STRESS-INDUCED REACTIVATION OF LATENT HERPES SIMPLEX VIRUS INFECTION IN LUMBAR GANGLIA OF RATS

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by

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JOSEPH MOSES BLONDEAU

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

To Deborah

It's because of you....

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III. ABSTRACT

It is generally accepted that herpes simplex virus (HSV) is capable of existing within natural or experimental hosts in a latent state. During the primary infection at peripheral sites, the virus enters nerve endings and migrates centripetally along the nerves to sensory ganglia of the peripheral nervous system. Within the neuronal cell bodies of ganglia, HSV becomes latent. Reactivation of latent virus either "spontaneously" or by defined reactivation stimuli may result in migration of the virus centrifugally along nerve axons to the periphery where a symptomatic or asymptomatic recurrent infection may take place. Data suggest that stress is associated with increased rates of recurrence of cold sores in patients. This is presumed to reflect a role for stress as a stimulus that reactivates latent ganglionic HSV infection. We wished to test the hypothesis that stress could activate latent HSV infection. Since this was impractical to study in patients, we chose to study this question in animals with latent HSV infection.

Since no current model satisfied the needs of the protocol developed to test our hypothesis, we established a new rat model of acute and latent HSV ganglion infections. Rats were tested for acute and latent HSV infections following inoculation at three different sites: ear pinna (Hill *et al*, 1975), sciatic nerve, and dorsum of the hind paw. We ultimately chose the dorsum of the rear paw as the preferred site

of inoculation based on ease of inoculation, volume to be inoculated, size of the lumbar dorsal root ganglia and ease of their removal.

We characterized the HSV infection in rats. Acute and latent HSV ganglion infection were studied using the immunohistological technique of peroxidase-anti-peroxidase (PAP) detection. We have found that rat ganglionic cells became positive for HSV antigens between two and three days post-inoculation. Cellular destruction was evident by days 3-4 post-inoculation but by day 6, no virus antigens were detectable. During the acute infection, we found that primarily the nerve cells of small type unmyelinated fibres contained virus antigens. Staining of latently infected ganglia revealed only one positive cell from only one section, but this one we believe to be present in the small type unmyelinated nerve cell. The difficulty of detecting HSV antigen in latently infected ganglia supports the theory that little HSV replication is occurring during the latent state. On the other hand, following <u>in vitro</u> reactivation of latent HSV, stained ganglionic sections revealed the presence of abundant HSV antigens primarily in the nerve cells of small type unmyelinated nerves. This observation also supported the idea that the latent state is not associated with productive infection.

To test our principal hypothesis, that stress could reactivate latent HSV infection in these animals, latently infected rats were cold restraint stressed for either 3 or 4

hours at 4°C. Cold restraint stress is a well defined type of experimental stress which results in stomach ulcers and increases in plasma corticosterone. When we compared stomach pathology, plasma corticosterone and recovery of HSV from stressed and unstressed controls, we observed that rats stressed for four hours had severe stomach ulcerations, high corticosterone levels and, at some time points, a faster time to the appearance of HSV CPE when compared to controls. We concluded that cold restraint stress could reactivate latent HSV lumbar ganglion infection in rats. These results suggested that this model may be valuable for further studies on the association between stress and recurrent HSV infections.

To provide some evidence, albeit indirect, on the pathogenesis of, and mechanism of, reactivation of latent HSV infection in rats, we undertook a further series of experiments. Acute and latently infected rats were treated with capsaicin (to destroy small type sensory neurons) in an attempt to modify acute infection and/or the establishment of latency. Topical capsaicin did not alter the pathogenesis of infection. Capsaicin applied directly to the sciatic nerve reduced the number of ganglia infected with HSV, but similar results were seen in rats treated with vehicle alone.

Acute and latently infected rat ganglia were cultured in vitro in the presence of hexamethylene-bis-acetamide, a demethylating agent, to test the ability of this compound to modify latency. The number of rats and ganglia positive for HSV and the time to CPE was not different between treated and

control ganglia removed during the acute stage of infection (4 days post-inoculation). However, latently infected rat ganglia (28 days post-inoculation) treated with hexamethylene-bis-acetamide had a faster time to CPE when compared to controls.

Experiments in which latently infected ganglia cultured in vitro in the presence of varying concentrations of nerve growth factor, epinephrine and norepinephrine as putative reactivating stress hormones were inconclusive.

IV. ABBREVIATIONS USED IN THIS MANUSCRIPT

ACTH	Adrenocorticotropin
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
C	Celsius
C_2	Second Cervical Ganglia
C_3	Third Cervical Ganglia
C_4	Fourth Cervical Ganglia
CMV	Cytomegalovirus
CPE	Cytopathic Effect
CRF	Corticotropin Releasing Factor
CNS	Central Nervous System
CS	Corticosterone
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
E	Epinephrine
EBV	Epstein-Barr Virus
FBS	Fetal Bovine Serum
GRS	Garamycin Reagent Solution
HEX	Hexamethylene-bis-acetamide
HSV	Herpes Simplex Virus
HSV-1	Herpes Simplex Virus Type 1
HSV-2	Herpes Simplex Virus Type 2
HSV-539	ATCC-VR No. 539 - HSV Type 1 Prototype
HSV-540	ATCC-VR No. 540 - HSV Type 2 Prototype
HSV-76	HS76 - HSV Type 1 Clinical Isolate

HSV-106	HSV Type 2 Clinical Isolate
L_4	Fourth Lumbar Ganglion
L_5	Fifth Lumbar Ganglion
L_6	Sixth Lumbar Ganglion
MEM	Minimal Essential Medium
mg	Milligram
ul	Microlitre
ml	Millilitre
NE	Norepinephrine
NGF	Nerve Growth Factor
pfu/ml	Plaque-Forming Units/Millilitre
PAP	Peroxidase Anti-Peroxidase
PBS	Phosphate-Buffered Saline
PGs	Prostaglandins
SD	Sprague Dawley Rats
UV	Ultraviolet Light
VZV	Varicella-Zoster Virus

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VI. INTRODUCTION

Recurrent herpes simplex virus (HSV) skin infection in otherwise healthy adults is an important problem because of its prevalence, the morbidity of the recurrent skin eruptions, the frequency of these recurrences, and the lack of uniformly reliable and efficacious control measures.

Both primary and secondary preventive measures, as well as better treatments, are required. It seems reasonable to expect that a better understanding of this recurrent infectious disease will enhance the development of control measures, be they vaccines, antiviral drugs or immune modulators.

HSV establishes a latent state in its host following primary infection. The virus migrates via nerve axons to ipsilateral sensory ganglia of the peripheral nervous system where it remains dormant (Klein, 1982). Appropriate stimuli reactivate the latent virus which migrates back along nerve axons to the periphery where a symptomatic or asymptomatic recurrent infection may take place.

It is, in part, because of latency that members of the Herpes virus group are so intriguing. It is reported that Herpesviruses cause more human disease than any other virus group (Crumpacker, 1981). In simple words, once an individual is exposed and infected with any member of the Herpesvirus group, he remains infected with the virus for life. It is interesting that an individual can be infected with more than one member of the Herpesvirus group (Lemon et al, 1979)

and that prior exposure to one member does not protect against infection with another Herpesvirus (Nahmias et al, 1981; Melnick, 1977). HSV is divided into two serotypes, HSV-type 1 (HSV-1) and HSV-type 2 (HSV-2). HSV-1 and HSV-2 share some antigenic cross-reactivity, however, antigenic cross-reactivity with the other members of the herpesvirus group does not appear to exist. Reports have shown that an individual can have concurrent infections with the same strain of HSV-1 at different sites (Embil et al, 1981) and an individual infected with HSV-1 can be subsequently infected with HSV-2 (Gerson et al, 1984).

In addition to those features already mentioned, HSV displays several other features that are unexplained. For example, why do some individuals, contracting HSV for the first time, not exhibit symptoms or signs of infection (Anderson and Embil, 1979; Blondeau and Embil, 1988)? Of those individuals that display symptoms of primary infection, what determines the severity of infection? Why are some individuals more prone to recurrent infections than others and what causes these recurrences (Chang, 1983)?

Our understanding of the pathogenesis of primary and recurrent HSV infections has been greatly enhanced by the development of numerous animal models mimicking various aspects of human herpesvirus infection. These models include the mouse ear model (Hill et al, 1975), the mouse foot pad model (Schwartz et al, 1978), the guinea pig vaginal model (Scriba, 1975, 1976, 1981a, 1981b; Stanberry et al, 1982, and

1985), and the rabbit ocular model (Gerdes and Smith, 1983; Green et al, 1981a). The usefulness of each of these systems cannot be overstated, but no one model fully reproduces what is currently thought to occur in human HSV infections. In part, for this reason, we have attempted to study acute and latent ganglionic HSV infections in a rat model.

Of the many questions that still remain unanswered regarding HSV infection in man, two are of particular interest since their answers may advance our understanding of latency and reactivation. These include defining the cell or cells that are involved with the propagation and/or replication of HSV within ganglionic cells. Is one cell primarily involved with both acute productive infection and subsequent latency or is one cell capable of allowing replication and another responsible for the establishment of the latent state. We hypothesize that, following peripheral inoculation in rats, the ganglionic cell(s) infected with HSV during the primary infection can be identified using the techniques of immunohistochemistry. As well, following reactivation of latent HSV in ganglia, the cell(s) can be characterized and the identity of the neuronal cell types supporting productive infection and latency, thus determined.

