INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®
NOTE TO USERS

Page(s) missing in number only; text follows. Page(s) were microfilmed as received.

114

This reproduction is the best copy available.

UMI
NEUROENDOCRINE MODULATION OF INNATE IMMUNITY DURING PRIMARY HERPES SIMPLEX INFECTION

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Griselle C. Ortiz, D.M.D., M.S.

The Ohio State University

2001

Dissertation Committee:

Dr. Phillip T. Marucha
Dr. John F. Sheridan
Dr. Keith Alley
Dr. David Padgett

Approved by

Adviser
Oral Biology Graduate Program
ABSTRACT

Herpes simplex viruses (HSV) are the cause of the most common clinically recognized herpesvirus infections. The severity and duration of the primary HSV infection have been correlated with the frequency and severity of subsequent recurrences. It is known that reactivation of latent HSV-1 can occur as a result of physical and/or emotional stress. However, the effects of stress on the modulation of the clinical pathophysiology of primary HSV-1 infections are not well understood. Although it is known that stress is immunosuppressive, we still do not understand the immunological mechanisms by which stress modulates early immune responses during a primary HSV-1 infection. Therefore, further information that could lead to the control of herpes virus infections in humans is necessary. Our understanding of how events that occur during a primary HSV infection are modulated by stress is of central importance to realizing this goal.
In this investigation, a cutaneous HSV-1 infection in the SKH-1 mouse model was characterized and utilized in order to study the effect of stress on the modulation of the clinical pathophysiology of cutaneous HSV-1. It was hypothesized that due to suppressed early immune responses, stress would increase the severity of a cutaneous primary HSV-1 infection. Despite increasing viral replication, RST decreased the clinical severity of primary HSV-1 in the skin of SKH-1 mice. Restrained animals also presented higher cellularity scores at the site of infection compared to controls. The mRNA levels of MIP-1 alpha and MCP-1, chemokines involved in leukocyte trafficking, were increased in RST animals when compared to controls. This increase correlated with the increase in cellularity observed. Restraint also increased the expression of IL-10 in the skin of mice acutely infected with HSV-1. Stress-induced modulation of innate immunity was also studied. A decrease in type I and type II IFN expression was found in the skin of acutely infected restrained mice when compared to controls. IFN-beta and INF-gamma expression levels were restored in restrained animals to control levels after treatment with RU486, while IFN-alpha levels remained suppressed. Treatment with RU-486 also increased the clinical severity of the cutaneous infection to control levels in restrained mice.
Dedicated to my husband Jeff Ramsey
ACKNOWLEDGMENTS

I thank my adviser, Dr. Phillip Marucha, and the members of my dissertation committee; Dr. John Sheridan, Dr. Keith Alley, and Dr. David Padgett for their guidance during this project.

I am grateful to Dr. John Horton and Dr. Keith Alley for giving me the opportunity to come to Ohio State and pursue the Dentist Scientist Program.

I thank Dr. Ana Mercado and Dr. Gina Rojas for their constant support and friendship. I thank Christine Daugherty for her friendship and for all the help she provided me. I thank Ray Tseng for his help conducting the disease severity scoring. I thank Dr. Michael Beck for providing some statistical advice. I also thank Dr. Christine Halket and Meghan Fox for their help in editing this dissertation.

I am especially grateful to my husband for his constant support and patience. I thank my parents and my siblings for their support.

This project was supported by a Mentored Clinical Scientist Development Award from the NIDCR.
VITA

January 13, 1968........................................................Born-Aibonito, Puerto Rico
1992..........................................................D.M.D., University of Puerto Rico
1995..........................................................M.S., Oral Biology, University of Alabama at Birmingham
1995-present.........................................................Graduate Teaching Associate, The Ohio State University
1998..........................................................Certificate in Periodontology, The Ohio State University, College of Dentistry

PUBLICATIONS


G. C. Ortiz, J. F. Sheridan, and P.T. Marucha. Stress-induced changes in the pathophysiology of primary HSV-1 infection. (In preparation)

FIELDS OF STUDY

Major Field: Oral Biology
Minor Fields: Virology, Immunology, and Molecular Biology
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>v</td>
</tr>
<tr>
<td>Vita</td>
<td>vi</td>
</tr>
<tr>
<td>List of Tables</td>
<td>ix</td>
</tr>
<tr>
<td>List of Figures</td>
<td>x</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xiii</td>
</tr>
</tbody>
</table>

## Chapters

1. Introduction......................................................... 1

   - Epidemiology of HSV-1 infections................. 3
   - Clinical presentation of HSV-1 infections.......4
   - Structure of HSV.............................................. 8
   - Viral attachment fusion and envelopment.......10
   - Viral replication...........................................12
LIST OF TABLES

Table                                                                 Page
1.1 Clinical presentation of primary and recurrent HSV-1 ...............5
2.1 Primer sequences used for RT-PCR .........................................57
2.2 Primer sequences used for competitive RT-PCR ........................58
2.3 Primers and probes sequences used for real-time PCR ...............59
3.1 Between group comparison of severity and dermatome involvement .................................................................88
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>9</td>
</tr>
<tr>
<td>1.2</td>
<td>18</td>
</tr>
<tr>
<td>1.3</td>
<td>21</td>
</tr>
<tr>
<td>1.4</td>
<td>23</td>
</tr>
<tr>
<td>1.5</td>
<td>27</td>
</tr>
<tr>
<td>1.6</td>
<td>28</td>
</tr>
<tr>
<td>2.1</td>
<td>56</td>
</tr>
<tr>
<td>3.1</td>
<td>77</td>
</tr>
<tr>
<td>3.2</td>
<td>79</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>3.3</td>
<td>The expression of IL-1β, IFN-α, IFN-β, and NGF at the site of primary infection one day post-cutaneous HSV-1 in SKH-1 mice</td>
</tr>
<tr>
<td>3.4</td>
<td>The kinetics of gB and gD mRNA expression during cutaneous HSV-1 infection in SKH-1 mice</td>
</tr>
<tr>
<td>3.5</td>
<td>The clinical course of cutaneous primary HSV-1 infection in restrained SKH-1 mice</td>
</tr>
<tr>
<td>3.6</td>
<td>Dermatome spread development during cutaneous HSV-1 infection in restrained SKH-1 mice</td>
</tr>
<tr>
<td>3.7</td>
<td>IgM response to primary cutaneous HSV-1 infection in restrained SKH-1 mice</td>
</tr>
<tr>
<td>3.8</td>
<td>IgG response to primary cutaneous HSV-1 infection in restrained SKH-1 mice</td>
</tr>
<tr>
<td>3.9</td>
<td>Effects of RST on the kinetics of infectious HSV-1 titers at the site of primary cutaneous infection</td>
</tr>
<tr>
<td>3.10</td>
<td>Effect of RST on the cellularity present at the site of primary cutaneous HSV-infection</td>
</tr>
<tr>
<td>3.11</td>
<td>The effect of RST on MCP-1 mRNA expression at the site of primary cutaneous HSV-1 infection in SKH-1 mice</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>3.12</td>
<td>The effect of RST on MIP-1 alpha mRNA expression at the site of primary cutaneous HSV-1 infection in SKH-1 mice</td>
</tr>
<tr>
<td>3.13</td>
<td>The effect of RST on IL-10 mRNA expression at the site of primary cutaneous HSV-1 infection in SKH-1 mice</td>
</tr>
<tr>
<td>3.14</td>
<td>Effect of RST on IFN-alpha gene expression during primary cutaneous HSV-1 infection</td>
</tr>
<tr>
<td>3.15</td>
<td>Effect of RST on IFN-beta gene expression during primary cutaneous HSV-1 infection</td>
</tr>
<tr>
<td>3.16</td>
<td>Effect of RST on IFN-gamma gene expression during primary cutaneous HSV-1 infection</td>
</tr>
<tr>
<td>3.17</td>
<td>Effect of glucocorticoid-receptor blockage on the clinical signs of a primary cutaneous HSV-1 infection in restrained mice</td>
</tr>
<tr>
<td>3.18</td>
<td>Effect of RU-486 on IFN gene expression during primary cutaneous HSV-1 infection in restrained mice</td>
</tr>
<tr>
<td>4.1</td>
<td>Possible mechanism for RST reduction of clinical severity during primary HSV-1 infection</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

ANOVA- Analysis of variance
BSA- Bovine serum albumin
DMEM- Dulbecco's modified Eagle medium
ELISA- Enzyme-linked immunosorbent assay
FWD- Food and water deprived
gB- Glycoprotein B
gD- Glycoprotein D
GC- Glucocorticoids
HSV- Herpes simplex virus
HSV-1 Herpes simplex virus type 1
Hve- Herpes virus entry mediator
HCF- Host cell factor
HPA- Hypothalamic-pituitary-adrenal axis
H&E- Hematoxylin and eosin
IFN- Interferon
IFNAR- Type I interferon receptor
IL- Interleukin
IP- Interferon gamma inducible protein
IPC- Infected cell polypeptide
LAT- Latency associated transcripts
Mig- Monokine induced by IFN-gamma
NGF- Nerve growth factor
NK- Natural killer cell
Oct- Octamer
PBS- Phosphate buffered saline
PMN- Polymorphonuclear cell
PCR- Polymerase chain reaction
RST- Restraint stress
RT- Reverse transcription
SNS- Sympathetic nervous system
TNF- Tumor necrosis factor
UV- Ultraviolet light
VP- Viral protein
CHAPTER 1

INTRODUCTION

Millions of people worldwide suffer from herpes simplex type I (HSV-1) infections (Liesegang, 2001). During the initial (primary) infection, HSV-1 invades the host through abrasions on the skin or mucous membranes and multiplies in the epithelium. The virus can evade the host immune system (Watanabe et al., 1999) and travel by retrograde axonal transport to neuronal cell bodies located in sensory ganglia (Penfold, 1994). Once in the sensory ganglia, the virus establishes a latent infection which could later reactivate, causing recurrent disease (Roizman and Sears, 1996, Watanabe et al., 1999).

The severity and duration of a primary HSV infection has been associated with the frequency and severity of recurrences (Pereira, 1996). Popular belief holds that acute and chronic psychological stress can trigger recurrences of HSV infections. Furthermore, many studies support the hypothesis that emotional stress is associated
with the development of recurrent HSV infection (Bierman, 1983, Friedman et al., 1977, Glaser et al., 1987, Katcher et al., 1973, Schmidt et al., 1985, Young et al., 1976). Although it is known that stress is immunosuppresive (Weizman and Bessler, 1999), we still do not understand the immunological mechanisms by which stress modulates early immune responses, such as expression of type I interferons during a primary HSV-1 infection. Many of the effects of stress on immune responses are mediated by glucocorticoids, which are released in response to the activation of the hypothalamic-pituitary-adrenal axis (Munk and Guyre, 1991). During primary HSV-1 infection, suppressed immune responses due to glucocorticoids and other stress-related hormones may impair the ability of the host to activate initial viral clearance, increasing the severity and duration of the infection.

Our laboratories have been interested in the study of the impact of stress on health and disease. For that purpose, various animal models have been developed specifically for the study of viral infections. In this body of work we utilized a murine cutaneous HSV-1 model with zosteriform spread to study the mechanisms in which a stressor, such as restraint stress (RST), modulates primary HSV-1. Understanding the events that modulate primary HSV infections is necessary for the
development of appropriate means of preventing primary infections and controlling recurrent disease. This chapter is intended as a review of the literature and will cover epidemiology, clinical presentation, general virology, and immune responses to HSV infection. The chapter concludes with a statement of purpose of this investigation.

**Epidemiology of HSV-1 infections**

Between 40 to 50 percent of adolescents and 60 to 90 percent of adults worldwide are infected with HSV-1 (Blackwelder et al., 1982, Gil et al., 1998, Liesegang, 2001, Rodu et al., 1992, Siegel et al., 1992). Clinical surveys greatly underestimate the incidence and prevalence of HSV-1 infections because more than two thirds of primary HSV-1 infections are asymptomatic (Gorbach et al., 1998). Other studies have shown an even lower prevalence of symptomatic primary HSV, reporting that only one to six percent of primary HSV-1 infections exhibit clinical symptoms (Umene and Sakaoka, 1999). It is known that the prevalence of HSV-1 can not be accurately determined by its clinical history. Serologic studies are usually favored to establish the prevalence of HSV-1
infections (Liesegang, 2001). Sero-epidemiologic studies have shown that 70-80 percent of the population in North America has antibodies against HSV (Liesgang, 2001, Royston and Aurelian, 1970).

The prevailing mode of HSV transmission is via asymptomatic shedding, since the number of people presenting asymptomatic disease is greater than the number of people that present clinical symptoms (Liesegang, 2001). It has been reported that asymptomatic HSV shedding happen in up to 80 percent of infected people (Liesegang, 2001).

Clinical presentation of HSV-1 infections

Most of the people that first encounter HSV-1 will develop antibodies against the virus through an asymptomatic infection (Royston and Aurelian, 1970). However, other people that become infected with the virus for the first time can develop typical herpetic infections such as herpetic gingivostomatitis. As summarized in Table 1.1 (Adapted from Ash and Ward, 1986, and Birnbaum and Dunne, 2000), acute or primary herpetic gingivostomatitis is characterized by the development of a high
### Primary Herpetic Gingivostomatitis

**Signs and symptoms:**
- High fever (102 to 104° F)
- Regional lymphoadenopathy
- Sore throat
- Occasional skin rashes
- Lips, gingiva, palate and tongue may be affected
- Erythematous and swollen gingiva
- Oral ulcers develop as vesicles rupture
- Ulcers
  - 2-3 mm in size
  - round and shallow
  - multiple and coalesce
- Duration
  - 7 to 10 days
  - 1 to 2 additional weeks if ulcers become secondarily infected

### Recurrent Infection

**Signs and symptoms:**
- Itching, burning or tingling sensation
- Tiredness and malaise
- Crops of vesicles appear after 24 hrs
- Lesions frequently present:
  - at the junction of the vermillion border and the skin of the lips
  - at nose and adjacent skin (mucocutaneous junction)
- Duration
  - 7-10 days

### Predisposing Factors:
- Sunlight and cold
- Psychological stress
- Trauma
- Menstruation and occasionally pregnancy
- Systemic illness: e.g. common cold
- Immune suppression

<table>
<thead>
<tr>
<th>Primary Herpetic Gingivostomatitis</th>
<th>Recurrent Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Signs and symptoms:</strong></td>
<td><strong>Signs and symptoms:</strong></td>
</tr>
<tr>
<td>High fever (102 to 104° F)</td>
<td>Itching, burning or tingling sensation</td>
</tr>
<tr>
<td>Regional lymphoadenopathy</td>
<td>Tiredness and malaise</td>
</tr>
<tr>
<td>Sore throat</td>
<td>Crops of vesicles appear after 24 hrs</td>
</tr>
<tr>
<td>Occasional skin rashes</td>
<td>Lesions frequently present:</td>
</tr>
<tr>
<td>Lips, gingiva, palate and tongue</td>
<td>- at the junction of the vermillion border and the skin of the lips</td>
</tr>
<tr>
<td>may be affected</td>
<td>- at nose and adjacent skin (mucocutaneous junction)</td>
</tr>
<tr>
<td>Erythematous and swollen gingiva</td>
<td>Duration</td>
</tr>
<tr>
<td>Oral ulcers develop as vesicles</td>
<td>7-10 days</td>
</tr>
<tr>
<td>rupture</td>
<td></td>
</tr>
<tr>
<td>Ulcers</td>
<td></td>
</tr>
<tr>
<td>2-3 mm in size</td>
<td></td>
</tr>
<tr>
<td>round and shallow</td>
<td></td>
</tr>
<tr>
<td>multiple and coalesce</td>
<td></td>
</tr>
<tr>
<td>Duration</td>
<td></td>
</tr>
<tr>
<td>7 to 10 days</td>
<td></td>
</tr>
<tr>
<td>1 to 2 additional weeks if</td>
<td></td>
</tr>
<tr>
<td>ulcers become secondarily infected</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1: Clinical presentation of primary and recurrent of HSV-1. (Adapted from the following sources: Ash and Ward, 1986 and Birnbaum and Dunne, 2000)
fever, swelling of the regional lymph nodes, and “sore” throat (Ash and Ward, 1986, Birnbaum and Dunne, 2000). Generalized pain in the oral cavity develops a few days after the initiation of the infection along with diffuse redness and swelling of the gingival tissues. After the development of “sore” mouth symptoms, the fever diminishes and typical herpetic vesicles develop. Ulcers form as the vesicles rupture making eating, drinking, and oral hygiene very painful. The infection is self-limiting and resolves in approximately seven to ten days (Ash and Ward, 1986, Birnbaum and Dunne, 2000). However, ulcers may become secondarily infected, prolonging the infection for another one to two weeks (Ash and Ward, 1986). The duration of a primary HSV infection has been associated with an increase in the frequency and severity of recurrences of the disease (Pereira, 1996).

