, the roles of this chemokine receptor include lymphocyte migration [Ansel et al., 1999]. It is therefore possible that the up-regulation of CXCR-5 could enhance lymphocyte migration to neurons.

Type 1 interferons are known to have antiviral [Mikloska & Cunningham, 2001] and anti-proliferative [Prejean & Colamonici, 2000] effects. IFN α/β Rβ is up-regulated
in response to HSV-2; this could inhibit viral spread in neurons [Mikloska & Cunningham, 2001]. MHCI presentation is also enhanced [Janeway et al., 1999]. Thus, signaling through the beta R of the Type I IFN receptor could enhance the host immune response.

Upon HSV-2 infection, ephrin-B1 is also up-regulated. In endothelium, it plays a role in angiogenesis [Mellitzer et al., 2000]. Enhanced ephrin-B1 expression could therefore influence the patterning of blood vessels in the area of infection, which could improve accessibility for immune cells.

Endothelin-2 is also up-regulated with HSV-2 infection. Immune and neuronal cells produce endothelins, which play roles in cell survival and proliferation [Damon, 1999] and angiogenesis [Gianessi et al., 2001]. Endothelin-2 up-regulation could enhance vessel formation, which could improve accessibility of the infected area for lymphocytes. As well, effects that benefit the virus could also occur, such as enhanced cell survival.

HSV-2 infection also results in CD6 up-regulation. Its expression in neurons has not been previously described. Roles of CD6 in the immune system include the co-stimulation of lymphocytes, including the activation of T cells. Its up-regulation could enhance host immunity in response to HSV-2 stimulation.

The IL-3 Rα is also up-regulated upon HSV-2 infection. It is expressed on differentiated neurons [Kannan et al., 2000] and is associated with a neuroprotective effect [Wen et al., 1998]. Because IL-3 is known to have proliferative effects on many immune cells [Frederk, 1992], the up-regulation of its receptor could enhance immunity.

The effect of HSV-2 infection thus includes the differential expression of many cell surface molecules, including cytokine receptors and co-stimulatory molecules. Also,
secreted factors are differentially expressed in cells of neural origin; this suggests a deeper role in the communication with the immune system and the interaction of viruses with host immunity.

The effect of acyclovir in the absence of HSV-2 has not been well examined. The results of these investigations suggest that acyclovir affects neural cells more than had been previously thought, and that these effects include the differential expression of chemokine receptors as well as adhesion molecules. CAD-5, CXCR-5, and ALCAM were found to be up-regulated in response to acyclovir stimulation.

There have been no previous reports of CAD-5 in neurons, but its roles in endothelial and hematopoietic cells involve barrier formation and vessel permeability [Cruz & DeFouw, 1999; van Buul, 2002]. The regulation of endothelial permeability could have implications for immune cell recruitment.

CXCR-5 is up-regulated in response to HSV-2 stimulation in the presence of acyclovir, as well as in response to acyclovir alone. It is therefore possible that the differential gene expression is due to acyclovir as well as to the virus. The extent of up-regulation is higher in response to HSV-2 than acyclovir, however; this suggests that the differential gene expression is not only due to the drug. The roles of CXCR-5 include immune cell recruitment [Ansel et al., 1999]. Therefore, it is possible that HSV-2 further enhances the extent of CXCR-5 expression, which could in turn heighten host immunity.

ALCAM was also up-regulated by acyclovir. This protein has been described in the brain, but its role in neuronal cells has yet to be determined [Aruffo et al., 1997]. In other cellular contexts, its roles involve cell migration and regulation of the inflammatory
response. Possible roles for ALCAM in acyclovir-stimulated neurons include the enhancement of host immune response.

Latent infection of HSV-2 has revealed the up-regulation of MDC, in comparison to productive infection. MDC is expressed in neurons [Meucci et al., 1998] but remains to be extensively studied. Roles of MDC may include migration of immune cells to the site of infection [Kunkel et al., 2002; Clemetson et al., 2000]. There are also conflicting reports that this chemokine may inhibit replication [Pal et al., 1997; Comment, 1998]. The up-regulation of MDC may therefore enhance the host antiviral response, but further studies should be conducted to investigate the nature of MDC as well as its role in HSV-2 infection.

The effect of temperature on productive and latent virus models includes the differential expression of molecules involved with immune cell chemotaxis. These results suggest that the induction and maintenance of viral latency involve the recruitment of host immune cells to infected neurons.

Experiments with cultured neurons, as well as in vivo models, should be used for future investigations. Because expression at the RNA level does not necessarily translate to protein expression, the genes found to be differentially expressed by nucleic acid microarrays should be verified by ELISA, flow cytometry, and/or immunoblot.
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