Second is the association that exists between stress and the development of recurrent HSV infections. It is well known that psychological stress such as traumatic life experiences (eg., death in the family) and/or stressful life experiences (eg., examinations) precede the development of

recurrent HSV infections in some individuals (Luborsky et al, 1976; Bierman, 1983; Schmidt et al, 1985; Silver et al, 1986). As well, it has been observed that serious illness with its attendant physiological stress may be associated with reactivation of latent HSV infection (eg., cold sores observed in patients with acute pneumococcal pnuemonia). To date, no experimental model has been developed to confirm the association between physiological stress and the development of recurrent HSV infections. We hypothesize that severe physiological stress of rats latently infected with HSV-1 will result in the reactivation of latent virus in dorsal root ganglia. To obtain a first estimate of the possible role of endogenous hormones on this process, we have tested various compounds in vitro for their ability to modify the reactivation pattern of latent HSV in rat lumbar dorsal root ganglia.

To this end, I have examined the effect of stress, perceived to be an important concomitant of, and antecedent of, recurrent eruptions in man, in rats with experimentally-induced latent HSV infection of sensory ganglion cells. Preliminary studies of HSV infection in rats confirmed that they have many characteristics of individuals infected with this virus, making it probable that our observations on the effect of stress have pertinence to the effects of stress and recurrent HSV skin disease in man. It is our contention that such a model of stress-induced reactivation of latent HSV ganglion cell infection will be useful to examine some

aspects of the pathogenesis and maintenance of latent infection and its reactivation in the future, and possibly lead thereby, to the development of better control measures.

In this review of the literature pertinent to my thesis work, the focus will be on our current understanding of recurrent HSV skin infection in man as derived from studies in patients and animal models of this infection. In addition, available data on stress-induced activation of recurrent HSV skin infection will be evaluated.

The literature review pertaining to recurrent cutaneous HSV infection is divided into four sections. These include 1) the basic properties of HSV, its mode of replication, epidemiology, pathology, pathogenesis, latency and virus reactivation; 2) current animal models of HSV infection and their contribution to the understanding of human HSV infection; 3) HSV infection of the nervous system and neuronal cells as it relates to recurrent skin infection; and 4) the association between stress and susceptibility to recurrent cutaneous HSV infection.

VII. LITERATURE REVIEW

1. HERPES SIMPLEX VIRUS: THE VIRUS

Detailed reviews of the properties and molecular genetics of HSV have been recently published (Schaffer, 1981). Briefly, HSV (Alphaherpesvirinae) belongs to the family Herpesviridae which also includes Cytomegalovirus (CMV) (Betaherpesvirinae), Epstein-Barr virus (EBV) (Gammaherpesvirinae), and Varicella-Zoster virus (VZV) (Alphaherpesvirinae).

The HSV virion has a diameter of 100 nm enclosing a double-stranded DNA. HSV DNA is approximately 96-98 x 10^6 daltons in mass. The icosahedral nucleocapsid, composed of 162 capsomers, is surrounded by a lipid-containing envelope derived from the host nuclear membrane, yielding a virion 180-200 nm in diameter. The area between the nucleocapsid and the envelope referred to as the tegument, (Roizman and Furlong, 1974) is fibrous in nature with no distinctive features. Specific members of the herpes virus group cannot be differentiated under the electron microscope.

HSV can be divided into HSV-1 and HSV-2. Our studies have focussed on HSV-1. There is approximately 49-50% homology between the DNA's of HSV-1 and HSV-2 (Pagano and Lemon, 1981). HSV-1 and HSV-2 can be differentiated by clinical (Nahmias, 1973), epidemiologic, biologic, serologic and DNA fragment characteristics. HSV-1 infects primarily non-genital sites (70-80% of isolates from orolabial lesions - cold sores - are type 1) and is transmitted primarily by

direct contact. In the laboratory, it causes small, rather than large, pocks to form on chick chorioallantoic membranes, is less neurotropic after genital or intramuscular infection in mice and produces larger plaques in certain cell monolayers (Smith et al, 1971). However, the definitive technique for distinguishing between HSV-1 and HSV-2 is by analysis of the DNA fragments after endonuclease digestion (Arens and Swierkosz, 1983; Roizman and Buchman, 1979; McFarlane and James, 1984).

2. CUTANEOUS HSV INFECTION IN OTHERWISE HEALTHY ADULTS

Cutaneous HSV infection can occur at any site in man. However, the majority of these infections are localized to the orolabial area (also called cold sores or labial herpes) or to the genital area (also called genital herpes). Orolabial infection may involve the entire oral cavity as well as lips (herpetic gingivostomatitis) or result in pharyngitis (Glezen et al, 1975), while recrudescent infection occurs on the lip and perioral skin (Overall Jr, 1984). HSV infection of the male or female genital tract can similarly occur either as a primary or recurrent infection (Corey et al, 1983).

A. Epidemiology

The percent of orolabial and genital HSV infections caused by HSV-1 and HSV-2 and their estimated frequencies are:

	HSV Type	Estimated Cases/ Year in USA
Primary gingivostomatitis	99% type 1	500,000
Recurrent orolabial infection	99% type 1	98 million
Primary or first episode genital herpes	50-92% type 2	60-500,000
Recurrent genital herpes	Mostly type 2	2-20 million

(Adapted from Overall Jr., 1981)

The greater frequency with which HSV-1 has been isolated from genital lesions in patients with primary or first infection compared to the rate in patients with recurrent genital lesions may suggest that HSV-1 becomes established in a latent form less frequently, is less prone to reactivate in spinal ganglia than in trigeminal ganglia, or that in spinal ganglia, reactivation of HSV-1 is somehow restricted and/or that of HSV-2 promoted. The converse may be true of HSV-2 infections in the orofacial area.

The substantial number of first and recurrent episodes of orolabial and genital HSV infection estimated to occur in the USA each year is paralleled by the prevalence of HSV-1 and HSV-2 infection as determined by serologic surveys. Overall, the prevalence of HSV antibody, without regard to type, approaches 40-65% in Americans by 16-20 years of age (Rawls et al, 1981). By middle age, greater than 90% of the

population have serological evidence of exposure to either HSV-1 and/or HSV-2 (Anderson and Embil, 1979). Prevalence is 16-65% greater in individuals from lower socioeconomic groups than in middle income groups for all age strata.

Seroepidemiologic studies relating prevalence of HSV-1 antibody to socioeconomic class within the same population have repeatedly demonstrated that infection with HSV-1 occurs at earlier ages among persons of lower socioeconomic classes (Scott, 1957; Porter et al, 1969). Seroepidemiologic studies of the prevalence of HSV-2 antibody in Americans reflects venereal acquisition of this virus: antibody prevalence is <10% until puberty (age 13) after which the prevalence increases to 10-70%, depending on sexual activity and socio-economic level (Nahmias et al, 1970; Rawls et al, 1974).

Man being the only natural host for HSV, the source of infection is restricted to infected individuals (Nahmias and Josey, 1981). Such individuals may transmit virus from overt lesions or asymptomatically in saliva or genital secretions. For example, Buddingh et al (1953) reported recovery of HSV from the mouths of 20% of asymptomatic children seven months to two years of age, 9% of children 3-14 years of age and 2.4% of adolescents and adults. Virologic studies in pregnant women of lower socioeconomic status revealed HSV in exocervical swabs in up to 0.01% (Josey et al, 1972). Rates of HSV-2 isolation from the exocervix as high as 12% have been reported in female prostitutes (Duenas et al, 1972). In asymptomatic males attending a Urology Clinic, HSV was iso-

lated from urethra, prostate or epididymis of 15% of the study group (Centifanto et al, 1972). It is clear that a substantial reservoir of HSV infected individuals exists in otherwise healthy adults.

B. Pathogenesis of Recurrent Cutaneous Infection

The pathogenesis of recurrent cutaneous human HSV infections has been extensively reported in the literature (Klein, 1982 and 1985; Jordan et al, 1984; Darby and Field, 1984).

The incubation period for HSV-1 or HSV-2 infection ranges from 2-12 days (Anderson and Embil, 1979). The mode of transmission is direct contact and the portal of entry, the skin or mucous membrane at the site of contact. It is assumed that initiation of infection requires inoculation of viable cells in the deeper layers of the epidermis or mucous membrane, presumably through (inapparent) nicks and breaks in the stratified, keratinized outer epidermis or outer layers of stratified squamous epithelial mucosal cells, respectively.

Following cutaneous infections, multiplication of virus at the inoculation site results in the uptake of virus by nerve endings innervating the affected area. Following uptake, the virus migrates centripetally along the axon to the contiguous ipsilateral ganglion where a state of latency is established. Latency persists for the life of the individual (Darby and Field, 1984). At certain times, however, latent virus reactivates following inapparent stimuli ("spontaneously") or following definable ones. Reactivated

virus is then thought to migrate centrifugally along axons to the periphery where it may result in the appearance of a clinically apparent recurrent infection. This hypothetical model is consistent with clinical observations but based largely out of necessity, on direct evidence obtained from studies in animals experimentally infected with HSV. Stimuli shown to reactivate the latent virus include physical trauma (sciatic nerve neurectomy, Klein, 1982; cellophane tape stripping of the epidermis of the ears of latently infected mice, Hill et al, 1978), sunlight (exposure of peripheral tissues of latently infected mice to UV light, Klein, 1982; Blyth et al, 1976), menstruation, stress (anecdotal and survey reporting, Klein, 1982) and immunosuppression (treatment of latently infected mice with cyclophosphamide, Kurata et al, 1978; Anderson and Embil, 1979; Hill et al, 1983).