Herpetic lesions are recognized histologically by distinct ballooning degeneration of the epithelium (Watanabe et al., 1999), which is characterized by enlarged and pale keratinocytes and acantholysis (Rapapini and Jordon, 1988). Watanabe et al. (1999) described that “epithelial hyperplasia with cell fusion of some cells and subsequent
necrosis and vasculitis in the upper dermis with granulocyte infiltration of the epidermis” (Watanabe et al., 1999) may also be observed histologically.

After initial exposure to the virus, it persists as a latent infection of the sensory ganglia, from where it can return unexpectedly to the epithelium to cause a recurrent infection (Pereira, 1996) (Hill, 1985). Recurrent HSV-1 infections can occur at epithelial sites such as the eyes, the skin, the oral cavity and the lips (Miller et al., 1998). Frequently, recurrent HSV-1 infection occurs at the vermillion border and skin of the lips or at the mucocutaneous junction of the nose (Table 1.1). As summarized in Table 1.1 (Adapted from Ash and Ward, 1986 and Birnbaum and Dunne, 2000), recurrent disease begins with a burning, tingling sensation (prodromal phase), followed by the development of herpetic vesicles (Ash and Ward, 1986, Birnbaum and Dunne, 2000). As in the primary infection the vesicles can rupture and coalesce before crusting occurs. Recurrent episodes of HSV-1 usually last seven to ten days. Some of the factors that have been associated with triggering recurrent HSV-1 infections are sunlight (UV light) and cold, psychological stress, mechanical trauma, menstruation and occasionally pregnancy,
systemic illness (e.g., the common cold), and immunosuppression (Birnbaum and Dunne, 2000). Mechanisms and mediators that could be involved in the reactivation of HSV-1 will be addressed later in this chapter.

Structure of HSV

Before we address the mediators that could be involved in triggering recurrent HSV-1 episodes, it is important to discuss some aspects of basic herpes simplex virology. First, HSV-1 is a member of the Herpesviridae alphaviridae family, classified on the basis of its architecture. The virus has four basic components: the capsid, tegument, envelope, and core (Flint et al., 2000, Ho, 1992, Pereira, 1996, Steiner and Kennedy, 1995). A diagram representing the structure of HSV-1 is presented in Figure 1.1 (Adapted from Flint et al., 2000). The core contains the virus linear double-stranded DNA (Pereira, 1996). The capsid protects the core and is composed of 162 identical capsomers (Pereira, 1996). The tegument is a structure without distinctive morphology found between the capsid and the envelope that contains important proteins for viral replication. Two of the proteins that are contained within the tegument are alpha-trans inducing factor (alpha-TIF
Figure 1.1: Structure of herpes simplex virus.  
(Adapted from Flint et al., 2000)
or VP16) and virion host shut off protein (VHS) (Kwong and Frenkel, 1988, Wu et al., 1994). The viral replication cascade is started by alpha-TIF, while VHS turns off the host-cell macromolecular system (Kwong and Frenkel, 1988, Wu et al., 1994). The envelope, the outer component of the HSV-1 virion, is derived from host-cell nuclear membranes and contains eleven glycoproteins present in its surface (Pereira, 1996). These glycoproteins have been described by Pereira as “the major target of humoral and cellular immune response and they mediate attachment, fusion, envelopment, and viral egress” (Pereira, 1996).

**Viral attachment, fusion, and envelopment**

For the virus to be able to infect a cell, it needs to attach to its cell membrane. The initial attachment of the HSV-1 virion to the cell membrane is mediated by the binding of glycoproteins B and C to cell surface glycosaminoglycans (Sears et al., 1991). Following initial attachment, interactions between the viral glycoproteins and cellular receptors allows for the virion to fuse with the cell membrane. The herpes virus entry mediator A (Hve-A), a member of the TNF superfamily
of receptors, has been identified as the glycoprotein D receptor (Sears et al., 1991). The lymphotoxin receptor and the polio-receptor related proteins have been identified as Hve-B and Hve-C, respectively (Sears et al., 1991).

Once the virus has entered the cell, the capsid and the tegument are released inside it allowing for the tegument protein VMS to enter the cytoplasm (Kwong and Frenkel, 1988, Schek and Bachenheimer, 1985). In the cytoplasm, VMS is involved in degrading and destabilizing the host's messenger RNA (mRNA), thereby interfering with host-cell protein synthesis (Kwong and Frenkel, 1988, Schek and Bachenheimer, 1985).

After the capsid reaches the cytosol, it associates to a microtubule and is transported close to the nucleus (Whittaker and Helenius, 1998). Dyenin, a minus-end-directed microtubule motor, has been described to mediate this transport (Sodeik, 2000). According to Whittaker and Helenius (1998), microtubule-associated transport is vital in the infection of neurons because the virus has to travel long distances to reach the neuronal nucleus (Whittaker and Helenius, 1998). As the HSV capsids are transported, they accumulate near the nuclear envelope and associate with the nuclear pore complexes. Once the virus is attached to the
nuclear pore, it can release its DNA inside the nucleus (Newcomb and Brown, 1994). Tegument proteins alpha-TIF (alpha transducing factor) and VHS (virion shutoff protein) also enter the nucleus, although they do not travel with the capsids (Batterson and Roizman, 1983).

**Viral Replication**

Three groups of viral genes are expressed during HSV-1 replication. These three groups of genes are classified as immediate-early (alpha), early (beta), and late (gamma) genes (Pereira, 1996). Once in the nucleus, alpha-TIF (viral tegument protein) binds to transcription factors from the host-cell initiating the transcription of viral immediate-early genes (McKnight et al., 1997). The complex formed between alpha-TIF and host-cell transcription factor binds to the promoter region of immediate-early genes (McKnight et al., 1997). This binding promotes the upregulation of immediate-early genes to produce five alpha-proteins. These proteins are known as infected cell polypeptides (IPCs). IPCs are potent activators of early and late genes (Pereira, 1996). The synthesis of alpha-proteins peaks approximately three hours after infection (Pereira, 1996).
The products of immediate-early genes initiate the expression of early genes (Miller et al., 1998). Early genes encode for polypeptides that are involved in DNA replication (Pereira, 1996). Thymidine kinase and DNA polymerase are produced by early genes, and their expression correlates with viral DNA synthesis. Expression of beta genes usually peaks six hours post-infection (Pereira, 1996).

The last viral genes to be expressed are the late genes. Late genes code for structural proteins such as VP16, VHS, capsid proteins, and glycoproteins (Pereira, 1996). Viral DNA synthesis directs gamma gene expression (Holland et al., 1980). The peak expression of gamma genes usually takes place nine hours after HSV infection (Pereira, 1996).

It has been described by Pereira (1996) that “viral DNA is synthesized in a rolling circle fashion” (Pereira, 1996). After its synthesis, the DNA is assembled and packaged into empty capsids (Pereira, 1996).

**Viral assembly and egression**

After the genome is packed, the capsids are ready to be assembled. HSV-1 capsids are assembled in the nucleus, but due to their large size (120nm), egression can not be accomplished via the nuclear pore complex (Whittaker and Helenius, 1998). In order to exit the
nucleus, the capsids bind to the inner nuclear membrane and bud into the lumen of the infected cell. Two possible mechanisms for the egression of the capsids have been proposed (reviewed by Whittaker and Helenius (Whittaker and Helenius, 1998)). In the first model, enveloped capsids travel via the endoplasmic and through the Golgi apparatus to reach the cell surface. The second model proposes that the virion looses its envelope in the cytoplasm and then passes through the Golgi apparatus. As the virion exits the Golgi apparatus, it gets re-enveloped. The virion finally fuses with the plasma membrane and is released to the extracellular space (Whittaker and Helenius, 1998). Once in the extracellular space, new virions can attach to neighboring cells and infect them.

**Viral Latency**

As previously described, HSV persists as a latent infection of the sensory ganglia after initial exposure to the virus (Pereira, 1996). According to Miller et al. (1998), latency is defined as “an infection in which the viral genome is present in a non-replicating state in an infected cell where the virus can intermittently reactivate” (Miller et al., 1998). HSV-1 DNA does not integrate into the infected cell genome during
latency. It exists in a circularized form and can be physically separated from the host's DNA (Deshmane and Fraser, 1989, Rock and Fraser, 1985, Wagner and Bloom, 1997). Sawtell (1997) stated that in infected neurons HSV DNA is associated with nucleosomes in a chromatin structure and only a small percentage of neurons in a ganglion are infected (Sawtell, 1997).

The concept of viral latency suggests the complete absence of viral transcription, but it is known that a group of genes identified as the latency associated transcripts (LATs) are transcribed during the latent state of the neuronal infection (Turner and Jenkins, 1997). Therefore, viral gene transcription is not completely dormant during latency. LATs are a group of RNAs that have been found in the nuclei of HSV-1 latently infected cells, and are transcribed during active and latent HSV-1 infections (Dobson et al., 1995, Nicosia et al., 1994, Wechsler et al., 1988). Induction of HSV-specific transcripts is not observed in cells that are infected with mutant LATs (Block et al., 1990), suggesting that they may play a role in the inhibition of viral replication. Viral deletion experiments indicate that LATs are not required for the establishment of a latent infection (Ho and Mocarski, 1989). Although the function of
LATs is still controversial, studies suggest that they could be involved in the down-regulation of immediate early genes and the thymidine kinase gene during acute productive infection (Chen et al., 1997, Garber et al., 1997).

Latency occurs because conditions exist within the neuronal nucleus, which inhibit the expression of genes important for HSV replication. During viral replication, a complex made of alpha-TIF and two cellular factors, octamer 1 (Oct-1) and a host cell factor (HCF), needs to be formed for alpha-TIF efficient DNA binding (Sadowsky et al., 1988, Wu et al., 1994). Oct-1 is a cellular transcription factor that, when in complex with alpha-TIF, recognizes and weakly binds to a sequence present on the promoter of viral immediate-early genes (Kristie and Sharp, 1990). HCF binding to the alpha-TIF/Oct-1 complex stabilizes and strengthens the complex (Figure 1.2 A). The alpha-TIF/Oct-1/HCF complex binds to the HSV genome and immediate-early (alpha) genes are expressed, starting viral replication (Figure 1.2 B).

Previous studies have demonstrated that octamer 2 (Oct-2) can repress alpha-gene expression (Lillycrop et al., 1991, Lillycrop et al., 1993). Studies by Lillycrop et al., established that Oct-2 has greater avidity than Oct-1 for the alpha-gene promoter region, but it is unable to
form a complex with alpha-TIF (Lillycrop et al., 1991, Lillycrop et al., 1993, Lillycrop et al., 1994). Therefore, when Oct-2 is present, expression of alpha genes does not occur and viral replication is inhibited, promoting the establishment of latency (Figure 1.2 C).
Figure 1.2: Transcription factors that modulate HSV replication. (Modified from Pereira, 1996)
HSV-1 reactivation

Once HSV invades the host through the skin or mucous membranes, the virus can evade the host immune system, establish a latent infection, and reactivate later to cause recurrent disease (Watanabe et al., 1999). HSV-1 reactivation involves a switch from viral dormancy to the initiation of viral replication within the neuron that may occur spontaneously or as a result of physical or emotional stress (Roizman and Sears, 1996).