C. Pathology of Cutaneous HSV Infection

Pathological changes in epithelial cells are common to both primary and recurrent infections. Lesions consist of intra-epithelial vesicles with epidermal cells showing ballooning degeneration. The basal epithelial cells are generally intact. Multinucleated giant cells arise from amitotic divisions of epidermal cells (Adam, 1982) and nuclear and cytoplasmic inclusions are almost always seen. HSV-altered cells may show chromatin margination and nuclear homogenization. Nuclei of these cells are said to have a ground glass appearance. This appearance may represent the early phase of viral replication (Adam, 1982). The presence

of inclusions may be more indicative of late stage viral replication. In the skin, mononuclear leukocyte infiltration is seen in the dermis. Following rupture of the vesicle, regenerated epithelial cells repair the damaged epithelium (Adam, 1982).

3. LATENCY

Latency is one of the central features underlying recurrent HSV infections. It is defined as a state in which the cell maintains the viral genome in a repressed or largely repressed state compatible with survival and normal activities of the cell; no virions are detectable morphologically and homogenized cells do not release infectious virus evidenced by the appearance of CPE in susceptible cell monolayers. This definition, substantiated largely from studies in mice, applies to all other animal model systems identified to date (Roizman and Sears, 1987). By this definition, HSV cannot be isolated from homogenized ganglia containing the cell bodies of nerves innervating the site of inoculation. This situation obtains in animals after all signs of lesion formation at the inoculation site have subsided or resolved. At this time, virus may be recovered from ganglia only by explantation culture of whole ganglia or culture of dispersed ganglionic cells (Roizman and Sears, 1987).

These differences in the state of HSV in the ganglia are reflected in the time for HSV induced CPE to appear in the test system. Productively infected ganglia usually manifest

specific CPE in 48 hours after initiation of culture. On the other hand, CPE in monolayers on which latently infected ganglia are cocultivated usually takes more than 6-7 days to appear (Stevens, 1975).

Two theories have emerged to explain the state of latent viruses: dynamic and static (Blyth and Hill, 1984; Roizman, 1986; Roizman, 1974).

The "dynamic state" hypothesis postulates slow continuous replication and release of viruses. This implies that latently infected animals can have, at any time, infectious virus present in either the "latently" infected cell or contiguous tissue. Support for this hypothesis came from an electron microscope study in which Baringer and Swoveland (1974) found viruses in a very small number of neurons in ganglia of latently infected rabbits. This interpretation is also supported by the observation that virus is shed spontaneously in rabbit eye secretions (Nesburn et al, 1967). However, this may simply be the result of the breakdown of "static" latency. Stevens and Cook have shown that 14 days after HSV infection of the hind footpad, virus can still be isolated in 10% of sciatic nerve and roots of mice by in vitro co-cultivation (Stevens and Cook, 1971). Moreover, HSV was not recovered directly from ganglia by homogenization on day 0 of reactivation studies but was after HSV reactivation induced by pneumococcal pneumonia (Stevens et al, 1975).

Blyth and Hill (1984) reported that <1% of cervical ganglia of mice contained infectious virus after apparent complete subsidence of primary infection of the ear pinna. As well, Schwartz et al (1978) observed that infectious HSV was found in 20% of dorsal root ganglia six months following peripheral inoculation of mice. These observations also may be interpreted as supporting the dynamic state hypothesis of latency.

According to the "static state" theory, viral specific DNA remains in the host neuron indefinitely in a non-productive, non-replicative state (Blyth and Hill, 1984). The cellular site and state of the viral DNA are unknown and HSV DNA may remain in the cytoplasm or nucleoplasm as a non-integrated entity (an episome) or it may be integrated into the host genome. The amount of viral DNA in the cell (Stevens, 1980) and the actual number of cells latently infected (Walz et al, 1976) are also not known precisely, although Klein (1982) has estimated that 0.1% of ganglionic cells are latently infected. However, he concedes that this is probably an underestimate. Other unknowns include the cell type(s) involved with the establishment of latency and the fate of the infected cell(s) following reactivation. However, the complete genome of the virus must be present in these cells since mature infectious virus can be recovered from ganglia explanted on susceptible cell lines.

The static state hypothesis of latency (Roizman, 1966) was based, in part, on observations that mice (Darvill and

Blyth, 1982) and patients with recurrent infections had high titres of HSV antibody (Douglas and Couch, 1970; Zweerink and Stanton, 1981). Since lytically infected cells express virus-specific glycoproteins on their cell surface and antibody plus complement are capable of lysing these infected cells (Roizman and Batterson, 1984), it was postulated that latently infected cells could not be productively infected since lytic cytolysis would then occur.

Experimental support for the static state hypothesis of latency comes from studies in which viral products can be detected, but complete virus cannot be isolated. However, it has not been possible to detect these products consistently in all *in vivo* systems (Blyth and Hill, 1984). Thus, Green *et al* (1981b) were unable to demonstrate viral antigens in mice ganglia by immunofluorescence. However, using specific monoclonal antibody, they were able to identify a viral specific polypeptide thereby indicating the presence of a viral specific component(s). Thymidine kinase, an HSV-coded enzyme, was found in ganglia of mice (Yamamoto *et al*, 1977) but not of guinea pigs (Fong and Scriba, 1980) and HSV mRNA was found in human trigeminal ganglia (Galloway *et al*, 1982) and guinea pigs (Tenser *et al*, 1982) but not in mouse tissue (Puga *et al*, 1978).

In mice infected via epithelial routes with HSV, infectious virus can be recovered by culture of homogenized sensory ganglia up to 14 days after inoculation. After 14 days, virus can no longer be detected by this method, but virus can

be demonstrated to exist in this tissue by the technique of cocultivation (Yamamoto et al, 1977). Yamamoto et al (1977) were interested in testing to see if HSV specific viral thymidine kinase activity could be detected in latently infected ganglia after virus could be no longer detected by homogenization (>14 days post-inoculation). They found that thymidine kinase activity could be detected for up to 60 days post-inoculation and speculated that this finding indicated that at least part of the viral genome was being continually or, at least, intermittently expressed by latently infected cells.

Puga et al (1978) addressed the issue of static versus dynamic state latency by measuring the amount of viral specific DNA and mRNA in mouse trigeminal ganglia during both the acute and latent stages of infection. Following corneal inoculation with HSV, trigeminal ganglia were extracted either 4-5 days (acute) or 6-10 weeks (latent) following inoculation. For hybridization experiments, whole ganglion DNA was extracted and reacted with the HSV-specific probes. Using ^{125}I-labelled viral probes (prepared from whole viral DNA), viral DNA was detected at both stages, but viral mRNA was only detected during the acute stage of infection when probed with either the same probes as for detecting DNA sequences or when probed with a ^{125}I-transcribed DNA probe selective for late HSV mRNA (Puga et al, 1978). These results were felt to support the static state hypothesis with the virus existing in a truly nonreplicating latent form.

Galloway et al (1979) probed human paravertebral ganglia obtained from cadavers for the presence of viral-specific RNA. Tissue was probed by in situ cytological hybridization with a nick-translated ^{3}H-labelled HSV-2 DNA probe. HSV RNA was detected in the ganglia of two out of seven cases studied. The autoradiographic images were found to be localized in neurons and only a fraction of the neurons within any one ganglion had a positive signal. From these data, it was postulated that the viral RNA detected might represent extensive transcription of the viral genome necessary for replication or alternatively, the RNA might represent a transcript from a region of the genome, with the expressions of one or more viral genes a necessary factor for the maintenance of the latent state.

Similarly, in another study, Galloway et al (1982) probed human sensory ganglia with fragmented HSV-2 DNA. Of the 40 cadavers from which ganglia were collected, 14 were positive for HSV RNA. All the transcripts detected were from the left hand end (30%) of the viral genome. From this study, they argued that specific transcription of the herpes simplex virus genome occurs in latently infected human ganglion cells.

Rock and Fraser (1983) reported that HSV-1 genomes residing in the latent state in mouse brains and trigeminal ganglia lacked free ends. This characteristic was proposed to be the result of deletion of the virion terminal sequences with integration of the virion DNA via its ends and joining

of these ends to form a linear concatamer or circles which may or may not integrate. Subsequent studies (Rock and Fraser, 1985) have shown that by Southern blot analysis, two copies of the genome joint fragment could be detected. Because of this, it was postulated that the absence of free ends is due to the joining of the termini.

At present, the consensus is that static state latency with limited or partial expression of the viral genome is probably the more correct hypothesis (Blyth and Hill, 1984).

A. Mechanism of Reactivation of Latent HSV

Although reactivation is poorly understood, models have been proposed to explain this phenomenon. Hill (1981) suggested that mechanisms of reactivation may act at either of two distinct sites or in combination. In the "ganglia trigger model", a reactivation stimulus acts directly on the ganglion harbouring the latent virus. Replication of HSV and release of virus from the ganglion is followed by centrifugal migration of the virus along the axon to the skin where a clinical lesion may become apparent as a result of local viral replication in epidermal cells. This process may also obtain despite the absence of overt clinical lesions (ie., asymptomatic viral shedding) (Hill, 1981).

In the "skin trigger model", a reactivation stimulus acts locally at the site of its cutanous application. In the skin, it is postulated that there exist microfoci of infection due to frequent release of the virus from the ganglia. The stimulus at the skin serves to increase the suscepti-

bility of the skin to the virus. This may result in the development of a clinical lesion due to an increase of virus spread and replication or subclinical microfoci of infection leading to asymptomatic viral shedding (Hill, 1981).

The third proposal assumes that HSV is continuously or frequently released from ganglia and that reactivation stimuli act at both the ganglia and the skin. This leads both to an increase of virus released from ganglia, and in the skin, to an increase in susceptibility of the skin to virus. This may lead to a clinical lesion.

Experimental evidence for one or all of these mechanisms comes from studies showing that agents such as cyclophosphamide (Openshaw et al, 1979; Blyth et al, 1980), prednisolone (Blyth et al, 1980), and antithymocyte serum (Blyth et al, 1980) can reactivate virus in the ganglia. However, since these substances cannot be considered to act exclusively at the peripheral (skin cell) or ganglion site, these studies do not provide definitive support for either the skin or ganglion trigger model.

Cyclophosphamide was injected intraperitoneally into mice latently infected with HSV-1. Treatment did not alter the proportion of mice that developed recurrent disease, but virus was isolated from the ears of a significantly higher proportion of mice treated with cyclophosphamide than from controls (Blyth et al, 1980). Prednisilone was injected intraperitoneally in combination with azathioprine and subsequently alone in mice latently infected (greater than four

weeks post-inoculation) with HSV-1. Treatment did not result in an increase in the duration of clinical recurrences nor did it result in an increase in the number of mouse ears from which HSV was recovered (Blyth et al, 1980). Antithymocyte serum (produced in rabbits) was injected subcutaneously into latently infected mice. Treatment increased the duration of clinical recurrence but did not significantly increase the proportion of mice from which virus could be isolated (Blyth et al, 1980). Based on these observations, Blyth et al (1980) postulated that control of HSV latency exists at various sites, particularly sensory ganglia, skin and perhaps other peripheral tissue. The controls in the ganglia might have different functions than those in the skin and latency might thereby be maintained by different processes in different sites. These controls may or may not act through the immune systems.

There exists a body of evidence supporting the skin trigger model (Harbour et al, 1983a).

Following cellophane tape stripping, 166 of 520 latently infected mice (32%) developed recurrent disease as judged by erythema. Virus was recovered from 0 to 9% of ganglia removed from mice one to five days after treatment, with the highest percentages occurring on days 1 and 2 (9 and 6%, respectively) after treatment. HSV was recovered from 0 to 40% of ear tissues tested with the highest percentages occurring on days 3 and 5 (36 and 40%, respectively) following treatment. It is difficult to deal exclusively

with a skin trigger model since peripheral stimulation undoubtedly stimulates sensory ganglia as well. However, peripheral stimulation may, in some way, increase the susceptibility of the skin to virus replication.

Topical application of 50% xylene to the ears of mice latently infected with HSV resulted in recurrent disease in 37% of mice as evidenced by erythema. Of 62 latently infected mice treated on the ear with xylene, 34 developed erythema and of these, 17 yielded virus from cultured ear tissue. Virus was recovered from 3 to 12% of mice ganglia removed 1 to 5 days following treatment, with the highest percentages occurring on days 2 and 3 (12% each) following treatment. HSV was recovered from 0 to 44% of sampled ear tissue with the highest percentages occurring on days 4 and 5 (44 and 24%, respectively) following treatment.

Application of DMSO to the ears of mice latently infected with HSV resulted in recurrent disease in 13% of mice as judged by erythema. Virus was isolated from 0-29% of mice ganglia that were removed from mice sacrificed 1-5 days following treatment (Harbour et al, 1983a) with the highest percentages of recovery (17 and 29%, respectively) occurring on days 1 and 2 following treatment. Virus was isolated from 4-28% of sampled ears with the highest percentages of recovery occurring on days 3 and 4 (26 and 28%, respectively) following treatment.

Following UV radiation to ears of latently infected mice, HSV was isolated from 20% of sampled ears (Harbour et al, 1983a).

Based on these observations, Harbour et al (1983a) postulated that DMSO was an efficient ganglion trigger stimulus since it can induce the appearance of infectious virus in ganglia without inducing much clinical disease. As well, they postulated that only some stimuli provided both the ganglion trigger and skin trigger that were needed together to induce recrudescent herpes. If such a dual trigger system occurred, virus would be reactived in ganglia by a stimulus to the skin. Virus would migrate to the skin and replicate under favourable conditions.

Stimuli acting on the skin appear to reactivate HSV by producing various types of inflammatory responses. Xylene results in rapid release of 5-hydroxytryptamine, histamine and neurogenic factors (Harbour et al, 1983a). Cellophane tape stripping causes release of these factors to a much less extent (Harbour et al, 1983a). Epidermal regeneration following cellophane tape stripping was observed by Hennings and Elgjo (1970). Increases in mitosis and DNA synthesis were seen in the stripped area 8-12 hours following cellophane tape stripping and this rose to 5-6 times that found in the control within 24 hours following treatment and returned to normal within four days. Treatment of this system with xylene blocked these increases (Hill et al, 1981). Common to all these peripheral stimuli of ear stripping, xylene or retinoic acid may be an increase in levels of prostaglandins since treatment of mice with indomethacin, a prostaglandin

inhibitor, greatly reduced the percentage of induced recurrent lesions (Hill, 1981).

B. Molecular Basis of Latency

Stevens et al (1987) used in situ hybridization to search for virally encoded RNA transcripts in latently infected sensory neurons. Transcripts that were detected mapped to the terminal repeat regions of the viral genome and encoded for a terminal protein ICP-0 (infected cell protein number 0). In acutely infected cells, the ICP-0 transcripts were found throughout the cell but in latently infected cells, the transcripts were localized in the nucleus. Northern blotting experiments revealed that the ICP-0 probe detected an RNA of 2.6 kilobases and when single stranded DNA from the ICP-0 region was used as a probe, RNA from the strand complementary to that encoding ICP-0 mRNA was the major transcript detected. The authors postulated that there are three possible ways in which this RNA species may play a role in HSV pathogenesis. First, the RNA may be an mRNA that encodes a protein that is important in initiating or maintaining the lytic cascade in infected cells (ie., an early alpha protein that may be necessary for the progression to the synthesis of beta products). Therefore, retention of this mRNA would prevent the lytic cascade from progressing. Second, the message may encode a protein necessary for maintenance of the latent state. Third, the "anti-ICP-0" transcript may not be a mRNA but rather a natural anti-sense RNA

that regulates expression of the ICP-0 gene product by lowering or eliminating the pool of functional ICP-0 mRNA.

The finding by Stevens et al (1987) was subsequently confirmed by Croen et al (1987). By in situ hybridization, Croen et al (1987) probed ganglia from human cadavers and found that transcription was detected from the region of the viral genome containing the gene that encodes the ICP-0 gene product. Sense transcripts were not detected, however, ICP-0 (anti-sense) transcript was found. The authors argued that this finding did not represent signals indicative of virus reactivation since no sense transcripts of immediate, early or late genes (characteristic of productive or acute infection) were detected. As well, these data were felt to argue in favor of the idea of an anti-sense transcript interfering with the functional mRNA and interrupting the ICP-0 message and lytic cascade.

4. ANIMAL MODELS FOR THE STUDY OF PRIMARY, RECURRENT AND LATENT HSV INFECTION

Experimental cutaneous or mucous membrane HSV infection has been studied in mice, rats, guinea pigs, rabbits, and monkeys (Roizman and Sears, 1987). A consistent feature of these animal models of human HSV infection is latent HSV infection at a predictable site (Roizman and Sears, 1987).

A. Infection of Mice in the Ear: Mouse Ear Model

Hill et al (1975) have described results from extensive studies on mice inoculated intradermally in the ear pinna

with HSV. Following inoculation, some mice develop paralysis, erythema and vesicular lesions at the inoculation site. Nineteen HSV clinical isolates and four laboratory strains of HSV (12, type-1 and 11, type-2) were tested for their ability to produce clinical signs following inoculation into the pinna. All viruses tested produced erythema at the inoculation site 2-5 days post-inoculation. The duration of erythema was generally 1-16 days, but persisted for 21 days in some. Titres of virus in the ear pinna were highest 5 days after inoculation. With HSV-1, virus was cleared from the skin by day 9, but with HSV-2, virus persisted for up to 12 days.

Mice either recovered or went on to develop signs of neurological involvement or ear paralysis. Differences were observed between the ability of HSV-1 and HSV-2 strains to produce neurological signs. A higher percentage of animals survived infection with HSV-1 than HSV-2. Neurological signs appeared after an average of 14.1 days in HSV-2 infected mice, but after an average of only 8.6 days in HSV-1 infected mice, suggesting greater neurotropism of HSV-1.

Mortality also varied depending on virus strains inoculated, but no virus tested killed more than 50% of inoculated mice. With one exception, all type 2 viruses were of greater virulence than type 1 at a factor close to 100-fold greater.