A series of biochemical events that precede HSV-1 reactivation can be activated by cellular stress (Miller et al., 1998). Tissue injury, UV radiation, and physiological stress are some of the events that are typically associated with the triggering of HSV-1 reactivation (Pereira, 1996). They do so by releasing mediators locally such as prostaglandins (Cherrick et al., 1992, Khyatti and Menezes, 1990) or systemically such as epinephrine and corticosteroids (Bloom et al., 1994). These mediators in turn affect the levels of intracellular messengers, such as cAMP and protein kinase C, which have been associated with triggering HSV-1 reactivation in vitro (Smith et al., 1992).
Nerve growth factor (NGF) has also been associated with HSV-1 reactivation (Hill et al., 1997). NGF is a protein released by target tissue innervated by sensory neurons, and when it binds to specific cell surface receptors, it mediates its effects through transcriptional regulation of genes (Wilcox et al., 1990). It has been demonstrated that HSV-1 latency occurs in neurons that are NGF dependent (Pereira, 1996). The removal of NGF from latently infected neurons in vitro causes HSV reactivation (Wilcox and Johnson, 1988, Wilcox et al., 1990). NGF causes an increase in the expression Oct-2, which represses immediate-early gene expression (Pereira, 1996). A decrease in the levels of Oct-2 in the nucleus of latently infected neurons caused by reduction of available NGF (Wood et al., 1992) has been suggested as a possible mechanism of HSV-1 reactivation (Figure 1.4) (Steiner and Kennedy, 1995).
Figure 1.3: Illustration of a possible role of NGF in HSV-1 reactivation. (Modified from Steiner and Kennedy, 1995)
Immune responses to HSV-1 infection

The immune system is in charge of eliminating virus-infected cells quickly and proficiently before damage caused by activated inflammatory cells causes tissue destruction. Inflammatory events usually result in viral clearance, although they do not prevent the entry of the virus into the nervous system (Roizman and Sears, 1990). Figure 1.4 (Adapted from Boss, 1997) summarizes some of the immune responses that take place on the skin during primary HSV-1. Initially, innate immune responses (proinflammatory cytokines, complement, type I interferons, and NK cells) limit the early viral spread. Later, antigen specific (T cell) and antibody responses are very important to the resolution of the infection. Cytotoxic T lymphocytes (CTL) are also important in the clearance of the virus. Primary exposure to the virus induces a memory response for protection against future infections with a similar virus (Bonneau, 1994). Thus, both innate and cell-mediated immunity are necessary for the resolution of the infection. In this section, the role of innate and cell-mediated immune responses in limiting and controlling viral infections is introduced.
Figure 1.4: Cutaneous Immunology during primary HSV-1 Infection (Modified from Bos D., 1997)
Innate Immunity

Type I Interferons

Interferon alpha (IFN-alpha) and interferon beta (IFN-beta) are a family of genes known as type I interferons (Stark et al., 1998). There are at least 15 molecular species of IFN-alpha (Cann, 1997). These IFN-alpha species are closely related. Some IFN-alpha species differ from each other by one amino acid (Cann, 1997). IFN-alpha is predominantly synthesized by leukocytes (Cann, 1997). The mature proteins contain 143 amino acids, with a minimum homology of 77 percent between the different types. All the genes encoding IFN-alpha are located on the human chromosome nine. There is only a single gene for IFN-beta also located on the human chromosome nine. In the mouse, IFN-beta and all the IFN-alpha species are located on chromosome four. The mature IFN-beta protein contains 145 amino acids. Unlike IFN-alpha, IFN-beta is glycosylated, with 30 percent homology to other interferons (Cann, 1997). Fibroblasts are the predominant synthesizers of IFN-beta (Cann, 1997).
Interferons alpha and beta bind to the same receptor, the Type I IFN receptor. Studies have demonstrated that the Type I IFN receptor has a multichain structure with two distinctive components: the IFNAR1 and the IFNAR2. (Platanias and Fish, 1999) (Uzé et al., 1995). The IFNAR1 chain is a 110-kDa protein. The IFNAR2 subunit occurs in two different forms: a long form IFNAR2c and a short form IFNAR2b. Both Type I interferon receptor types are capable of transducing signals, and both mediate the biological effects of alpha and beta interferon (Colamonici et al., 1994). A major pathway for the generation of interferon signals involves activation of tyrosine kinases of the Janus family (Jak kinases) and tyrosine phosphorylation/activation of Stat-proteins (signal transducers and activators of transcription).

Three mechanisms have been described to cause the upregulation of interferon genes (Cann, 1997). First, interferon gene transcription is increased when a virus inhibits cellular protein synthesis, reducing the amount of intracellular repressor proteins that would suppress gene expression. Second, double-stranded (d/s) RNA is also a potent inducer of interferon. The mechanism of induction of d/s RNA depends on the secondary structure of the RNA rather than any particular nucleotide sequence. It has been suggested that as a result of d/s RNA binding,
protein kinase R is activated to phosphorylate a negative regulator of the NF-κB transcription factor, IkB, and thus promote IFN-beta gene expression (Biron, 1999). Third, metabolic inhibitors such as actinomycin D, which inhibits cellular transcription, or cycloheximide which inhibits protein translation, also upregulate the expression of type I interferons (Cann, 1997).

The antiviral effects of type I IFN are mediated by IFN-induced proteins (Roitt et al., 1996). IFNs induce the transcription of a cellular gene encoding the enzyme 2', 5'-oligoadenylate synthetase (Der et al., 1998). This enzyme activates RNase L which digests viral genomic RNAs, viral and cellular mRNAs, and cellular ribosomal RNAs (Figure 1.5) (Adapted from Cann, 1997). Due to the degradation of mRNAs and rRNAs, the end result of this mechanism is a reduction of protein synthesis. Thus, the cell is protected from viral damage. IFNs also cause the activation of RNA-activated protein kinase (PKR) (Clemens and Elia, 1997) (Der et al., 1998). The substrate of PKR is the small subunit of the eukaryotic initiation factor 2 (eIF2α) (Gil and Esteban, 2000). eIF2α
Interferons

\[ 2' 5'\text{-oligoadenylate synthetase} \]

\[ (n+1) \text{ATP} \rightarrow (2' 5') \text{pppA(pA)n+ nPPi} \]

\[ \text{RNAase L (active)} \quad \text{RNAase L (inactive)} \]

RNA degradation

**Figure 1.5:** Mechanism of induction of 2' 5'-oligoadenylate synthetase by interferons.
(Modified from Cann, 1997)
Figure 1.6: Mechanism of induction of PKR by interferons. (Modified from Cann, 1997)
is required by ribosomes for the initiation of protein translation. PKR inhibits translational initiation by phosphorylating eIF2α (Levine and London, 1978) (Figure 1.6, Adapted from Cann, 1997). Like the 2', 5'-oligosynthetase mechanism, this mechanism also results in the inhibition of protein synthesis.

Natural killer cells

NK cells are of major significance during HSV-1 infections because of the ability of the virus to alter the metabolism of the cells that it infects (Welsh and Vargas-Cortes, 1992). Also, viruses are inducers of IFN, which increases the cytotoxicity and proliferation of NK cells (Welsh and Vargas-Cortes, 1992). NK cells are described as large granular lymphocytes which mediate non-major histocompatibility complex (MHC)-restricted cytotoxicity without any specificity (O'Shea and Ortado, 1992).

The NK cell response develops within the first two days of a viral infection in parallel with the induction of type I interferons (Gidlund et al., 1978, Roitt et al., 1996). The turnover rate for endogenous NK cells is relatively low (Birion et al., 1983). NK cell activation itself leads to an increase in NK cell numbers because their activation promotes an increase
in their proliferation (Welsh and Vargas-Cortes, 1992). NK cells can be activated by cytokines such as IL-2 and IFN-alpha, IFN-beta, and IFN-gamma (Kuribayashi et al., 1981). Activated NK cells release large amounts of IFN-gamma and other cytokines such as IL-1 which are important in the regulation of following immune responses (Roitt et al., 1996).

**Interferon gamma**

As reviewed in the previous section, activated NK cells release large amounts of IFN-gamma during viral infections (Roitt et al., 1996). IFN-gamma activates macrophages, promotes T and B lymphocyte differentiation, and stimulates NK cell cytolytic activity (Abbas et al., 1994) (Heise and Virgin, 1995). There is a single gene for IFN-gamma on the human chromosome 12 (Cann, 1997). In the mouse the IFN-gamma gene is located on chromosome 10. The mature IFN-gamma protein contains 146 amino acids, is glycosylated, and has very low homology to other interferons (Cann, 1997). Ultimately, IFN-gamma promotes Th1 and macrophage-rich inflammatory reactions while suppressing Th2 and eosinophil reactions. IFN-gamma is also involved in the creation of antiviral and anti-proliferative states. During HSV infections, IFN-gamma
shuts off viral replication by inhibiting alpha-TIF mediated transactivation of immediate-early and early gene transcription (Raneiro de Stasio and Taylor, 1990). TNF-alpha, a cytokine produced primarily by activated mononuclear phagocytes and NK cells (Rinaldo and Torpey, 1993), has direct antiviral activity against HSV, inhibiting viral replication via synergism with interferons (Mestan et al., 1986, Wong and Goeddel, 1986). IFN-gamma is important in both macrophage activation and control of HSV replication. Decreased IFN-gamma expression may reduce NK cell and macrophage activation, which are necessary responses for the resolution of HSV infections. Yu and co-workers, using mice that express the knockout phenotype for IFN-gamma (GKO mice), have demonstrated that IFN-gamma is required to contain a primary HSV infection in the skin (Yu et al., 1996). In their study, animals lacking the ability to produce IFN-gamma were more susceptible to a cutaneous HSV infection than wild type animals (Yu et al., 1996).
Cell mediated responses to HSV

Th1 and Th2 responses

T-helper (Th) cells are lymphocytes that modulate antigen-specific immune responses to HSV (Abbas et al., 1994). Th cells are identified by the presence of a specific surface marker called CD4. Because of the presence of this cell surface marker, Th cells are also identified as CD4+ cells. Th cells are classified into two subsets: Th1 and Th2. Th1 cells are involved in cell-mediated immunity. They produce cytokines such as IFN-gamma and IL-2, which are necessary for the activation of macrophages and NK cells (Abbas et al., 1994). Th2 cells are important in the development of antibody-mediated immunity. Th2 cells produce cytokines such as IL-4 and IL-10 that are involved in the activation and proliferation of B cells to produce antibodies against the virus (Abbas et al., 1994). It has been reported that HSV infections are characterized with mainly Th1 type responses (Carmack et al., 1996). Many studies have revealed that, because Th1 cells and cytotoxic T lymphocytes play a role in the protection of host against herpes infections, a shift from a Th1 to a Th2 response will lead to increased susceptibility to HSV infections (Del Prete and Romagnani, 1994, Scott and Kaufman, 1991). Jayaraman and collaborators reported that the susceptibility of mice to herpes stromal
keratitis, a severe complication of HSV-1 infection in the cornea of the eye, increased when a change from a Th1 response to a Th2 response occurs (Jayaraman et al., 1993). Ikemoto and co-workers demonstrated that suppressor T-cells, which they defined as CD8+ type 2 cells, have the potential to increase the severity of an acute HSV infection through the release of IL-4 and the production of Th2 cytokines (Ikemoto et al., 1995). IL-4 inhibits macrophage activation and blocks most of the macrophage activating effects of IFN-gamma, including increased production of cytokines, such as IL-1 and prostaglandins (Abbas et al., 1994).

Most of the effects of IL-4 are similar to the effects of IL-10, another cytokine produced by Th2 cells. Activated macrophages and other cells, such as keratinocytes, produce IL-10. IL-10 has three major biological activities: first, it inhibits cytokine production by macrophages (e.g., TNF-alpha, IL-1), second, it inhibits the accessory functions of macrophages in T-cell activation; and third, it has stimulatory actions toward B-cells. A gene homologous to the IL-10 gene has been found in the genome of the Epstein-Barr virus (Moore et al., 1990, Suzuki et al., 1995), which is a herpesvirus family member (HSV-4). Viral IL-10 has
similar activity with the cytokine, suggesting that the virus may have gained the human gene as a resource for inhibiting antiviral activity (Suzuki et al., 1995).

Chemokines

Chemokines are mediators that perform many important roles in the generation of innate and acquired immune responses (Lusso, 2000), such as the margination, adhesion, and extravasation of leukocytes from circulation to areas of HSV-1 infection (Baggiolini, 1998). The majority of chemokines belong to the CXC (alpha) chemokine family, identified by a two-cysteine motif with an intervening amino acid, or to the CC (beta) family, characterized by two adjacent cysteine residues (reviewed by Lusso, 2000). Two other chemokine families, the C or gamma family presenting a single cysteine motif, and the CX3C family, in which three amino acids are located between two cysteine residues, have also been described (Lusso, 2000). Chemokines appear to be encoded by single copy genes (Sprenger et al., 1998). The majority of the physiological activities of chemokines are mediated by cellular receptors coupled to G-proteins (Sprenger et al., 1998). Chemokine gene expression after
stimulation with proinflammatory cytokines is at least partly due to transcriptional activation (Sprenger et al., 1998). It has been reported that glucocorticosteroids can inhibit CXC and CC-chemokine gene expression by regulating transcription factors that are important mediators of their expression (Smith and Herschman, 1996, Sprenger et al., 1998).

Chemokines mediate the migration of leukocytes to sites of HSV-1 infection (Mahalingam et al., 2001). The induction of mononuclear leukocyte attracting chemokines (CC), such as MCP-1 and MIP-1 alpha, facilitates the recruitment of inflammatory cells to HSV infected tissues helping to control the infection (Sprenger et al., 1998). Also, chemokines can modulate Th cytokine-secretion patterns. For example, the expression of CC chemokines has been shown to mediated Th1 polarized immune responses (Lusso, 2000, Schrum et al., 1996), which are important for the control of an HSV-1 infection.

*Glucocorticoids and immunomodulation*

The sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis mediate the physiologic responses to stress (Johnson et al., 1992, Solomon, 1987). Activation of the SNS results in
the production of plasma catecholamines, which have been associated with increased mobilization of lymphocytes and neutrophils from the spleen (Ernstrom and Sandgerh, 1973, Hay, 1977, Ottaway and Husband, 1992). A catecholamine-mediated increase in blood flow has been shown to increase the availability of immune cells that can be recruited to areas of viral infection (Ernstrom and Sandgerh, 1973, Hay, 1977, Ottaway and Husband, 1992). Catecholamines are also involved in the suppression of CTL responses during primary HSV-1 infection (Dobbs et al., 1993).

The HPA axis consists of the hypothalamus, the anterior pituitary, and the adrenal cortex. These tissues produce and secrete hormones that serve as the primary signals of the axis (McEwen et al., 1997). One of these hormones is corticotropin-releasing hormone (CRH). CRH, synthesized by a subset of parvocellular neurons in the paraventricular nucleus of the hypothalamus (Vale et al., 1985), induces the secretion of adrenocorticotropic hormone (ACTH) (Imura, 1985). ACTH is produced by a subset of anterior pituitary cells known as corticotrophs (McEwen et al., 1997). ACTH induces the synthesis and secretion of glucocorticoid steroids (GC). Produced and secreted by cells in the adrenal cortex, GC are the final hormones produced by the activation of the HPA axis. GC are lipophilic (De Bosscher et al., 2000), hence they can enter cells...
passively through the cell membrane. Once in the cell's cytoplasm, GC can bind to the glucocorticoid receptor (De Bosscher et al., 2000). The glucocorticoid receptor then can move into the cell nucleus where it regulates gene transcription through binding to glucocorticoid receptor elements (GRE) present in the genome of GC-regulated genes (De Bosscher et al., 2000).