Depending on the strain of virus tested and the dose inoculated, 10-50% of mice developed lesions on the pinna 1-3

weeks post-inoculation and these lesions lasted from 1-7 days.

To study latency, ganglia innervating the ear (cervical 2nd, 3rd, 4th) were removed and co-cultivated with Vero cells (African green monkey kidney cells) and observed for 21 days for the appearance in the Vero cell monolayer of HSV characteristc cytopathic effect (CPE). CPE was detected 7-21 days following the start of co-cultivation.

i) Recurrent HSV Infections in the Mouse Ear. To study the possibility that spontaneous recurrent infection could occur in mouse ears following recovery from primary infection, 60 female mice inoculated with HSV-1 (2×10^4 pfu/mouse) were observed daily for 150 days. During the first two weeks following primary infection, 40 mice developed erythema and of these 40, three had ear paralysis. Erythema recurred in all mice, except one, that showed erythema during the primary infection. HSV was isolated from 7/25 ears after homogenization and culture in Vero cells, supporting the conclusion that virus infection had recurred in ear tissue (Hill et al, 1975).

ii) Induced Recurrent HSV Infections. In a subsequent study, female Swiss white mice were inoculated in the right ear pinna with 6×10^4 pfu of HSV-1 (Blyth et al, 1976) to examine induced HSV reactivation. Only mice developing inflamed ears were used. At the time potential reactivating stimuli were applied mice were clinic-

ally normal. Signs of reactivation included erythema, lesions and/or ear paralysis. Mice with these signs were sacrificed and the ear pinna removed and cultured for HSV.

Intraperitoneal administration of immunosuppressive therapy (prednisone, cyclophosphamide, cortisone acetate) did not result in reactivation of HSV. Ultraviolet (UV) irradiation to the pinna caused erythema that persisted for eight days. In randomly sacrificed mice, HSV could be isolated from mouse ears 2-3 days following treatment but at no other time. Since UV light was hypothesized to trigger recurrent HSV skin infection in man by induction of prostaglandins (PGE), PGE-2 was inoculated into the pinna. Erythema occurred within 10 minutes and persisted for 3-5 hours. HSV was isolated in 10-30% of pinna cultured 2, 3, 4 days after PGE-2 treatment and was related, in part, to the number of PGE-2 doses administered.

Trauma (ear stripping with cellophane tape six times in succession) applied to the ear of latently infected mice resulted in 30% of mice showing reactivation (erythema and/or lesions) (Hill et al, 1978). A further increase in the number of strippings did not increase the percentage of mice showing reactivation. Ear stripping of control mice produced erythema within several minutes, but this disappeared within 10 hours in 60% of mice. After 24 hours, 20% had erythema; after 48

hours, 60% had erythema; and after 72 hours, 2% had erythema. Ear stripping of latently infected mice resulted in 15% having erythema for an average of 5.5 days (longer than control groups). Hence, erythema persisting beyond 72 hours was interpreted as being due to HSV infection. Virus cultures from latently infected mice whose ears had been stripped showed that, of 31% with clinical reactivation, 73% were positive for HSV compared to 2.5% from mice not showing reactivation following stripping.

Based on the above studies, Hill et al (1978) suggested criteria by which symptomatic reactivation could be considered significant. First, continuous erythema after stripping should last longer than three days. Second, late developing erythema should last for two consecutive days. Third, erythema occurring later than seven days following stripping was not thought to be a result of the treatment. Using these criteria, induced reactivation was observed in 22% of mice with a range of 7-32% for individual groups. Virus isolation from stripped ears peaked at 4-5 days following stripping with no virus isolation on day 0 of stripping or day one post-stripping.

Subsequent experiments have shown that HSV could be isolated from the ear pinna of undisturbed, latently infected, clinically normal mice four weeks following inoculation. By co-cultivating ear sections with Vero

cells, Hill et al (1980) recovered virus from 8-9% of mice tested. This isolation rate was significantly higher than the rate of 3.5% associated with spontaneous recurrences. They argued that the 8-9% from which virus was isolated might represent animals about to develop lesions. However, the authors did not exclude the possibility of latency in the ear skin accounting for the findings, in which instance these results might represent a manifestation of reactivation according to the "dynamic state" hypothesis.

iii) Ear Inoculation in Different Strains of Mice.

Harbour et al (1981) inoculated five inbred and two outbred strains of mice in the ear pinna with one strain of HSV-1 using the method of Hill et al (1975) and observed evidence of primary infection in all strains of mice tested. Inbred strains were more uniform in their response than outbred strains. One strain of inbred mice was highly susceptible to HSV infection with a 39% death rate compared to 2-19% in the other 6 strains. It was inferred that this susceptibility to a lethal effect of HSV may be genetically based.

The propensity to develop erythema in the ear after cellophane stripping also differed between strains. Mice were classified as either suitable or unsuitable depending on the presence and severity of erythema during the primary infection. Based on this criterion, 2/2 outbred strains had suitable mice with 38 and 42%

reactivation following stripping (six weeks post-inoculation) while unsuitable strains had 19 and 31% reactivation, respectively. Of the 5 inbred strains stripped 6 weeks after inoculation, 20-34% of suitable mice showed reactivation while 10-30% of unsuitable mice showed reactivation. Mice were also ear stripped at 10 and 14 weeks post-inoculation (10 weeks suitable outbred, 19 and 21% reactivation; unsuitable, 8 and 13% reactivation; inbred suitable, 0-24% reactivation; unsuitable, 5-19% reactivation). The average duration of erythema, from any group, was 2.5-4.5 days (Harbour et al, 1981). Mice were more likely to develop recurrent disease following the first stripping than the second, and on the second than after the third, and so on. HSV was recovered from homogenized ear tissue and cervical ganglia more frequently in mice that were susceptible to erythema induced by ear stripping. It was concluded that both inbred and outbred strains of mice developed recurrent clinical herpes after trauma to the skin. Repeated trauma may act as a "ganglion trigger" and thereby eliminate latently infected neurons, and this could account for decreasing reactivation after each treatment.

B. Infection of Mice in the Footpad: Mouse Footpad Model

The mouse footpad model was described by Stevens and Cook (1971). Following inoculation in the footpad, mice develop acute inflammation accompanied by lesions. These

lesions healed by 14 days post-inoculation (Cook and Stevens, 1973). In 90% of mice, flaccid paralysis developed in the inoculated leg by the eighth day following inoculation. Approximately 30% of the paralysed mice died by day 12 with evidence of encephalitis and paralysis in the contralateral leg. Within three weeks, about 40% of the paralysed mice recovered while the remainder were left with permanent paralysis.

Virus cultures of feet, sciatic nerves, dorsal roots, dorsal root ganglia, spinal cords and brains showed that peak titres were obtained at day 2-3, 5-6, 4-5, 4-5, 7-8, 8-10, respectively. Virus could not be recovered from ventral roots or draining lymph nodes. Based on these data, Cook and Stevens (1973) postulated that centripetal infection through the nervous system was the pathway whereby encephalitis developed in mice after HSV footpad inoculation.

C. Infection of Guinea Pigs: Guinea Pig Models

Guinea pigs have been used to develop an animal model of female human genital infection by intra-vaginal inoculation with HSV-2 (Scriba, 1975 and 1976) and human skin infection by footpad injection of HSV-1 and HSV-2. HSV-2 infection of guinea pigs differs from experimental infections of other species in that virus persists in this model not only in neurons, but also in peripheral tissues (Scriba and Tatzber, 1981).

The results of HSV-2 and HSV-1 inoculation subcutaneously in footpads, of intradermal footpad inoculation and

vaginal inoculations have been described in 2-3 month old Hartley guinea pigs.

i) Subcutaneous Inoculation. Following subcutaneous inoculation in the footpad with 10^4 PFU of HSV-2, local inflammation accompanied by vesiculation developed in 60% of animals (Scriba and Tatzber, 1981). Lesions generally persisted for 14-20 days and the acute phase of infection was therefore taken to be between days 0-20 after inoculation. Recurrent vesicular lesions on the footpad developed at random thereafter and were considered to be manifestations of the chronic phase which was arbitrarily considered to start on day 21. Of 124 female guinea pigs infected and monitored for 100 days after infection, 58% of animals developed footpad vesiculation during primary infection and 62% developed footpad recurrent lesions. However, the development of recurrent lesions was not related to prior development of primary lesions since the rate of animals showing recurrent herpes was not significantly different between the groups with or without primary lesions. All HSV-2 strains tested (including laboratory adapted strains and recent clinical isolates) induced primary and recurrent lesions, whereas none of the HSV-1 strains caused recurrent HSV infections.

ii) Spread of HSV-2 During Acute Infection. The spread of the virus during the acute phase of infection was determined from studies of the isolation of virus from

various organs and tissues. HSV-2 was isolated from footpad skin in all animals up to two days following inoculation and thereafter, in a declining proportion of animals, to day 11 (Scriba, 1975). However, co-cultivation of footpad tissue revealed that virus could be recovered from the inoculation site greater than 30 days after inoculation (Scriba and Tatzber, 1981).