GC have immunosuppressive actions which can affect the host defense mechanisms against an HSV-1 infection (Munk and Guyre, 1991). Stress-induced increase in GC levels can suppress the production of important mediators involved in the control of viral infections. For example, monocytes and macrophages are vital for the initial response of the host to a viral infection. GC inhibit many monocyte and macrophage functions such as chemotaxis, antigen presentation, phagocytosis, and cytokine release (Munk and Guyre, 1991). GC suppress cellular infiltrate to the lymph nodes during HSV-1 infections (Bonneau et al., 1993, Dobbs et al., 1993). GC also play a role in the suppression of CTL responses caused by restraint stress during primary HSV-1 infections (Bonneau et al., 1993, Dobbs et al., 1993). GC have been shown to inhibit the production of cytokines such as IFN-gamma during viral infections (Sheridan et al., 1998).
Models for cutaneous HSV infection

The skin is the most frequent site not only for entry of HSV but also for recurrent HSV infections. Therefore, it is important to understand the HSV-1 modulating immune mechanisms that are specific to the skin. Although immunity to viral infections in experimental animals has been extensively investigated, little information is available on the in vivo expression of cytokines in skin during an HSV-1 infection. Teague and Goodpasture reported a zosteriform cutaneous murine model in which viral inoculation is performed in the dorsal surface of the animal (Teague and Goodpasture, 1923). The virus then travels from the inoculation site to sensory neuron cell bodies and then returns to the skin of the innervating dermatome resulting in a zosteriform rash (Teague and Goodpasture, 1923). Further characterization of this model by Simmons and Nash has shown that after inoculation the virus returns from the infected sensory neurons to the skin 72 hours post-infection. At that time, the virus can be detected distant from the inoculation site (Simmons and Nash, 1984, Simmons and Nash, 1985). This same phenomenon has been reproduced in our laboratories (Sheridan et al., 1997) and also by other investigators (Dillard et al., 1972, Nagafuchi et al., 1979, Simmons and Nash, 1984, Simmons and Nash, 1985, Sydiskis 38
and Schultz, 1965). The model established in our laboratory uses the SHK-1 hairless mouse (Sheridan et al., 1997). The model was established for the study of the effects of antioxidants on the healing of cutaneous herpetic lesions (Sheridan et al., 1997). An advantage that the use of the hairless SKH-1 mice provides is that it allows the easy monitoring and manipulation of the herpetic lesions because the pretreatment of the skin to remove hair is not necessary. Pretreatment of the skin for depilation purposes has been shown to influence the susceptibility of mice to the formation of cutaneous HSV-1 lesions (Sydiskis and Schultz, 1965).

**Purpose of this investigation**

Both popular opinion and scientific evidence (Bierman, 1983, Friedman et al., 1977, Glaser et al., 1987, Katcher et al., 1973, Schmidt et al., 1985, Young et al., 1976) support the hypothesis that acute and chronic stressors are involved in triggering the development of recurrent HSV infections in humans. The immune response has been pointed out as an important component of the host's defense against HSV. Furthermore, it has been demonstrated that activation of the HPA axis by stressful events results in elevation of corticosteroids in the serum, which may
impair immune function. Although the skin is the primary site for recurrent HSV-1, we still do not understand the immunological mechanisms in which stress modulates immune responses to HSV in this organ.

This investigation had two main purposes. The first purpose was to use our model for cutaneous HSV-1 infection in SKH-1 mice to study how stress modulates the clinical presentation of a primary HSV-1. The second purpose was to use the cutaneous HSV-1 model to investigate the effects of stress on innate immune responses such as, type I interferon gene expression at the site of primary HSV-1. The hypothesis was that stress-induced suppression of early immune responses increases the severity of primary HSV. Because the severity and duration of a primary HSV infection increases the frequency and severity of subsequent recurrences (Pereira, 1996), the study of the events that modulate primary HSV is critical to understand the impact that those events can have on later episodes of reactivation and disease recurrence.

Preliminary studies were conducted to characterize our cutaneous HSV-1 model at both the clinical and molecular levels. The role of stress in the modulation of clinical signs, viral load, and immune responses was studied. The effects of stress-induced glucocorticoids on the clinical
Pathophysiology of cutaneous HSV-1 were studied, providing important insights on how stress can modulate the pathogenesis of viral infections. Stress-induced modulation of interferon-alpha and interferon-beta gene expression was explored at the mRNA level using competitive RT-PCR at the site of primary infection. Also, the stress-induced modulation of type II interferon gene expression was studied using competitive RT-PCR. Stress-induced modulation of chemokines and of cytokines involved in cell-mediated immunity at the mRNA level was examined using real time PCR. To study the effect of GC on stress-induced modulation of interferon expression pharmacologic blockage experiments were performed, using the type II glucocorticoid-receptor antagonist RU-486.

The data obtained from these studies will provide an important basis for future studies related to viral reactivation and disease recurrence, and for the development of appropriate mechanisms of prevention and control of HSV and other viral infections.
Animals

Virus-antibody-free SKH-1 male mice, 6-8 weeks of age, were obtained from Charles River, Inc (Wilmington, MA). The animals were allowed to adapt to their surroundings for at least seven days before the initiation of any experimental procedure. In-house bred mice were used for molecular studies. All animals were housed at a facility accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). The mice were maintained on a 12 hour light/dark cycle.

Restraint stress paradigm

Experimental animals were placed in well-ventilated, loose-fitting 50-ml centrifuge tubes for three cycles prior to HSV-1 infection and for five cycles after. A cycle consisted of restraint for 15 hours,
starting at 6:00 PM (lights out) and ending at 9:00 AM (lights on at 6:00 AM) as described by Padgett et al. (Padgett et al., 1998). Since restrained animals (RST) do not have access to food or water during restraint, unrestrained control animals (FWD) were deprived of food and water while experimental animals were restrained.

**Viral inoculation**

The infection of both experimental and control mice was achieved under anesthesia using a 0.3-ml intraperitoneal injection of 0.44 mg/ml Xylazine (Phoenix Scientific, St. Joseph, MO) and 7.8 mg/ml Ketamine (Phoenix Scientific, St. Joseph, MO). A centrally located epidermal 1cm² abrasion was performed using a template and a 25-gauge needle on the dorsal surface of each mouse. Abrasions placed at this location can not be reached by the animals for self-licking. The abraded surface was then inoculated with 10 µl of a 1 x 10⁹ PFU/ml HSV-1 stock McIntyre strain as described by Sheridan et al. (Sheridan et al., 1997).
Scoring of herpetic lesions

The stage and severity of the infection was recorded daily and scored using the following classification system as modified from Sheridan et al. (Sheridan et al., 1997): 0 - no signs of infection, 1 - redness and/or swelling, 2 - single lesion, 3 - multiple lesions, 4 - necrosis and coalescence, 5 - spread in dermatome pattern. The course of the infection was monitored and disease scores were recorded daily. A single examiner blinded as to the treatment groups conducted the scoring.

ELISA

Enzyme linked immunosorbent assay (ELISA) was performed in order to monitor the development of antibodies against HSV-1 in the experimental and control animals. Blood samples were collected via tail bleeding at 0, 7, 14, and 21 days after infection. ELISA plates (Fisher Scientific, Co.) were coated overnight at 4°C with a 100 ul of a 1:70 dilution of HSV-1 stock in carbonate coating buffer (0.01 M Na₂CO₃ and 0.03M NaHCO₃, pH 9.6) per well. The plates were washed three times with PBS-Tween 20 and were blocked for one hour at room temperature with 100 ul per well of a 0.1% bovine serum albumin (BSA) (Sigma) in PBS-Tween 20 solution. After the plates were washed three times with
PBS-Tween 20, samples were diluted 1:3 serially from 1:25 to 1:625 and 100 ul of each dilution was added to the plates, which were incubated at 37°C for one hour. After incubation, the plates were washed three times with PBS-Tween 20 and 100 ul of a horseradish peroxidase-conjugated goat anti-mouse IgG or IgM antibody (ICN Pharmaceuticals, Inc.) 1:1000 dilution was added to the wells. The plates were incubated overnight at 4°C. The next morning the plates were washed six times and 100 ul of an ABTS solution was added to the wells. The plates were incubated at 37°C for one hour. Absorbance was read at 405 nm using an automated ELISA plate reader (Molecular Devices, Inc.). Titer data were determined at the highest dilution that had an OD reading above the negative control. Because the samples were diluted 1:3, the reciprocal value of each dilution was log transformed (base 3) for statistical analysis.

Analysis of infectious HSV-1 titers

Skin samples were excised on days 1, 3, 5, 7, and 9 post-infection, placed in 0.75 ml of ice-cold supplemented DMEM (Gibco BRL, Grand Island, NY), and stored at -70°C until assayed for infectious HSV-1. Samples were thawed quickly and homogenized with a Tissue Tearor (Biospec Products, Bartlesville, OK). The homogenates were clarified by
centrifugation at 1,000 g at 4°C for five minutes. Detection of infectious HSV-1 was performed according to the method of Bonneau et al. (Bonneau et al., 1991). Serial 10-fold dilutions of the supernatants were prepared and assayed in duplicates on confluent monolayers of Vero cells. Virus was absorbed for one hour at 37°C in 5% CO₂ followed by the addition of methylcellulose overlay media. The overlay media was prepared by autoclaving 3.75 g of methylcellulose (Sigma) in a 500 ml glass bottle. While the bottle was still hot, 180 ml of sterile water were added and the solution was stirred overnight. The next morning, 70 ml of sterile water, 17.5 ml of 10x DMEM, 5 ml of FBS (Biocell, Rancho Dominguez, CA), 2 ml of 3 % glutamine (Gibco BRL, Grand Island, NY), 1.5 ml Pen-Strep (Gibco BRL, Grand Island, NY), 7 ml sodium bicarbonate, and 0.2 ml fungizone (Gibco BRL, Grand Island, NY) were added. The methylcellulose media was stored at 4°C. After the methylcellulose media was added, the plates were incubated for three to five days and the overlay media was removed. The cell monolayers were fixed with 5% (wt/vol) formaldehyde for 30 minutes and then stained with 0.5% (wt/vol) crystal violet. A single observer blinded as to the treatment groups counted the number of plaques formed.
**Staining for immune cells**

Skin samples were formalin-fixed and sectioned. Hematoxylin and eosin (H&E) staining was performed on tissue sections that were mounted on chrome alum gelatin-coated slides. Samples were incubated with Gill Hematoxylin #3 (undiluted) for one to two minutes, then rinsed with lukewarm running water for five minutes. The tissues were then stained with 0.5% eosin for one minute and incubated for two minutes in ethanol gradient solutions of 95% and 100% concentrations. Finally, the slides were placed in xylene for two minutes and coverslipped using permount® solution (Fisher Scientific).

**Histologic analysis**

Skin samples were assessed for cellular infiltration on days 3, 5, 7, and 9 post-infection. H&E stained slides were rated on a scale of 0 to 5. A sample receiving a rating of 0 had an appearance similar to normal skin, while a sample rated as 5 had massive infiltration of PMNs and mononuclear cells. Intermediate samples received ratings of 1, 2, 3, or 4 depending on the magnitude of cellular infiltration observed (Cook et al., 1995, Padgett et al., 1998). A single examiner blinded as to the treatment groups scored the samples.
Treatment with RU486

One day before the initiation of restraint, experimental animals were injected subcutaneously with 0.1 ml of 25 mg/kg of the Type II glucocorticoid receptor inhibitor RU486 (Sigma Chemical, St. Louis, MO) dissolved in polyethylene glycol 400 vehicle (Sigma Chemical, St. Louis, MO). Control animals were injected with 0.1 ml of the vehicle only. Mice were treated with RU486 or the vehicle each day of the stress paradigm, one hour prior to the initiation of restraint.

Total RNA extraction

Skin samples excised at different time points during the infection were submerged in 1-ml TRIzol reagent (Life Technologies, Rockville, MD) and stored in 5-ml polypropylene tubes at −80°C. Samples were homogenized using a Tissue Tearor (Biospec Products, Bartlesville, OK). Total RNA was extracted according to the manufacturer’s protocol for the TRIzol reagent. Briefly, after homogenization the samples were incubated for five minutes at room temperature. The samples were then centrifugated at 9,500 rpm for 10 min at 4°C. The supernatant was transferred to a new tube and 200 ul of chloroform (Fisher Scientific) was added. The tube was then vortexed and incubated for three minutes.
The samples were centrifuged at 9,500 rpm for 15 min at 4°C and the aqueous layer was removed. The aqueous layer was mixed with 1 ml of isopropanol (Fisher Scientific) and incubated at room temperature for 10 minutes. Samples were centrifuged at 9,500 rpm for 10 minutes at 4°C. The RNA pellet was washed once with 1 ml 75% ethanol and was centrifuged at 8,200 rpm for 5 minutes at 4°C. The RNA pellet was partially dried and dissolved in RNA water. To determine both the purity and the concentration of RNA present in solution, spectrophotometry of 10 ul of each sample was performed at absorbances of 260 and 280 nm. The 260/280 ratio was used to determine nucleic acid purity.

Purification of mRNA from isolated total RNA

Selection of mRNA from total RNA was performed using magnetic oligo (dt)25 beads and a magnetic particle concentrator (Dynal AS, Oslo, Norway). Twenty μg of total RNA sample was heated to 65°C for two minutes. After heating, the solution was added to an oligo (dt)25 bead solution in binding buffer (20 mM Tris-HCl pH 7.5, 1.0 M LiCl, 2 mM EDTA). Hybridization was allowed to proceed for three to five minutes at room temperature. The tube was then placed in a magnetic column and washed twice using a washing buffer (10 mM Tris-HCl pH 7.5, 0.15 M LiCl,
1 mM EDTA). An elution buffer (2 mM EDTA) was added and the sample was heated to 65°C for 2 min. The tube was then placed in a magnetic column and the eluted mRNA was collected and transferred to a new tube.

Reverse Transcription (RT)

To prepare complementary DNA (cDNA), a solution containing mRNA, oligo-dt primer, dNTP mix, ribonuclease inhibitor RNasin (Promega, Madison, WI), and 15 U of AMV reverse transcriptase (Promega, Madison, WI) in reaction buffer (250 mM Tris-HCl pH 8.3, 250 mM KCl, 50 mM MgCl₂, 50 mM DTT and 2.5 spermidine) was incubated at 42°C for 60 minutes. The solution was then heated to 90°C for five minutes to destroy AMV-RT, and finally the cDNA was cooled to 4°C. The cDNA was then stored at -80°C.

Polymerase Chain Reaction (PCR)

PCR was performed in a 25 μl reaction mixture (10 mM Tris-HCl, 50 mM KCl, 3 mM MgCl₂, 0.2 mM dNTPs, 0.1-1.4 μM 5' and 3' primers of each cytokine, and 1 U of Taq DNA polymerase) (Gibco BRL, Gaithersburg, MD). The cDNA was amplified for 35 cycles with each cycle consisting of
a 94°C/45 seconds denaturation step, an annealing step at 60°C/45 seconds, and a primer extension step at 72°C/two minutes. Before amplification, reactions were incubated at 94° C for one minute. After amplification, extension was performed at 72°C for seven minutes. Samples were electrophoresed in a 1.8% agarose gel stained with ethidium bromide and visualized in an UV transiluminator. Primer sequences are shown in Tables 2.1 and 2.2. The primer sequences were obtained or designed according to the following sources: NGF (Ullrich et al., 1983), gD (Kudelova et al., 1995), gB (Stuve et al., 1987), IFN-alpha consensus sequence (Hughes et al., 1994), G3PDH (Sabath et al., 1990), and IFN-beta (Clontech, Palo Alto, CA).

**Competitive RT-PCR**

To quantitate the expression of IFN-alpha, IFN-beta, and IFN-gamma, internal standard DNA sequences (competitors) which compete with target cDNA for the same primers during PCR amplification were designed and constructed according to the PCR MIMIC construction kit (Clontech, Palo Alto, CA). PCR products generated with the competitors differed from the target cDNA in length and were distinguished based on molecular weight size. A molecular weight correction factor was
calculated for each target cDNA/competitor pair and was used to adjust
the densitometric ratios between the co-amplified competitor and the
target cDNA. The primer sequences used to amplify each target gene and
competitor, along with the sizes of their respective PCR products, are
shown in Table 2.2.

For each gene, standard cDNA samples containing high amounts of
the gene of interest were quantitated using serial two-fold dilutions of
competitor in the presence of a single dilution of the standard cDNA. The
ratio of standard cDNA product versus competitor product was
determined for each competitor dilution. The concentration of
competitor that resulted in a target cDNA/competitor ratio of 1.0 was
determined. At this ratio, the concentration of competitor is equivalent
to the concentration of the specific cDNA being amplified. A standard
curve was generated for each gene by amplifying serial two-fold dilutions
of the standard cDNA with a fixed amount of competitor. A sample of one
of the standard curves generated for G3PDH is presented in Figure 2.1
(A). Experimental cDNA samples were amplified with the fixed competitor
concentration. The cDNA/competitor ratios of the standard and
experimental samples were determined by densitometric analysis (NIH
Image 1.61, Bethesda, MD), followed by adjustment with the
The amount of specific cDNA in each experimental sample was determined by polynomial regression analysis (Figure 2.2 B) and expressed as attomoles of mRNA per µg of total RNA. The values generated for each sample were adjusted by the corresponding amounts of G3PDH gene expression. Peak expression in control (FWD) animals was determined for each gene and was used to calculate the relative percentage of expression \[
\frac{\text{Gene of interest expression}}{\text{control mean peak expression}} \times 100
\] at each time point.

**Real Time RT-PCR**

Real time PCR was carried out in a PE Biosystems AB1 Prism 7700 Gene Amplification machine. The fluorogenic 5' nuclease assay, or TaqMan, uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR. The relative quantity of IL-10, IL-2, IL-4, MCP-1, MIP-1 alpha, and GAPDH was determined using gene specific fluorogenic probes. Primers and probes were obtained from PE Biosystems (Table 2.3). Two different dyes were used to determine the relative amount of the gene of interest and GAPDH in the samples simultaneously; VIC was used for GAPDH and FAM for IL-10, IL-2, IL-4,
MCP-1, and MIP-1 alpha. Each cDNA sample (2 ul) was mixed with the TaqMan Universal PCR Master Mix (PE Biosystems, Branchburg NJ). The primer concentrations utilized were 0.9 mM for IL-2, IL-4, IL-10, MCP-1 and MIP-1alpha and 1uM for GAPDH. The probe concentrations were 100nM for IL-2, IL-4 and IL-10; 250 nM for MCP-1 and MIP-1alpha; and 2 

µM for GAPDH. The final volume of each reaction was 25 µl. The settings for the thermal profile were as follows: 50°C for two minutes for denaturation, 95°C for ten minutes to activate AmpliTaq Gold DNA polymerase, then 40 amplification cycles each of 95°C for 15 seconds followed by 60°C for one minute to anneal/extend. The relative amount of specific cDNA in each sample was determined by measuring the fluorescence of the probe specific for each gene (FAM). The generated values were adjusted by subtracting the corresponding GAPDH values determined by measuring the fluorescence of VIC (FAM – VIC = delta Ct value). The value determined for uninfected skin was used as a control and was subtracted from the delta Ct value of each reaction to determine the fold-increase in the expression of each gene compared to normal skin.
Statistical Analysis

Statistical analyses were performed using SAS version 6.03 (SAS Institute Inc., Cary NC), JMP IN version 3.2.1 (SAS Institute Inc., Cary NC) and Statview 5.0.1 (SAS Institute Inc., Cary NC). Mean values were compared and statistical significance was assessed using a one-tailed Mann-Whitney-Wilcoxon test for clinical severity data; Student’s t-test, for antibody data. ANOVA, Student’s t-test, and Post-hoc Tukey-Kramer test were used to analyze viral titers and molecular data using JMP-IN 3.2.1 (SAS Institute, Cary, NC). Results were considered significant if p<0.05.
Figure 2.1: Example of a gel for competitive RT-PCR of G3PDH (A) and Polynomial regression plot for G3PDH standard curve (B).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGF</td>
<td>3' ACC GGA GCA AGC GCT CCA TCC</td>
<td>389</td>
</tr>
<tr>
<td></td>
<td>5' CGC AGT GAG GTG CAT AGC GT</td>
<td></td>
</tr>
<tr>
<td>GD</td>
<td>5' AAC TAC CCC GAT CAG TTT ACC T</td>
<td>420</td>
</tr>
<tr>
<td></td>
<td>3' GAT GGT CAG GGT GTA GGG TTG TTT</td>
<td></td>
</tr>
<tr>
<td>GB</td>
<td>3' TTT TTT TTT ATT GGG AGG GGC GGG</td>
<td>238</td>
</tr>
<tr>
<td></td>
<td>5' GCA TGC GCA TCT TTT GGT TTT TTT G</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>5'-ATG GCA ACT GTT CCT GAA CTC AAC T</td>
<td>563</td>
</tr>
<tr>
<td></td>
<td>5'-CAG GAC AGG TAT AGA TTC TTT CCT TT</td>
<td></td>
</tr>
<tr>
<td>G3PDH</td>
<td>5'-TGA AGG TCG GTG TGA ACG GAT TTG GC</td>
<td>983</td>
</tr>
<tr>
<td></td>
<td>3'-CAT GTA GGC CAT GAG GCT CAC CAC</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1: Primer sequences used for RT-PCR. Sequences were obtained from the following sources: NGF (Ullrich et al., 1983), gD (Kudelova et al., 1995), gB (Stuve et al., 1987), IL-1β (Gray et al., 1986), and G3PDH (Sabath et al., 1990).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Target</td>
</tr>
<tr>
<td>IFN-α</td>
<td>5'-ATC GCT AGG CTC TGT GCT TTC CT</td>
<td>520</td>
</tr>
<tr>
<td></td>
<td>3'-AGG GCT CTC CAG ACT TCT GCT CTG</td>
<td></td>
</tr>
<tr>
<td>IFN-β</td>
<td>5'-CCA CCA CTC ATT CTG AGG CAT CAA</td>
<td>509</td>
</tr>
<tr>
<td></td>
<td>3'-CAG CTC CAG CTC CAA GAA ACG AAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATT CG</td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5'-TGA ACG CTA CAC ACT GCA TCT TGG</td>
<td>436</td>
</tr>
<tr>
<td></td>
<td>3'-CGA CTC CTT TTC CGC TTC CGC TTC CTG AG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>983 606</td>
<td></td>
</tr>
<tr>
<td>G3PDH</td>
<td>5'-TGA AGG TCG GTG TGA ACG GAT TTG GC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3'-CAT GTA GGC CAT GAG GCT CAC CAC</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2: Primer sequences used for competitive RT-PCR. Sequences were obtained from the following sources: IFN-alpha consensus sequence (Hughes et al., 1994), IFN-gamma (Dijkmans et al., 1985), G3PDH (Sabath et al., 1990), and IFN-beta (Clontech, Palo Alto, CA).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer and probe sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4</td>
<td>Forward- GGC ATT TTG AAC GAG GTC ACA</td>
</tr>
<tr>
<td></td>
<td>Reverse- AGG ACG TTT GGC ACA TCC AT</td>
</tr>
<tr>
<td></td>
<td>Probe- CTC CGT GCA TGG CGT CCC TTC T</td>
</tr>
<tr>
<td>IL-2</td>
<td>Forward- CTC CTG AGC AGG ATG GAG AAT T</td>
</tr>
<tr>
<td></td>
<td>Reverse- CGC AGA GGT CCA AGT TCA TCT</td>
</tr>
<tr>
<td></td>
<td>Probe- CTG AAA CTC CCC AGG ATG CTC ACC TTC</td>
</tr>
<tr>
<td>IL-10</td>
<td>Forward- TTT GAA TTC CCT GGG TGA GAA</td>
</tr>
<tr>
<td></td>
<td>Reverse- ACA GGG GAG AAA TCG ATG ACA</td>
</tr>
<tr>
<td></td>
<td>Probe- TGA AGA CCC TCA GGA TGC GGC TG</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Forward- TTG GCT CAG CCA GAT GCA</td>
</tr>
<tr>
<td></td>
<td>Reverse- CCT ACT CAT TGG GAT CAT CTT GC</td>
</tr>
<tr>
<td></td>
<td>Probe- AAC GCC CCA CTC ACC TGC TGC TACT</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Forward- CAA GTC TTCTCA GCG CCA TAT G</td>
</tr>
<tr>
<td></td>
<td>Reverse- TCT TCC GGC TGT AGG AGA AGC</td>
</tr>
<tr>
<td></td>
<td>Probe- AGC TGA CAC CCC CAC TGC CTG C</td>
</tr>
</tbody>
</table>

Table 2.3: Primer and probe sequences used for real-time PCR.
CHAPTER 3

RESULTS

1. Preliminary experiments

Many studies support the hypothesis that emotional stress is associated with the development of recurrent HSV infections (Bierman, 1983, Friedman et al., 1977, Glaser et al., 1987, Katcher et al., 1973, Schmidt et al., 1985, Young et al., 1976). Our understanding of the mechanisms by which stress can modulate HSV infections is of central importance for the development of the appropriate therapies for prevention and control of the disease. Thus, one of the objectives of this investigation was to characterize a murine model of cutaneous HSV-1 infection that could be utilized to study the mechanisms by which stress may be involved in the modulation of primary HSV-1
infection. The data obtained will help to provide a better understanding of the ways that stress could modulate the development of primary and recurrent HSV infections.

It is known that the severity of a primary HSV infection is associated with the frequency and severity of recurrent episodes of the disease (Pereira, 1996). Thus, it is important to understand how stress modulates primary HSV in order to assess the impact that different stressors may have on later episodes of reactivation and disease recurrence. For this purpose, preliminary experiments were designed in order to characterize the clinical presentation of the infection in the model. Initial experiments also dealt with the establishment of a methodology for molecular studies of key genes that could be modulated by stress during HSV infections.

The first studies were conducted in order to characterize the clinical pathophysiology of a cutaneous HSV-1 infection with secondary zosteriform spread in the SKH-1 mouse. Mice were infected with HSV-1 via an abrasion at the dorsal surface of the animal. The clinical severity of the infection was recorded daily for 21 days using the clinical severity score described by Sheridan et al. (Sheridan et al., 1997). Animals were considered clinically infected if they presented a score greater than or
equal to two for three consecutive days. Animals were clinically infected in all initial experiments. Herpetic symptoms started to appear by five to six days post-infection and the animals presented the highest severity scores by days seven to nine. From the data obtained in the initial experiments the clinical severity scores were modified to consider the coalescence and necrosis of the lesions as a separate score (Figure 3.1).

Preliminary experiments were conducted to establish the methodology and to optimize primers that will allow for the study of the mRNA expression of the targeted genes during primary cutaneous HSV-1 infection. For this purpose, skin samples at various points during the infection were collected. Tissues were homogenized and total RNA was isolated. The mRNA was selected, cDNA was prepared, and RT-PCR was performed. In Figure 3.2, the gene expression of IL-1β, NGF, gB, and gD in the skin of HSV-1 infected and control mice was studied at day 3 post-infection. (Wood et al., 1992). It is known that stress can suppress IL-1β expression (Marucha et al., 1998) and that IL-1β could be involved in the modulation of NGF expression (Wood et al., 1992). Some IL-1β expression was detected in infected animals, whereas no expression was observed in control mice. NGF expression was detected in the skin of both infected and non-infected animals. Infected mice presented a slight
increase in NGF expression when compared to control mice. The expression of HSV-1 glycoproteins gB and gD was selected as markers for viral replication. Both gB and gD were detected in the skin of infected mice whereas no expression was detected in non-infected control samples. Preliminary experiments were also conducted to detect the expression of IFN-alpha and IFN-beta in the skin of SKH-1 mice acutely infected with HSV-1. The expression of type I IFNs was targeted because they are important in limiting early viral spread (Roitt et al., 1996). IFN-alpha and IFN-beta gene expression was detected at day 1 post-HSV-1 infection using RT-PCR (Figure 3.3).

The kinetics of gB and gD mRNA expression through the course of a primary HSV-1 infection in SKH-1 mice were analyzed in order to establish the time points where viral replication was occurring during the infection. Using four animals per time point the kinetics of gB and gD were followed (Figure 3.4). Both gB and gD were strongly expressed at days 3 and 5. The expression of gB and gD decreased by day 7 post-infection, and no expression was observed by day 21 post-infection. Both glycoproteins were expressed at the site of secondary dermatome spread at days 5 and 7 post-infection. No expression was found at later time points.
In summary, in this series of preliminary studies we characterized the clinical presentation of a primary HSV-1 infection and established the methodology necessary to study the expression of several genes that could play key roles in the mechanisms by which stress modulates HSV-1 reactivation and recurrence.

II. Effect of restraint stress on the clinical course of a primary cutaneous HSV-1 infection

After the cutaneous HSV-1 infection model was characterized, it was utilized to study how stress can modulate the clinical pathophysiology of a primary HSV-1 infection. It has been demonstrated that stress-induced activation of the HPA axis results in elevation of corticosteroids that may impair immune function. Thus, it was hypothesized that due to stress-induced suppression of immune responses toward HSV-1, RST would increase the clinical severity of a primary HSV-1 infection.

A series of experiments were designed to test that hypothesis. In those experiments animals were restrained for eight 15-hr cycles in well-ventilated 50-ml conical tubes. Control animals were deprived of food and water for the same time period (RST mice do not have access to food or
water while restrained). After the end of the third cycle, RST and control mice were infected with HSV-1 on the dorsal area. Disease scores were recorded daily thereafter. The course of the development of herpetic symptoms is shown in Figure 3.5 and the course of development of dermatome formation is presented in Figure 3.6. Herpetic vesicles developed between days 5 and 6 post-infection. Animals that presented a score greater than or equal to 2 for three consecutive days were considered clinically infected. Because the appearance of symptoms did not occur simultaneously in all animals, the data are presented as the mean score during peak infection for each group. For statistical purposes, peak infection was defined as the range of time in which severe symptoms developed during preliminary studies using home-cage animals. Contrary to the hypothesis, RST animals presented lower peak mean disease scores than FWD control animals (Table 3.1) (p<0.05, Wilcoxon test). RST animals also presented a lower percentage of secondary dermatome involvement than the FWD controls (Table 3.1).
To examine the possible reasons for the unexpected decrease in clinical signs observed in restrained animals, a series of experiments focusing on how RST modulates immune responses toward HSV-1 were designed. The results obtained in those experiments are presented in the following sections.