By day 2, virus was found in the popliteal, deep and superficial inguinal lymph nodes, and ipsilateral but not contralateral dorsal root ganglia. Virus was recoverable from proximal sections of the sciatic nerve by day 4. Virus was recovered from skin and limbs, popliteal lymph nodes, sciatic nerve and dorsal root ganglia 16 days post-inoculation (25-100%, 25%, 50%, and 75%, respectively).

HSV-2 could be isolated from healthy animals between 20-300 days post-inoculation by co-cultivation of tissues. Virus was recovered from 55% of dorsal root ganglia, 32% of the lumbrosacral segments of the spinal cord, 43% of sciatic nerves and 95% of footpad skin specimens (Scriba and Tatzber, 1981). No virus was detectable in the draining lymph nodes. By contrast, only 8% of homogenized footpad skin specimens yielded positive cultures while all nerve segments and ganglia cultured were sterile. These results demonstrated that latent infection of footpad skin is characteristic of this model of human HSV skin infection and that, as in

mice, spontaneous activation of latent infection in the skin or in dorsal root ganglia of nerves subserving footpad skin, also occurs (8%).

On the other hand, subcutaneous footpad inoculation of guinea pigs with HSV-1 revealed that HSV-1 caused local inflammation and remained localized within the skin near the inoculation site. Virus could be recovered by co-cultivation from footpad explants as frequently as after infection with HSV-2, but HSV-1 could not be recovered from ganglia or central nervous system (CNS) tissue (Scriba and Tatzber, 1981).

iii) Intradermal Inoculation of HSV-1 or HSV-2. Injection of HSV-1 or HSV-2 intradermally into the footpad demonstrated that primary lesions could be induced by both virus types. Generally, these lesions were more severe than following subcutaneous inoculation in this site. Spread of virus following HSV-2 inoculation intradermally was, for the most part, as previously mentioned for subcutaneous inoculation. A higher percentage of dorsal root ganglia became latently infected following intradermal inoculation than after subcutaneous inoculation. Also, recurrent lesions occurred in 38% of animals following recovery from primary infection with HSV-1.

iv) Vaginal Infection. Following vaginal inoculation with HSV-2, guinea pigs developed severe primary infection (Scriba, 1976; Scriba and Tatzber, 1981; Stanberry

et al, 1982) beginning 2-3 days following inoculation with vesicles evident at 3-5 days; the vesicles developed into confluent pustules 5-7 days after infection. Between day 7 and 9, the pustules ulcerated and the perineum became purulent with, in some cases, extensive necrosis (Scriba, 1976). Hind limb paralysis developed and death of some animals occurred 9-20 days post-inoculation. Animals recovering from this infection did so by 2-4 weeks after infection.

Recurrent herpetic lesions (1-2 vesicles surrounded by a red halo) developed on the vulva, in animals observed for 320 days, were less severe than the primary infection and generally persisted for 2-4 days. Recurrent herpetic lesions were not restricted to the genital area and were also found on the skin of the hind foot pads as early as two weeks after primary infection (Scriba, 1976).

In contrast, vaginal infection with a strain of HSV-1 led to very mild vulvovaginitis 3-6 days following inoculation with the appearance of one or two vesicles on the vulva. Recurrent herpes was not seen following HSV-1 inoculation (Scriba and Tatzber, 1981).

HSV-2 could be recovered from the vagina but not from the uterus up to seven days post-inoculation. Invasion of the nervous system occurred as early as 2 days post-inoculation (Scriba, 1976) and occurred more commonly than following subcutaneous footpad inoculation

since all animals yielded virus from dorsal root ganglia and spinal cord during the acute phase of the infection (Scriba and Tatzber, 1981). By tissue explantation, latent virus was recoverable from the vagina 200 days post-inoculation.

D. Infections of Rabbits - Ocular Inoculation

The understanding of the natural history of human eye infections with HSV has been greatly enhanced by studies of HSV eye infections in rabbits. These studies have also supported the concepts of latent ganglion infection following initial peripheral infection, and reactivation. As early as 1920, Gruter (1920) had shown that inoculation of HSV onto rabbit cornea resulted in acute keratitis with epithelial lesions most intense 5 to 7 days after inoculation. Goodpasture and Teague (1923) subsequently reported that during the acute phase of the disease, HSV spreads along the fifth cranial nerve to the ipsilateral trigeminal ganglion. Cocultivation of trigeminal ganglion leads to the recovery of HSV but virus could not be recovered from cell free homogenates of ganglionic tissue (Stevens et al, 1972; Stevens, 1975). These observations are consistent with the presence of latent HSV in the ganglion. Reactivation of latent virus may lead to periodic recurrent infections with detectable virus found in the eye (Stevens, 1975; Nesburn et al, 1967; Gerdes and Smith, 1983).

Following the acute phase of keratitis, disciform edema develops and persists for several weeks (Centifanto-

Fitzgerald et al, 1982). The stromal layer eventually becomes opaque with infiltration of lymphocytes, macrophages and polymorphonuclear leukocytes. Vascularization of the cornea and tissue necrosis occur and it appears that damage is primarily due to the immune response since virus is generally not recoverable from the eye at this time (Centifanto-Fitzgerald et al, 1982). This is consistent with the clinical situation.

The previous sections on animal models of HSV infections have reviewed the contributions that each has made to the overall understanding of HSV infections.

5. ANATOMY AND PHYSIOLOGY OF DORSAL ROOT GANGLIA OF HUMANS

The following sections review our current knowledge of dorsal root ganglia and studies undertaken to try and elucidate the pathogenesis of HSV infections within the cells of these structures.

A ganglion is an aggregate of cell bodies located outside the central nervous system (Matzke and Foltz, 1983). Dorsal root ganglia, also referred to as sensory, spinal or posterior root ganglia, are found on the posterior roots of the spinal nerves (House et al, 1979). Cells in sensory ganglia are of the monopolar type (one process). The cell nucleus is large with a distinct nucleolus and the cytoplasm contains finely granular Nissl substances. The cells within a ganglion tend to be arrayed near the periphery, the central portion being occupied by myelinated fibers. Nerve cells are

surrounded by a single layer of flattened cells which form a capsule. These flattened cells are referred to as satellite cells and the capsule they form becomes continuous with the Schwann cell sheath that runs along the single process of the nerve cell body (House et al, 1979). The ganglion is surrounded by a dense connective tissue capsule that is continuous with the epineurium of the spinal nerves (Carpenter, 1976).

In spinal ganglia, interneural spaces contain large and small axons, satellite cells, Schwann cells and blood vessels (Carpenter, 1985). The unipolar neurons are either ovoid or spherical in shape with cell diameters from 20 to over 100 um. By light microscopy, two types of ganglion cells are detected based on their staining properties (Haematoxylin and Eosin), large neurons appearing light while the smaller neurons stain dark.

Within dorsal root ganglia, the cell bodies of neurons subserving pain and temperature are of the small unmyelinated type (Carpenter, 1985). In the dermis, cold sensation is detected by a specialized structure known as the bulb of Krause while heat is detected by the organ of Ruffini. These specialized structures consist of nerve endings (receptors) covered with a complex capsule (House et al, 1979). The receptors for pain consist of naked, free, terminations of the peripheral nerve fibers (House et al, 1979). These fibers are spread out among the epithelial cells of the epi-

dermis and are uniformly distributed over the body surface (including orifices) (House et al, 1979).

Within the dorsal root ganglia, the cell bodies of nerve fibers subserving light touch and pressure are large and well myelinated (House et al, 1979). Receptors for these nerve endings terminate on specialized epithelial cells in the epidermis.

6. INTRA-AXONAL MOVEMENT OF HSV

In 1923, Goodpasture and Teague (1923) postulated, and Kristensson et al (1971) demonstrated experimentally, that HSV spreads within axon(s) to the central nervous system from skin or mucosal sites of infection. Since that time, numerous investigators have studied HSV spread within the peripheral nervous system (PNS) and the CNS in animals experimentally infected with this virus.

Hill et al (1972) inoculated mice in the rear food pad with either HSV-1 or HSV-2 and then searched for evidence of virus within the sciatic nerve, spinal cord and brain by electron microscopy 6-7 days after infection. At 6 days post-inoculation, mice with unilateral or bilateral hind limb paralysis had widespread infection of neurons and glial cells in the spinal cord. In some cases, virus particles were seen in unmyelinated and myelinated axons. Virus was also demonstrated in the brain and ipsilateral sciatic nerve.

They proposed three explanations for the occurrence of HSV particles intraaxonally. First, virus particles entered

the axon directly from adjacent cells, which were not identified; second, virus particles were travelling from the periphery towards the CNS; and third, virus particles were travelling within the perikaryon of an infected neuron. Hill et al (1972) concluded that of the three possibilities, the first seemed the least likely because of the perceived difficulty of HSV in penetrating the myelin sheath. The second and third explanations seemed more plausible. Enveloped virus particles were found to lie within cisternae of the agranular endoplasmic reticulum. These cisternae were suggested to be part of a continuous system extending along the length of the axon and therefore, a possible channel for intra-axonal movement of HSV particles.

Cook and Stevens (1973) also inoculated mice in the hind footpad with HSV-1 and then studied the subsequent movement of virus intraaxonally by immunofluorescent and electron microscopy.

Using the technique of immunofluorescent microscopy, virus specific fluorescence was observed in nerve trunks, but the cell type(s) involved were not identified. Within sacrosciatic spinal ganglia, neurons and satellite cells showed virus-specific fluorescence. No samples of nerves or ganglia fluoresced more than 6 days after inoculation (Cook and Stevens, 1973).

Electron microscopy revealed virus particles within neuronal cells, demonstrating that neurons in ganglia were productively infected.

Kristensson et al (1978) inoculated mice in the hind footpad and cornea with HSV-1 or HSV-2 and studied the neural spread of virus to the trigeminal and spinal ganglia by peroxidase antiperoxidase (PAP) method and electron microscopy. No difference was noted between HSV-1 and HSV-2 in their neurotropic characteristics and both types rapidly invaded the nervous system. To demonstrate transport of HSV from the periphery to ganglia, horseradish peroxidase (HRP) was injected into the foodpad or cornea and 24 hours later, ganglia were collected and stained to detect the HRP, and histologic changes. Neurons showing altered morphology consistent with virus infection corresponded to those which took up the HRP. It was postulated that HRP was taken up at axonal nerve endings near the site of infection and then transported by retrograde axonal transport to the nerve cell body and that HSV was transported in a similar way.

PNS destruction was limited, but CNS destruction was extensive. The area of special note was a region defined as the transitional zone within the trigeminal root where the CNS and PNS converged. These changes were confirmed in another study (Townsend and Baringer, 1976). To explain this, Walz et al (1976) postulated that the immune response may limit virus-induced damage within ganglia while the blood-brain barrier may preclude the operation of such a virus-containing mechanism in the CNS (Brightman et al, 1970).

The most elaborate and detailed study on axonal HSV transport was that of Lascano and Berria (1980). They inoculated mice in the hind footpad and traced virus movement utilizing the unlabelled antibody enzyme PAP. Following inoculation, virus multiplied locally in epidermal cells and in the connective tissue of the dermis. It sequentially spread into small nerve branches innervating the local area, the sciatic nerve, its spinal branches, the spinal ganglia, the cord and then the CNS. Within the sciatic nerve, infection was localized to Schwann cells. They did not observe intra-axonal virus particles within the sciatic nerve. Based on these results, they suggested that neural transport of HSV depended on at least two mechanisms: active transport between Schwann and connective tissue cells of the sciatic nerve and meningeal cells, neurons and glial cells within the CNS and passive movement of the virions along the intercellular spaces within the neural structures.

However, these observations were at variance with others in the literature. Unlike Lascano and Berria, Cook and Stevens (1973) observed intra-axonal HSV particles. Entry of virus into the spinal cord was found to occur by the ventral and dorsal roots according to Lascano and Berria (1980), but this contrasts with a report indicating entry via dorsal roots only published by Kristensson (1970).

In addition to the above results, Lascano and Berria (1980) found staining of the walls of some blood vessels of the footpad and lungs of HSV infected mice, suggesting that

hematogenous infection of the CNS following skin inoculation was theoretically also possible.

Corneal or footpad inoculation of mice with HSV-1 results in encephalitis or myelitis, respectively. Schwann cells become infected but there is not destruction or apparent demyelination in the PNS (Townsend and Collins, 1986). Demyelination has previously been reported within the CNS (Kristensson et al, 1979). CNS demyelination was thought to occur by one of two mechanisms, first, lytic destruction by HSV of myelin producing oligodendroglial cells and second, immune destruction, possibly due to cross-reactive antigens shared between HSV and myelin (Kristensson et al, 1979).

7. GANGLION AND NEURONAL CELL TYPES HARBOURING LATENT VIRUS

Whereas it is accepted on the basis of studies in experimentally infected animals and naturally infected patients that lytic and latent HSV-1 and HSV-2 can be cultured from DRG, it is unclear whether the infected cell types are the same ones in both situations, what proportions are infected and whether reactivation of latent infection results in cell destruction. Studies in animals with experimental HSV infection in peripheral tissues have yielded considerable pertinent data.

To determine which ganglionic cell(s) are infected during acute and latent stages of HSV infection following cutaneous or other peripheral tissue inoculation, different approaches have been used to examine this question. Itoyoma

et al (1984) used the PAP technique to study the transition from the acute to the latent stage of trigeminal ganglion infection during primary HSV infection of the cornea in mice. They found that by 2 days post-inoculation, isolated neurons within the trigeminal ganglion were positive for HSV antigens and that by 4 days, more cells in the centre of the ganglion stained for HSV antigen. This location was interpreted as indicating retrograde axoplasmic transport since the central portion of ganglia contained the neurons with terminals that ended in the periphery (cornea). Also, at 4 days, infected satellite cells were present in a ring-like array around infected neurons. This was felt to indicate that replicating virus spread from neurons to neighbouring satellite cells. By 8 days post-inoculation, HSV antigen was detected in neurons but not in satellite cells, indicating that infection producing HSV antigen in a neuron can occur without cytolysis (Itoyoma et al, 1984). These neurons which switched from the HSV-permissive to non-HSV-permissive state (Itoyoma et al, 1984; Openshaw et al, 1981) may be the ones that harbour latent HSV.

Ljungdahl et al (1986) used an indirect approach to further our understanding of the cellular localization of latent HSV infection in DRG. They treated mice with capsaicin, the pungent agent in hot pepper, in an attempt to destroy the ganglionic cell population that may be involved in the spread of HSV into the brain and the development of acute and latent infections. Capsaicin, administered intraperitoneally, at

the neonatal age, causes loss of chemosensitive pain (Jancso et al, 1968) and degeneration of small sized sensory neurons (some of which can be identified because they contain substance P) (Jessell et al, 1978; Jancso et al, 1981).

Control, untreated mice infected with HSV-1 by injection into the snout were shown to have HSV infected neurons of both the small and large type, aggregated in groups. Neurons containing substance P-like immunoreactivity (SPLI) as well as other neurons contained HSV antigens. Following capsaicin treatment, only single neurons or none at all contained SPLI. After HSV infection, HSV antigens was present in many of the remaining neurons (Ljungdahl et al, 1986).

Other observations included a lower mortality rate in capsaicin treated mice, a lower percentage of capsaicin treated mice harbouring latent virus, a longer time needed to detect virus from cultured ganglia in capsaicin treated mice than in controls (ie., 8.1 days vs 7.0 days, respectively). From these studies, it was concluded that HSV can be transported in both capsaicin sensitive and insensitive neuronal cell types. The decrease in the number of sensory neurons may have accounted for the decrease in reactivateable virus.

Although much has been learned from the studies cited here, it still remains uncertain whether cells involved with acute and latent HSV infection are the same in both cases. As well, the proportions of cells that become infected has not been fully elucidated and following reactivation, the fate of the cell(s) harbouring HSV is unclear.

8. INJECTION OF HSV DIRECTLY INTO THE SCIATIC NERVE OF MICE

In an attempt to increase the percentage of ganglia that become acutely infected and subsequently latently infected, we injected HSV directly into the sciatic nerve. As well, we were interested in studying (immunohistologically) the cell(s) involved with primary and recurrent HSV ganglionic infection and it was postulated that direct sciatic nerve inoculation would facilitate these studies. Therefore, a review of the literature relating to HSV inoculation directly into the sciatic nerve is presented.

Townsend and Collins (1986) injected HSV-1 directly into the sciatic nerve of mice and used the contralateral nerve as a control (injected with inactivated HSV or sterile saline). Additionally, some mice were infected in the hind footpad. Of the 20 mice inoculated, 13 survived until analyzed (7 days post-inoculation). Eight of these showed sciatic nerve lesions whereas none was present in controls. No lesions were seen in the sciatic nerves of mice inoculated in the footpad. Lesions consisted of (focal) demyelination of a few axons, myelin debris, mononuclear infiltrations and macrophages. Scattered Schwann cells contained both intranuclear virions and in the cytoplasm, enveloped nucleocapsids. Based on these findings, Townsend and Collins (1986) suggested that demyelination following HSV infection into the sciatic nerve may be due to the presence of extracellular virus. Extracellular virus may attract nonspecific macrophages similar to that seen in the CNS (Townsend and Collins, 1986). Alterna-

tively, extracellular virus could infect Schwann cells which, in turn, are destroyed, leading to segmental demyelination and a macrophage response (Townsend and Collins, 1986).

9. CNS INFECTION BY HSV VIA THE ADRENAL GLAND

More indirect proof that HSV enters the CNS by retrograde neuronal movement is provided by a study demonstrating prevention of CNS infection after adrenalectomy (Hill et al, 1986). Mice were inoculated intravenously via the lateral tail vein or facial vein. Following inoculation, virus was found to replicate within the adrenal cortex and medulla, and the lower thoracic spinal cord 2-3 days after infection. Bilateral hind limb paralysis was noted in mice by day 5 after inoculation. Following unilateral adrenalectomy, all animals showed unilateral paralysis while control animals were uniformly paralysed bilaterally. It was postulated that virus entered the spinal cord via the adrenals by retrograde movement along sympathetic nerves.