**III. The effect of RST on antibody responses to HSV-1 during a primary cutaneous infection**

It is known that stress can alter the peak of antibody responses during viral infections (Sheridan et al., 1998). Thus, the effect of restraint stress on antibody responses to primary HSV-1 infection was studied. Blood samples were obtained from the animals at 0, 7, 14, and 21 days post-infection by tail bleeding. Plasma from those samples was analyzed for the development of antibodies against HSV-1 using ELISA. The results shown in Figure 3.7 represent the IgM response to primary cutaneous HSV-1 in SKH-1 mice. Compared to FWD controls, a statistically significant decrease in the IgM response was found in RST mice at day 7 post-infection (p<0.05, Student's t-test). The results shown in Figure 3.8 represent the IgG response to primary cutaneous HSV-1 infection in RST. Restrained animals presented decreased IgG
titers at day 14 using the Student’s t-test ($p<0.05$), as compared to controls. Although initial IgG and IgM titers were suppressed in RST animals, by the end of the infection the titers were similar. Thus, RST does not affect the final antibody titer.

**IV. Effect of restraint stress on infectious HSV-1 titers during primary cutaneous HSV-1**

During herpes labialis, the clinical severity of the infection parallels the kinetics of viral replication (Rytel et al., 1978). Therefore, a stress-induced decrease in viral replication at the site of primary infection could explain a decrease in clinical signs. To explore if RST could induce a change in the kinetics of HSV-1 replication at the site of primary infection, skin samples from restrained and control mice were excised at days 3, 5, 7, and 9 post-infection. The samples were analyzed for the presence of infectious HSV-1 using a plaque formation assay. The HSV-1 titers for the FWD mice peaked at day 3 and decreased thereafter. By day 7, the levels were barely detectable (Figure 3.9). The titers for the RST group peaked at day 5 (statistically significant increase compared to FWD, ANOVA, $p=0.03$; Post-hoc Tukey-Kramer, $p<0.05$) and continued to be increased even by day 7 (statistically significant increase compared to
FWD, ANOVA p=0.008; Post-hoc Tukey-Kramer, p<0.05) (Figure 3.9). At day 9 post-infection, replicating virus was undetectable in both the RST and the control groups. These findings suggest that the clinical severity of a primary HSV-1 infection does not parallel the kinetics of viral replication during stress. RST can change the kinetics of HSV-1 replication, altering the course of the primary infection and prolonging its infectious period.

V. Effect of restraint stress on cellular infiltration during cutaneous HSV-1 infection

Leukocytes have the potential to induce tissue damage in areas of inflammation and viral infection (Schmid and Rouse, 1992, Yu et al., 1996). Stress has been associated with decreased cell trafficking to the lungs during an influenza infection (Sheridan, 1998). Thus, it was hypothesized that stress-induced suppression of leukocyte trafficking to the area of primary HSV-1 infection could contribute to a decrease in the clinical severity of the infection.

Studies were conducted to determine if RST has an effect on the amount of cellular infiltrate present at the site of primary HSV-1 infection. Skin samples were collected at days 3, 5, and 7 post-infection and were
stored in formalin. Histologic analysis of the samples was conducted using a cellularity score described by Cook et al (Cook et al., 1995). Scoring was performed in areas of the slide compatible with herpetic infection (e.g. presence of acatholysis and keratinocyte ballooning). Leukocytic infiltration increased in correlation with disease progression. RST animals showed a higher cellularity score than controls at day 5 post-infection (Figure 3.10). By day 7 the scores were comparable between the control and RST animals. These findings suggest that RST could increase cellular trafficking at the site of primary infection in SKH-1 mice and that the severity of the clinical presentation of primary HSV-1 did not correspond to the amount of cellular infiltrate present.

VI. Effect of RST on C-C chemokine expression at the site of primary cutaneous HSV-1 infection.

Chemokines are mediators of leukocyte migration during inflammation. It was hypothesized that the increase in the amount of cellular infiltrate at the site of primary HSV-1 infection in RST mice could be modulated by an increase in chemokine expression. For that purpose, the effects of restraint stress on the expression of MCP-1 and MIP-1 alpha were studied. Skin samples were collected at days 1, 3, 5, and 7 post-
HSV-1 infection. The tissues were homogenized, total RNA was isolated, and mRNA was selected. After cDNA was prepared from the isolated mRNA, real-time RT-PCR was performed. The levels of MIP-1 alpha and MCP-1 were below the detection levels of assay for non-infected skin. The expression of MCP-1 peaked at day 1 in the control group and remained at lower levels at days 3-7 (Figure 3.11). RST changed the kinetics of MCP-1 expression during primary cutaneous HSV-1. RST animals presented lower MCP-1 levels at day 1 compared to the control group. However, MCP-1 levels were increased in the skin of HSV-1 infected RST mice thereafter. A 5-fold increase in MCP-1 expression in RST mice was found at day 5 post-infection (Figure 3.11) when compared to control mice (ANOVA, p=0.01; Post-hoc Tukey-Kramer test, p<0.05). Likewise, while MIP-1 alpha expression kinetics at days 1 and 3 post-infection were similar in RST and control mice (Figure 3.12), however the expression of MIP-1 alpha in RST mice was 7-fold higher at day 5 post-infection (ANOVA, p=0.01; Post-hoc Tukey-Kramer, p<0.05). In summary, both MCP-1 and MIP-1 alpha gene expression increased by day 5 post-infection in RST, mice resulting in the increase in cellularity observed at the site of primary HSV-1 infection.
VII. Effects of RST on the expression of cytokines involved in cell-mediated immunity during primary cutaneous HSV-1 infection.

Cell-mediated immunity has a central role in protection against primary and recurrent HSV infections (Catin et al., 1995 Babu, 1995, Rinaldo and Torpey, 1993, Schijns et al., 1995, Sharma et al., 1996). Defects in cell-mediated immunity can result in an enhanced risk of primary and recurrent infections with HSV (Rinaldo and Torpey, 1993).

To study the effects of restraint stress on the gene expression of IL-2, IL-4, and IL-10, skin samples were collected at days 3, 5, 7, and 9 post-infection. The tissues were homogenized, total RNA was isolated, and mRNA was selected. After cDNA was prepared from the isolated mRNA, real-time RT-PCR was performed. The expression of IL-2 and IL-4 in the skin from RST and control mice was undetectable by real-time PCR during the time points studied. In control mice, IL-10 expression peaked at day 5 post-infection and decreased thereafter (Figure 3.13). In RST mice, IL-10 expression increased by day 5 post-infection and remained elevated as of day 9 post-infection (Figure 3.13). Compared to gene expression in control mice, the expression of IL-10 in the skin of RST mice showed a statistically significant increase at day 9 post-infection (ANOVA, p=0.002; Post-hoc Tukey-Kramer, p<0.05) (Figure 3.13).
VIII. Effects of RST on the kinetics of type I expression during primary cutaneous HSV-1 infection.

Innate immunity is important to control acute viral infections. To assess the effects of RST on mRNA expression of IFN-alpha and IFN-beta, RNA was isolated from the skin samples of restrained and FWD control mice excised at various time points during the infection. Competitive RT-PCR was performed. The levels of IFN-alpha and IFN-beta gene expression were undetectable in uninfected RST and FWD skin samples. In the FWD group, the expression of IFN-alpha and IFN-beta peaked at day 3 post-infection, decreasing thereafter until becoming undetectable or nearly undetectable by day 7 post-infection (Figures 3.14-3.15). In RST animals, peak IFN-alpha expression occurred at day 3 post-infection (Figure 3.14); IFN-beta peak expression occurred at day 5 post-infection (Figure 3.15). At day 3 post-infection, a 66.14% decrease in IFN-alpha expression was found in the RST group by when compared to control animals (ANOVA, p=0.04; Post-hoc Tukey-Kramer p<0.05) (Figure 3.14). Also at day 3 post-infection, 68.05% decrease in IFN-beta expression was found in the skin of restrained mice when compared to controls (ANOVA,
These findings suggest that RST can alter the gene expression of IFN-alpha and IFN-beta, suppressing the ability of the host to effectively control viral replication.

**IX. Effects of RST on the kinetics of IFN-gamma expression during primary cutaneous HSV-1 infection.**

IFN-alpha and IFN-beta are involved in the activation of NK cells (Biron et al., 1999). When activated, NK cells produce high amounts of IFN-gamma during viral infections (Biron et al., 1999). Thus, it was hypothesized that the stress-induced suppression of type I interferons could lead to suppressed IFN-gamma expression during the early stages of the primary HSV-1 infection. To assess the effects of RST on mRNA expression of IFN-gamma, RNA was isolated from the skin samples of RST and FWD mice excised at various time points during the infection. Competitive RT-PCR was performed. The levels of IFN-gamma gene expression were undetectable in uninfected RST and FWD skin samples. In the control group, IFN-gamma expression peaked at day 3 post-infection, decreasing thereafter (Figure 3.16). IFN-gamma expression decreased by 4-fold in the skin of HSV-1 infected RST mice when compared to controls.
(ANOVA, p=0.008, Post-hoc Tukey-Kramer, p<0.05) (Figure 3.16). Thus, RST can alter the expression of IFN-gamma in the skin of mice during a primary HSV-1 infection.

X. Effects of GC receptor blockage on HSV-1 clinical signs

Previous studies have demonstrated that restraint stress in mice results in sustained increases in plasma corticosterone levels (Bonneau et al., 1991, Feng et al., 1991, Hermann et al., 1994, Iwakabe et al., 1998, Padgett et al., 1998, Sheridan et al., 1991). Since GC are potent anti-inflammatory agents, we hypothesized that GC could be involved in the reduction of the clinical signs observed in restrained mice during cutaneous HSV-1 infection. In order to study the effects of GC in the clinical severity of a primary cutaneous HSV-1 infection, we used the glucocorticoid receptor antagonist RU486. RST animals treated with RU-486 presented a statistically significant increase in clinical symptomatology (3.35 ± 0.28) when compared to RST mice treated with vehicle (2.19 ±0.25) (p<0.001, Wilcoxon test) (Figure 3.17). These findings suggest that GC could be a factor involved in the stress-induced modulation of the clinical presentation of primary HSV-1.
XI. Effects of GC receptor blockage on type I and type II IFN gene expression

To evaluate the effect of RST-induced GC on the expression of IFN-alpha, IFN-beta, and IFN-gamma, mice were treated with the GC receptor antagonist RU486. RNA was isolated from the skin samples of restrained and control mice at day 3 post-infection. Competitive RT-PCR was performed. At day 3 post-infection, treatment with RU-486 restored the mRNA levels of IFN beta and IFN gamma in restrained mice to control levels (Student's t-test, p<0.05), but did not restore IFN-alpha levels (Figure 3.18). The findings suggest that the stress-mediated suppression of IFN-beta and IFN-gamma levels could be GC mediated.
Figure 3.1: Clinical signs of a primary cutaneous HSV-1 infection in SKH-1 male mice. A 1-cm² abrasion was made on the dorsal surface of each mouse. Virus was applied to the abraded area. The development of herpetic symptomatology was monitored and scored daily. The numbers in the figure represent the following scores: 1- redness and/or swelling, 2- single lesion, 3- multiple lesions, 4- necrosis and coalescence, 5- spread in dermatome pattern.
Figure 3.1 Clinical signs of primary cutaneous HSV-1 infection in SKH-1 male mice.
Figure 3.2: The expression of IL-1 beta, NGF, gB, and gD mRNA at the site of a primary HSV-1 infection three days post-cutaneous HSV-1 in SKH-1 mice. Mice were either infected with HSV-1 or mock-infected with saline via an abrasion at the dorsal surface of the skin. Skin samples from the site of infection were collected at day 3 post-infection. Tissue was homogenized and total RNA was isolated. From the total RNA, mRNA was selected and cDNA was prepared. The cDNA for IL-1 beta, NGF, gB, gD and G3PDH was amplified for 35 cycles, then samples were electrophoresed in a 1.8 % agarose gel stained with ethidium bromide and visualized on a UV transiluminator.
Figure 3.2: The expression of IL-1p (563), NGF (389), and gB (238) following post-cutaneous HSV-1 in SKH-1 mice.
Figure 3.3: The expression of IL-1 beta, IFN-alpha, IFN-beta, and NGF mRNA at the site of primary infection three days post-cutaneous HSV-1 in SKH-1 mice. Mice were either infected with HSV-1 or mock infected with saline via an abrasion at the dorsal surface of the skin. Skin samples from the site of infection were collected at day 3 post-infection. Tissue was homogenized and total RNA was isolated. From the total RNA, mRNA was selected and cDNA was prepared. The cDNA for IL-1 beta, IFN-alpha, IFN-beta, NGF, and G3PDH was amplified for 35 cycles, then samples were electrophoresed in a 1.8 % agarose gel stained with ethidium bromide and visualized on an UV transiluminator.
Figure 3.3: The expression of IL-1β (563), IFN-α (520), IFN-β, (509), and NGF (389) at the site of primary infection one day post-cutaneous HSV-1 in SKH-1 mice.
Figure 3.4: The kinetics of gB and gD mRNA expression during cutaneous HSV-1 infection in SKH-1 mice. Mice were infected with HSV-1 via an abrasion at the dorsal surface of the skin. Skin samples from the primary site of infection were collected at days 3, 5, 7, 14, and 21 post-infection. Total RNA was extracted from the samples, mRNA was selected, and cDNA was prepared. The cDNA for gB and gD was amplified for 35 cycles, then samples were electrophoresed in a 1.8 % agarose gel stained with ethidium bromide and visualized on an UV transiluminator. Samples were obtained from 4 individual animals.
Figure 3.4: The kinetics of gB and gD mRNA expression during cutaneous HSV-1 infection in SKH-1 mice.
Figure 3.5: The clinical course of cutaneous primary HSV-1 infection in restrained SKH-1 male mice. Animals were either restrained or food and water deprived for 3-cycles prior to infection and then for 5 additional cycles. A 1-cm² area was abraded on the dorsal surface of each mouse; virus was then applied to the abraded area. Animals that had a score greater than or equal to 2 for three consecutive days were considered clinically infected. Measurements represent mean scores ± SEM. n=13-14 mice/group.
Figure 3.5: The clinical course of cutaneous primary HSV-1 infection in restrained mice.
Figure 3.6: Dermatome spread development during a cutaneous HSV-1 infection in restrained SKH-1 mice. Animals were restrained for 3-cycles prior to infection and for 5 cycles post-infection. A 1-cm² abrasion was made in the dorsal surface of each mouse; virus was then applied to the abraded area. The percentage of animals presenting secondary herpetic spread was recorded daily. Data points represent the mean percentage of animals presenting secondary spread at each of the time points.
Figure 3.6: Dermatome spread development during cutaneous HSV-1 infection in restrained SKH-1 mice.
<table>
<thead>
<tr>
<th>Exp</th>
<th>Variable</th>
<th>n</th>
<th>FWD</th>
<th>n</th>
<th>RST</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Peak\textsuperscript{a} mean disease score</td>
<td>13</td>
<td>3.88 ± 1.40</td>
<td>14</td>
<td>2.53 ± 1.20</td>
<td>0.0159*</td>
</tr>
<tr>
<td>2</td>
<td>Peak\textsuperscript{a} mean disease score</td>
<td>18</td>
<td>4.57 ± 0.71</td>
<td>20</td>
<td>3.90 ± 0.06</td>
<td>0.0032*</td>
</tr>
<tr>
<td>1</td>
<td>Peak\textsuperscript{b} mean percentage dermatome involvement</td>
<td>13</td>
<td>71.2%</td>
<td>14</td>
<td>21.4%</td>
<td>0.0063*</td>
</tr>
<tr>
<td>2</td>
<td>Peak\textsuperscript{b} mean percentage dermatome involvement</td>
<td>18</td>
<td>79.6%</td>
<td>20</td>
<td>48.3%</td>
<td>0.0069*</td>
</tr>
</tbody>
</table>

* Statistically significant difference among groups using Wilcoxon Test
* Days 6-11  \textsuperscript{b} Days 7-10

Table 3.1: Between-group comparison of severity and dermatome involvement.
Figure 3.7: IgM response to primary cutaneous HSV-1 infection in restrained SKH-1 mice. Animals were either RST or FWD for 3-cycles prior to infection. A 1-cm² abrasion was made in the dorsal surface of each mouse; virus was then applied to the abraded area. Blood was collected via tail bleeding at various time points. The serum obtained was diluted serially 1:3 and was assayed for HSV-1 specific IgM by ELISA. The data points represent the mean antibody titer ± SEM.

n=11-13 animals per group.

* Statistically significant difference by Student’s t-test (p=0.036).
Figure 3.7: IgM response to primary cutaneous HSV-1 infection in restrained SKH-1 mice.
Figure 3.8: IgG response to primary cutaneous HSV-1 infection in restrained SKH-1 mice. Animals were either RST or FWD for 3-cycles prior to infection. A 1-cm² abrasion was made in the dorsal surface of each mouse; virus was then applied to the abraded area. Blood was collected via tail bleeding at various time points. The serum obtained was diluted serially 1:3 and was assayed by ELISA for HSV-1 specific IgG. The data points represent the mean antibody titer ± SEM.

n=11-13 animals per group.

* Statistically significant difference by Student’s t-test (p<0.05).
Figure 3.8: IgG response to primary cutaneous HSV-1 infection in restrained SKH-1 mice.
Figure 3.9: Effects of RST on the kinetics of infectious HSV-1 titers at the site of primary cutaneous infection. Animals were RST for 3-cycles prior to infection. A 1cm² abrasion was made in the dorsal surface of each mouse; virus was then applied to the abraded area. Skin samples from the infected area were excised and stored in ice-cold DMEM. Tissues were homogenized. Viral titers were determined using a standard plaque-formation assay. Data represent mean ± SEM of three pooled experiments (n=10-12 mice/group) (day 9, n=4 mice/group).

* p=0.03 (ANOVA), p<0.05 (Post-hoc Tukey-Kramer test) as compared to FWD mice.

** p=0.008 (ANOVA), p<0.05 (Post-hoc Tukey-Kramer test) as compared to FWD mice.
Figure 3.9: Effects of RST on the kinetics of infectious HSV-1 titers at the site of primary cutaneous infection.
Figure 3.10: Effect of RST on the cellularity present at the site of primary cutaneous HSV-1 infection. Animals were RST for 3-cycles prior to HSV-1 infection. Skin samples were excised and stored in formalin at days 3, 5, and 7 post-infection (n= 4-5 mice/group). Slides from each specimen were rated on a score of 1 to 5 for cellularity.
Figure 3.10: Effect of RST on the cellularity present at the site of primary cutaneous HSV-1 infection.
Figure 3.11: The effect of RST on MCP-1 mRNA expression at the site of primary cutaneous HSV-1 infection in SKH-1 mice. Animals were infected with HSV-1 after the end of the third RST cycle. Skin samples were excised at various time points during the infection. Tissue was homogenized, RNA isolated, mRNA was selected, and prepared cDNA was subjected to real-time PCR. MIP-alpha expression in each sample was adjusted by its corresponding GAPDH. The adjusted value was compared to expression levels obtained from uninfected control samples and fold increase expression was calculated as described in the Materials and Methods section. \( n = 4 \)

* \( p=0.01 \) (ANOVA), \( p<0.05 \) (Post-hoc Tukey-Kramer test)
as compared to FWD group.
Figure 3.11: The effect of RST on MCP-1 mRNA expression at the site of primary cutaneous HSV-1 infection in SKH-1 mice.
Figure 3.12: The effect of RST on MIP-1 alpha mRNA expression at the site of primary cutaneous HSV-1 infection in SKH-1 mice. Animals were infected with HSV-1 after the end of the third RST cycle. Skin samples were excised at various time points during the infection. Tissue was homogenized, RNA isolated, mRNA was selected, and prepared cDNA was subjected to real-time PCR. MIP-alpha expression in each sample was adjusted by its corresponding GAPDH. The adjusted value was compared to expression levels obtained from uninfected control sample and fold increase expression was calculated as described in the Materials and Methods section. \( n=4 \) mice/group.

* \( p=0.01 \) (ANOVA), \( p<0.05 \) (Post-hoc Tukey-Kramer test) as compared to FWD group.
Figure 3.12: The effect of RST on MIP-1 alpha mRNA expression at the site of primary cutaneous HSV-1 infection in SKH-1 mice.
Figure 3.13: The effect of RST on IL-10 mRNA expression at the site of primary cutaneous HSV-1 infection in SKH-1 mice. Animals were infected with HSV-1 after the end of the third RST cycle. Skin samples were excised at various time points during the infection. Tissue was homogenized, RNA isolated, mRNA was selected, and prepared cDNA was subjected to real-time PCR. IL-10 expression was adjusted by the its corresponding GAPDH expression (used as housekeeping gene). The adjusted value was compared to expression levels obtained from uninfected control sample and fold increase expression was calculated as described in the Materials and Methods section. n=4 mice/group.

* p=0.002 (ANOVA), p<0.05 (Post-hoc Tukey-Kramer test) as compared to FWD group.
Figure 3.13: The effect of RST on IL-10 mRNA expression at the site of primary cutaneous HSV-1 infection in SKH-1 mice.
Figure 3.14: Effect of RST on IFN-alpha gene expression during primary cutaneous HSV-1 infection. Tissue was excised, homogenized, and polyA-tailed RNA was subjected to competitive RT-PCR. Gene expression was calculated as attomoles of IFN-alpha mRNA divided by the corresponding attomoles of G3PDH mRNA expressed in each sample. Peak IFN-alpha expression in control (FWD) animals was determined and the relative percentage of IFN-alpha expression was calculated \[
\left( \frac{\text{IFN-alpha expression}}{\text{control mean peak expression}} \right) \times 100
\]. Bars represent mean ± SEM.

Days 1-3 (n=8-12 mice/group), Days 5-7 (n=4 mice/group).

* p=0.04 (ANOVA), p<0.05 (Post-hoc Tukey-Kramer test) as compared to FWD group.
Figure 3.14: Effect of RST on IFN-alpha gene expression during primary cutaneous HSV-1 infection.
Figure 3.15: Effect of RST on IFN-beta gene expression during primary cutaneous HSV-1 infection. Tissue was excised, homogenized, and polyA-tailed RNA was subjected to competitive RT-PCR. Gene expression was calculated as attomoles of IFN-beta mRNA divided by the corresponding attomoles of G3PDH mRNA expressed in each sample. Peak IFN-beta expression in control (FWD) animals was determined and the relative percentage of IFN-beta expression was calculated \[
\frac{\text{IFN-beta expression}}{\text{control mean peak expression}} \times 100
\]. Bars represent mean ± SEM.

Days 1-3 (n=8-12 mice/group), Days 5-7 (n=4 mice/group).

* p=0.03 (ANOVA), p<0.05 (Post-hoc Tukey-Kramer test) as compared to FWD group.

ND- Not detected
Figure 3.15: Effect of RST on IFN-beta gene expression during primary cutaneous HSV-1 infection.
Figure 3.16: Effect of RST on IFN-gamma gene expression during primary cutaneous HSV-1 infection. Tissue was excised, homogenized, and polyA-tailed RNA was subjected to competitive RT-PCR. Gene expression was calculated as attomoles of IFN-gamma mRNA divided by the corresponding attomoles of G3PDH mRNA expressed in each sample. Peak IFN-gamma expression in control (FWD) animals was determined and the relative percentage of IFN-gamma expression was calculated \[\left(\frac{\text{IFN-gamma expression}}{\text{control mean peak expression}}\right) \times 100\]. Bars represent mean ± SEM.

Days 1-3 (n=8-12 mice/group), Days 5-7 (n=4 mice/group).

* p=0.008 (ANOVA), p<0.05 (Post-hoc Tukey-Kramer test) as compared to FWD group.
Figure 3.16: Effect of RST on IFN-gamma gene expression during primary cutaneous HSV-1 infection.
Figure 3.17: Effect of glucocorticoid-receptor blockage on the clinical signs of a primary cutaneous HSV-1 infection in restrained mice. Beginning one day prior to the initiation of RST and throughout the duration of the restraint paradigm, mice were injected subcutaneously with 25mg/kg of either the glucocorticoid-receptor antagonist RU486 or control vehicle. Mice were treated with RU-486 or vehicle 1 hour prior to the initiation of RST. Mice were infected with HSV-1 after the end of the third RST cycle. Disease scores were recorded daily. \( n=7-8 \) mice/group.

In the figure, square points represent the scores distribution, bars inside the diamonds represent +/- standard deviation of the mean, middle lines and the circle at the center of the diamonds represent the mean of each group, overlap marks in the diamonds correspond to a Student’s t-test degree of separation, and the diamonds represent the 95% confidence interval.

* \( p<0.001 \), Wilcoxon test as compared to RST-RU 486, FWD-vehicle, and FWD-RU486.
Figure 3.17: Effect of glucocorticoid-receptor blockage on the clinical signs of a primary cutaneous HSV-1 infection in restrained mice.
Figure 3.18: Effect of RU-486 on IFN-alpha, IFN-beta, and IFN-gamma gene expression during primary cutaneous HSV-1 infection in restrained mice. Tissue was excised at day 3 post HSV-1 infection, homogenized, and polyA-tailed RNA was subjected to competitive RT-PCR. Gene expression was calculated as attomoles of each interferon mRNA divided by the corresponding attomoles of G3PDH mRNA expressed in each sample. Peak expression of each gene in control (FWD) animals was determined and the relative percentage of the expression of each gene was calculated $[(\text{Gene expression} / \text{control mean peak expression}) \times 100]$. Bars represent mean ± SEM. n=8 mice/group.

* p<0.05, Student's t-test as compared to FWD group.

** p<0.05, Student's t-test as compared to RST-RU group.
Figure 3.18: Effect of RU-486 on IFN-alpha, IFN-beta, and IFN-gamma gene expression during primary cutaneous HSV-1 infection in restrained mice.
It is well-documented that stress can suppress immune responses to viral infections (Bonneau, 1994, Bonneau, 1996, Bonneau et al., 1991, Bonneau et al., 1993, Dobbs et al., 1996, Dobbs et al., 1993, Sheridan, 1998, Sheridan et al., 1998, Sheridan et al., 1991, Weizman and Bessier, 1999). Previous studies also support the hypothesis that emotional stress is a triggering factor for the development of recurrent HSV infections (Bierman, 1983, Friedman et al., 1977, Glaser et al., 1987, Katcher et al., 1973, Schmidt et al., 1985, Young et al., 1976). Increased severity of primary HSV could lead to more frequent and severe recurrent episodes of the disease (Pereira, 1996). In this study it was hypothesized that stress could suppress early immune responses (e.g. type I IFN gene expression), impairing the ability of the host to activate initial viral clearance and thereby increasing the severity and duration of
NOTE TO USERS

Page(s) missing in number only; text follows. Page(s) were microfilmed as received.

114

This reproduction is the best copy available.
the infection. Indeed, in this study stress suppressed interferon gene expression during primary HSV. But despite suppressed immune responses, stress failed to increase the clinical severity of the infection, although it did increase viral load. In this chapter the findings of this study will be discussed in relation to the current knowledge of the modulatory effects of stress on viral infections.

Our laboratory has been interested in the study of the impact of stress on health and disease (Marucha et al., 1998, Mercado et al., 2001, Padgett et al., 1998a, Padgett et al., 1998b, Sheridan, 1998, Sheridan et al., 1998, Sheridan et al., 1991, Sheridan et al., 2000). For that purpose, various animal models for the study of viral infections (such as HSV and influenza) have been developed. These models have been helpful in providing knowledge of the ways in which stress modulates the nature of viral infections. For example, using a murine model of influenza, it was shown that stress could alter the pathophysiology of infection (Sheridan, 1998). In this model, stress decreased cell trafficking to the infected lungs and while increasing corticosterone levels (Sheridan, 1998). IFN-gamma production in the lymph node cells and in splenocytes obtained from stressed mice infected with influenza was suppressed in vitro (Dobbs et al., 1996). A model for acute HSV-1 in the footpad of
mice has also been used in the laboratory. In this model, it was demonstrated that stress suppressed lymphoproliferative responses against HSV, such as the development of HSV-1 specific cytotoxic T lymphocytes (CTL), and natural killer (NK) cell activity (Bonneau et al., 1991). Stress also increased HSV-1 titers in the footpad of HSV-1 infected mice (Bonneau et al., 1991). Due to anatomical reasons, the footpad HSV-1 infection model is not representative of the natural course of human cutaneous HSV-1. The size of the lesions presented is smaller than that of human cutaneous HSV-1 infections, and the infection does not result in a clearly distinct zosteriform spread. Therefore, the effects of stress on the clinical severity of primary HSV could not be studied using the footpad model.

One of the objectives of this investigation was to characterize a cutaneous HSV-1 model that would allow us to study the effects of stress on the clinical presentation of HSV-1 infection. For that purpose, we chose a murine cutaneous infection model with zosteriform spread in which the clinical signs of the infection can be clearly identified. Reported originally by Teague and Goodpasture in 1923, viral inoculation in this model is performed on the back of the animal (Teague and Goodpasture, 1923). The virus travels from the inoculation site to the sensory nervous...
system (SNS) and then returns to the skin of the whole innervating
dermatome, resulting in a zosteriform rash (Teague and Goodpasture, 1923, Dillard et al., 1972, Nagafuchi et al., 1979, Simmons and Nash, 1984, Simmons and Nash, 1985, Sydiskis and Schultz, 1965). This model has been used in our laboratory with SKH-1 hairless mice to evaluate the use of antioxidant healing formulations on topical therapy of cutaneous HSV-1 infections (Sheridan et al., 1997).

Contrary to our hypothesis, which was that stress increases the severity of a primary HSV-1 infection, RST decreased the clinical severity of primary HSV-1 in the skin of SKH-1 mice. Restrained mice presented lower disease scores during mean peak infection than controls. Even among RST and control mice presenting the same score of three, RST mice presented fewer and smaller lesions than controls. The scoring system used in this study was not able to detect this difference. Future studies should be designed in which the number of individual lesions and their size are analyzed. While the lesions in control mice usually coalesced and necrosed (a score of four), the lesions in RST mice were circumscribed to the area of the original vesicle. In addition, the lesions in RST mice generally healed faster than the lesions in controls.
The presence of secondary dermatome spread received the highest score in the classification system. The fact that RST mice presented a lower percentage of dermatome spread than control mice suggests that restraint stress could be modulating HSV transport. For dermatome spread to occur, the virus must travel by anterograde axonal transport to the sensory ganglia and return by retrograde axonal transport to the skin innervated by the infected neurons. It is known that axonal transport of HSV-1 can be inhibited (Kristensson et al., 1986, Miranda-Saksena et al., 2000). For example, nocodazole, a microtubule depolymerizer, can inhibit capsid, tegument, and glycoproteins transport; brefeldin A, an inhibitor of Golgi function, can inhibit the anterograde transport of tegument and glycoproteins (Miranda-Saksena et al., 2000). The retrograde transport of HSV-1 can also be inhibited with the use of nocodazole (Topp et al., 1994). It is unknown whether or not stress can affect HSV-1 transport.

Thus, further studies should be designed to address the possibility of an RST-induced modulation of HSV-1 transport.

Surprisingly, despite decreased clinical signs, RST animals presented increased and prolonged viral replication at the site of primary infection. Herpetic lesions initially represent viral-induced injury to the infected epithelial cells but later represent damage caused by inflammatory...
reactions mediated by inflammatory cells (Schmid and Rouse, 1992). It
has been reported that during herpes labialis the clinical severity of the
infection correlates with the kinetics of viral replication (Rytel et al.,
1978). In this study the kinetics of viral replication did not parallel the
severity of the infection. The results of the present study suggest that
stress-induced neuroendocrine activation could be an underlying
mechanism involved in the modulation of both the clinical and sub-clinical
pathophysiology of a primary HSV-1 infection.

It is well documented that restraint stress induces a sustained
increase in plasma corticosterone in mice (Bonneau et al., 1991, Feng et
al., 1991, Hermann et al., 1994, Iwakabe et al., 1998, Padgett et al.,
has been demonstrated in mice that the topical application of 0.5%
hydrocortisone during primary cutaneous HSV-1 reduced the clinical signs
of the infection, while increasing the titer and extending the presence of
infectious virus (Awan et al., 1998). This study demonstrated that RST
applied during a primary HSV-1 infection produces effects similar to the
effects observed by Awan and collaborators in their model. The results
from both studies suggest that a stress-mediated increase in endogenous
GC could be mediating the decrease in clinical severity observed during
primary HSV-1 infection in stressed mice. Also, the fact that topical application of hydrocortisone can prolong and increase viral replication during primary HSV-1 (Awan et al., 1998), strongly suggests that stress-induced GC could also be mediating the increased and prolonged HSV replication during primary HSV-1 found in this study. A glucocorticoid response element (GRE) has been recently identified in the HSV-1 genome (Hardwicke and Schaffer, 1997). This finding suggests that GC could directly modulate the replication of the virus. Indeed, in vitro, dexamethasone can enhance HSV-1 replication in latently infected neurons acting through the GRE (Hardwicke and Schaffer, 1997). An increase in GC levels induced by the activation of the HPA axis due to restraint could lead to increased viral replication during stress. A stress-induced increase in HSV-1 titers has been reported previously in the footpad model (Bonneau et al., 1991, Kunescov et al., 1992). Thus, stress-induced GC could increase viral load directly by enhancing viral replication through the GRE in the HSV-1 genome and indirectly by suppressing immune responses.

Our results indicate that stress can both impair immune responses and increase viral replication during primary HSV, supporting the findings of other investigators (Bonneau et al., 1991, Brenner and Moynihan,
1997, Kunescov et al., 1992). For example Bonneau et al. (1991), demonstrated that repeated cycles of RST suppressed lymphoproliferative responses, the development of HSV-1 specific cytotoxic T lymphocytes (CTL), and NK cell activity and increased HSV-1 titers (Bonneau et al., 1991). Kunescov et al. (1992) demonstrated that mild electric footshock decreased spleen and lymph node cellularity CTL responses and increased infectious HSV-1 in mice acutely infected with HSV-1 (Kunescov et al., 1992).

Because the effects of stress on the expression of type I interferons during primary HSV-1 have not been explored in this model, we studied the effects of stress on the expression of IFN-alpha and INF-beta gene expression at the site of primary HSV-1 infection. During an HSV-1 infection, IFN-alpha and IFN-beta are produced in great amounts in order to mediate and regulate immune responses against the virus (Biron, 1998). Our results showed that RST modulated the kinetics of interferon gene expression. At day three post-infection, RST decreased the expression of IFN-alpha and IFN-beta in the skin of animals infected with HSV-1. Immune responses mediated by type I interferons are important for the development of adaptive immune responses against HSV-1 (Kadowaki et al., 2000). Thus, the RST-induced reduction of INF-alpha
and IFN-beta gene expression in the skin during primary HSV-1 could impact the nature of subsequent immune responses necessary for the control of the infection such as IFN-gamma expression.

In this study, blocking the glucocorticoid-receptor with RU486 restored IFN-beta expression in restrained animals to control levels. Studies in vitro have demonstrated that the synthetic glucocorticoid analogue dexamethasone inhibits IFN-beta synthesis by decreasing its mRNA levels in both human and murine fibroblasts induced either with double stranded RNA or by infection with the Newcastle disease virus (Gessani et al., 1988). Data from this study support those findings in vivo and suggest that endogenous glucocorticoids are involved in the decrease of INF-beta mRNA expression observed in the skin of stressed mice during primary HSV. Recently, a possible GRE sequence was identified on the murine IFN-beta gene in L929 cells (Soury et al., 1995). Although Soury et al. (1995) reported that dexamethasone could directly induce the expression of IFN-beta in L929 cells (Soury et al., 1995), they did not examine the effect dexamethasone on virally induced IFN-beta expression. In the present study, no detectable levels of IFN-beta expression were observed in uninfected skin samples obtained from
stressed animals. This suggests that in the absence of viral infection there is a differential modulation of INF-beta expression by glucocorticoids in vivo.

As previously stated, herpetic lesions, which initially represent viral-induced damage, subsequently represent damage caused by inflammatory reactions directed by immune cells (Schmid and Rouse, 1992, Yu et al., 1996). Leukocytes mediate tissue damage by the production of reactive oxygen species and the release of their lysosomal contents (Kapp and Zeck-Kapp, 1990). NK-cells mediate tissue damage through their cytotoxic activity, which plays an important role in protection against viral diseases (Rafi-Janajerh et al., 1998). Thus, leukocytes and/or NK-cells could be responsible for tissue destruction during primary HSV-1. Restraint stress could be suppressing the recruitment of cells involved in early-non-specific immunity to the site of the primary infection, decreasing the amount of tissue damage while promoting prolonged viral replication. The decrease in the expression of IFN-alpha, which is synthesized predominantly by leukocytes, suggests that stress could be either altering cell trafficking to the skin or reducing the expression of the gene. A decrease in the amount of leukocytes recruited to the site of viral infection could explain the reduction in tissue damage observed in
RST animals. It has been demonstrated that stress can reduce cellular infiltration to the lungs and suppress lymphadenopathy in the draining lymph nodes during an influenza infection (Sheridan et al., 1998). In contrast, this study showed an increase in the cellular infiltrate present at the site of primary HSV-1 infection. The fact that the glucocorticoid receptor antagonist RU-486 increased clinical severity in RST animals without restoring IFN-alpha expression levels indicates that the observed tissue damage may not be completely leukocyte induced. In RST mice, increased expression MIP-1 alpha and MCP-1 (chemokines involved in leukocyte trafficking) correlated with an increase in cellular infiltrate observed at the site of primary HSV-1 infection. Regardless of increased cellular infiltrate, the expression of IFN-alpha was reduced in the skin of restrained mice infected with HSV-1 strongly suggesting that the decrease in IFN-alpha expression is due to stress-induced suppression of leukocyte function, not leukocyte trafficking. These findings also suggest that the observed IFN-alpha downregulation is not GC-mediated.

Interferon gamma is a cytokine associated with cell-mediated immunity. Mainly produced by CD4+ Th1 lymphocytes, IFN-gamma is also secreted by activated NK cells (Roitt et al., 1996). In the present study, IFN-gamma expression peaked at day three post-infection. Because the
frequency of virus-specific CD4+ T-cells is generally low at that point (Roitt et al., 1996), it is unlikely that the IFN-gamma expression observed could be T-cell derived. Active NK cells are detected within 2-3 days of a viral infection (Roitt et al., 1996). Thus, the fact that the peak of IFN-gamma expression occurred by day three post-infection suggests that NK cells are the primary source of IFN-gamma in the skin during primary HSV-1.

Although we did not directly study NK cell function in this study, our data suggests that stress could be suppressing NK cell activity in the skin during primary HSV. Type I interferons are involved in the activation of NK cells and when activated, NK cells produce great amounts of IFN-gamma during HSV-1 infections (Biron et al., 1999). Because suppressed IFN-gamma expression was found at a time point when NK cells were most likely the major producers of IFN-gamma, it is highly possible that stress is suppressing either the trafficking or activity of NK cells in the skin during primary HSV-1 infection.

The results from this study also indicate that the stress-induced suppression of type I interferon found in the skin of mice acutely infected with HSV-1 could be mediating the suppression on NK cells. Type I INFs are critical for the activation of NK cells. They induce NK cell-mediated
cytotoxicity (Biron et al., 1999), which has been implicated in causing non-specific tissue injury (Rafi-Janajerh et al., 1998). NK cells also have an important role in the pathogenesis of tissue necrosis associated with EBV-positive pathology (Teruya-Feldstein et al., 1997). Stress-mediated suppression of NK cell activity can reduce the non-specific tissue damage, which is necessary for the control of HSV-1 replication at the site of primary infection. Thus, stress could be mediating the increase in viral load with decreased clinical signs observed in restrained mice during acute cutaneous HSV-1 infection by suppressing either the trafficking or the function of NK cells. The data from this investigation also suggest that GC could be mediating these stress-induced responses because the treatment with RU-486 restored both IFN-beta and IFN-gamma expression levels in RST mice acutely infected with HSV-1, while increasing the clinical severity of the infection. Thus, GC-mediated suppression of IFN-beta expression could be involved in suppressing vital NK cell responses that are necessary to control HSV-1 replication but that result in some tissue damage. Further studies should be designed in order to study the effects of stress-induced GC suppression of NK cell responses in the skin during primary HSV-1.
The following mechanism is proposed as a way to explore how NK cell suppression could modulate these responses (Figure 4.1). The decrease in IFN-gamma expression observed in the skin of restrained mice infected with HSV-1 could be involved in the modulation of subsequent immune responses against the virus. For example, the monokine induced by IFN-gamma (Mig) and IFN-gamma inducible protein-10 (IP-10) are related members of the CXC chemokine subfamily and are produced by different cell types in response to IFN-gamma (Gasperini et al., 1999). IP-10 and Mig are potent NK cell chemoattractants (Romagnani et al., 2001, Taubb et al., 1995, Teruya-Feldstein et al., 1999). IP-10 can augment NK-cell cytotoxicity in a dose-dependent fashion (Taubb et al., 1995). Mig and IP-10 play an important role in the pathogenesis of tissue necrosis associated with some cases of EBV-positive pathology (Teruya-Feldstein et al., 1997). Suppression of IFN-gamma expression cause a decrease in the expression of IP-10 and Mig leading to a decrease in NK-cell trafficking and NK-cell cytotoxicity (Gasperini et al., 1999). Stress-induced decreases in type I interferon gene expression could be suppressing NK-cell mediated responses that are essential to control viral replication but that involve a certain amount of tissue damage to be effective. A decrease in type I interferon gene expression can cause a
decrease in the amount of IFN-gamma produced by NK-cells at the site of HSV-1 infection. Decreased IFN-gamma production will lead to a decreased expression of IP-10 and Mig, interferon-induced chemokines important in NK cell recruitment and activation. A decrease in IP-10 and Mig expression could lead to a decrease in NK-cell recruitment and cytotoxicity, which in turn could lead to increased and prolonged viral replication at the site of viral infection with minimal tissue damage. Further studies should be designed to address this hypothesis.

HSV-1 reactivation has been correlated with the number of latently infected neurons in the ganglia (Sawtell, 1998). A direct correlation has been also established between an increase in viral load and the number of neurons in which HSV-1 latency is established within a ganglion (Sawtell, 1997). Because RST can increase and prolong viral replication during primary HSV-1 infection, stress-induced neuroendocrine activation could cause an increase in the number of neurons that become latently infected, increasing the opportunity for later episodes of reactivation and disease recurrence. Thus, this type of stressor could be masking the severity of an HSV-1 infection by decreasing its clinical signs while
Figure 4.1. Possible mechanism for RST reduction of clinical severity during primary HSV-1 infection.
impairing the ability of the host to control viral replication, prolonging the
time in which the individual is able to spread the virus to uninfected
individuals.

Many different stimuli have been identified to induce HSV
recurrences. Stress is one such stimulus that can trigger HSV-1
reactivation. However, because the mechanisms by which stress
modulates HSV-1 reactivation are not completely understood, clinical
treatments that would predictably prevent and control stress-induced
disease recurrences are still unavailable. According to Leung and Sacks,
the incidence of genital herpes infections is increasing in an “epidemic-like
fashion” (Leung and Sacks, 2000). All persons infected with HSV are
potentially contagious, whether or not lesions are visible (Pereira, 1996).
Because HSV is mainly disseminated through asymptomatic shedding
during both primary and recurrent infections, transmission usually occurs
unbeknownst to anyone involved (Mertz et al., 1992). The prolongation
of viral replication with reduced symptomatology induced by the stressor
observed in this model, has important public health implications. To be
able to design strategies for the control of the infection, further research
is needed to understand the molecular mechanisms that can cause the
asymptomatic (sub-clinical) spread of the disease (Wald, 1999). The
murine model utilized in this investigation could be used to determine some of the mechanisms that modulate viral replication with reduced clinical signs. Understanding the mechanisms involved in the differential regulation of the clinical versus the sub-clinical pathophysiology of HSV infections could help in the development of successful strategies for the control of the disease in human populations.
LIST OF REFERENCES