10. HERPES SIMPLEX VIRUS: SPECIFIC IMMUNE RESPONSE

Rouse (1984) and Lopez (1984), in discussing immunity to HSV in man, discussed the immune response to the different phases of HSV infection. These phases and the major immune mechanisms involved include establishment of primary infection in naive individuals with recovery mediated by NK cells, macrophages and early interferon production (natural resistance mechanism), dissemination from the site of primary

infection to other organs, but especially the CNS with recovery mediated by NK cells, macrophages, early interferon production, antibody production and cytotoxic T lymphocytes (adaptive immune mechanism), and termination of infection at the primary and disseminated sites involving the same mechanisms as those for primary infection and dissemination. Establishment and maintenance of latency was postulated to involve both natural and adaptive immune mechanisms but is not fully understood. Similarly, the contributions and roles of natural and adaptive immunity in the breakdown of latency and development of recrudescent disease, and recovery from recrudescent disease, are not well understood.

Following infection of a susceptible individual with HSV, the initial host response consists of macrophages, interferon and NK cells which operate prior to the activation of the immune system (Jayasuriya and Nash, 1985). The importance of macrophages was demonstrated following intraperitoneal infection of HSV in mice and localization of HSV antigens in peritoneal macrophages (Johnson, 1964). Elimination of macrophages prior to HSV inoculation lead to increased organ infection and early death.

Interferon is known to be produced from activated lymphocytes and macrophages. Administration, early in infection, of anti-interferon increases the severity of infection (O'Reilly et al, 1977; Gresser et al, 1976), whereas administration, late in infection, has no effect. Interferon has also been shown to stimulate NK cells. Mouse strains

exhibiting resistance to HSV have been shown to have enhanced interferon and NK activity (Lopez, 1980). There are at least three mechanisms by which interferon might play a role in resistance to herpesvirus infections. Interferon can inhibit replication of herpesviruses (Rasmussen and Farley, 1975) and may thus act by limiting the quantities of infectious virus produced (Lopez, 1984). It may augment the lytic capacity of NK cells (Ullberg and Jondal, 1981) or it could function by activating macrophages so that they sequester the replication of HSV-1 (Huang et al, 1971) or lyse virus infected cells (Stanwick et al, 1980).

The role of NK cells in early resistance to HSV infection has been shown (Lopez, 1980). Indirect evidence comes from patients suffering from Wiscott-Aldrich syndrome in whom there is a low NK cell count compared to healthy individuals. These patients have a high mortality rate from HSV infections (Merigan and Stevens, 1971).

The role of HSV antibody in restricting virus spread during primary infection remains controversial (Jayasuriya and Nash, 1985). As in other first infections, they are not detectable until 10-14 days after the initiation of HSV infection. Once present, herpes-specific antibodies appear to operate at at least two levels during primary infection. They neutralize free virus, thereby reducing access of the virus to the nervous system and preventing haematogenous dissemination. They may also mediate the destruction of

virus-infected cells by complement or killer cells (Jayasuriya and Nash, 1985).

Evidence for the role of antibodies in protecting against primary HSV infection was shown by Worthington et al (1980), who demonstrated that passive transfer of antibody mediated recovery from HSV infection in immunosuppressed mice and was more effective than the transfer of immune spleen cells. Kapoor et al (1982a) found that mice deficient in humoral immunity but with intact cellular immune responses, were able to clear local virus from ear tissue following ear pinna inoculation within 10 days. However, mice developing CNS infection had more severe disease and a higher percentage of mice developed latent infection. Evans et al (1946) found that immune serum arrested the progression of HSV within the neurons system of mice. Also, McKendall et al (1979) found that administration of anti-HSV antibody, within 48 hours of infection, prevented neurological symptoms and restricted latent infection. Jayasuriya and Nash (1985) suggest that these results indicated the antibody plays a critical role in restricting spread of HSV to the CNS and in limiting the establishment of latency. The exact mechanism of how this occurs is not fully known, but it is assumed that the antibody functions to inactivate the virus before it has access to peripheral nerve endings. This, in turn, could restrict access of the virus to the nervous system and thus spread within the CNS.

The role of HSV-specific antibody in the genesis and maintenance of latency remains a controversial issue. Despite high levels of serum antibody, recurrent infections continue to occur in humans (Nahmias and Roizman, 1973). Reeves et al (1981) have observed an association between an increased risk of recrudescence with high titres of neutralizing serum antibody in convalescent-phase serum in patients. Stevens et al (1972) demonstrated that passive immunization of mice maintained HSV ganglion infection in a latent state. Part of the difference in the association between HSV-antibody and maintenance of latent infection may be species specific.

T-cells appear to be the more important arm of the immune response in terms of immunity to and recovery from HSV infections. It is known that individuals with T-cell immunodeficiency suffer from more severe recurrent disease and that patients with B-cell deficiency are no more susceptible to HSV than normal individuals (Merigan and Stevens, 1971). Mice that have undergone neonatal thymectomy have an increased susceptibility to HSV infection despite the presence of HSV specific antibody (Mori et al, 1967; Jayasuriya and Nash, 1985). Zisman et al (1970) found that mice treated with anti-lymphocyte serum developed persistent viremia culminating in fatal encephalitis. Using nude mice, Kapoor et al (1982b) showed an increased susceptibility to fatal HSV infection in such T-cell deficient animals and a protective passive effect of antibody by restriction of spread to and

within the CNS. However, passive antibody did not aid in resolution of local infection.

T-cells, which mediate delayed-type hypersensitivity (DTH), recruit monocytes, macrophages and other effector cells that possess antiviral activity into the area of infection (Jayasuriya and Nash, 1985). The recruitment is mediated by lymphokines which are released by antigen-activated DTH T-cells. To study the importance of the DTH response to HSV, mice were infected in the ear pinna (Nash et al, 1981a; Nash et al, 1981b). In this system, draining lymph nodes (DLN) near the site of infection contained HSV specific DTH T-cells 4-10 days post-inoculation. These cells were capable of transferring both DTH and anti-viral immunity to challenged syngeneic mice (Jayasuriya and Nash, 1985). Suppression of HSV specific DTH T cells (draining lymph node cells from tolerized mice failed to adoptively transfer DTH) resulted in failure to inactivate local virus indicating the importance of these cells in recovery from infection.

Cytotoxic T-cells (CTL's) provide protection against viral infection and are generated early in HSV infection, prior to the appearance of neutralizing antibody (Pfizenmaier et al, 1977). CTL's can be generated against type-common and type-specific antigen determinants. Following mouse ear inoculation, CTL's appear in DLN four days post-inoculation, reach maximum activity by 6-9 days, and are present but non-cytotoxic responsive after 12 days post-inoculation (Nash et

al, 1980b). Challenge of sensitized mice leads to a rapid CTL response (Nash et al, 1980b).

Suppressor T-cells may be involved with HSV pathogenesis, inhibition of immune surveillance precipitating recurrent HSV infections (Jayasuriya and Nash, 1985). Nash et al (1981b) had previously shown that specific tolerance to HSV indicates DTH can be established in mice. This tolerance appears to be mediated by T-cells, however, the exact lineage has not been elucidated. To date, very little is known about the role of suppressor cells in HSV pathogenesis. However, Jayasuriya and Nash (1985) postulated that since DTH appeared to be important in controlling the primary phase of HSV infection, suppressor T-cells may facilitate recurrence by suppression of the DTH mechanism.

The previous sections on the immune system and HSV specific immunity have reviewed available data relative to HSV infection. The review is intended to show the complex nature of the HSV-immune response and in the following sections on stress, how the immune responses may be affected by acute stress.

11. STRESS, RECURRENT HSV INFECTION AND IMMUNITY

Data on stress, HSV infection and/or HSV-immunity are not abundant. One basic problem in this area may be the absence of suitable in vivo models for carrying out such research. However, available physiological data and

anecdotal literature relevant to stress, HSV and/or HSV-immunity are reviewed.

Stress can be defined as "the reactions of the animal body to forces of a deleterious nature, infections, and various abnormal states that tend to disturb its normal physiologic equilibrium (homeostasis)" (Stedman, 1982).
Stress is capable of activating the neuroendocrine system, one result being the release of catecholamines from the adrenal medulla (Schmidt et al, 1985).

There is a general perception (Drob et al, 1985) that stress is a concomitant of, and precipitating factor in, recurrent orolabial and genital HSV infection. The effect of stress may be mediated by changes in the immune system.

The exact mechanism(s) how stress may modify disease pathogenesis and recovery from disease is unknown. However, Macek (1982) presented evidence that emotional stress and anxiety surrounding disease appear integrated through the hypothalmic centres in the brain. This in turn triggers neurohumoral mediators that may react with lymphoid cell receptors to suppress the immunological capacity of the host (Macek, 1982).

According to Axelrod and Reisin (1984), release of ACTH from the anterior pituitary gland stimulates synthesis of glucocorticoids from the adrenal cortex. Glucocorticoids, in turn, stimulate the synthesis of norepinephrine and epinephrine from the adrenal medulla. During times of stress, plasma levels of ACTH, norepinephrine and epinephrine are

elevated (Axelrod and Reisin, 1984) and these neurohormones have been shown to suppress an immunological response including lymphokine synthesis and antibody responses (Johnson et al, 1982 and 1984).

There have been numerous accounts of altered immunological parameters in humans during either times of stress or during times of loneliness, which may be a less obvious form of stress. Psychosocial factors like emotional states, events in our lives, and the social context in which we live are often thought to affect our health (Jemmot and Locke, 1984). For example, blood samples taken from medical students prior to, and during, exams showed a significant reduction in natural killer (NK) cell activity (Kiecolt-Glaser et al, 1984b) compared to matched controls. Similarly, patients defined as high loneliness subjects or having recently experienced stressful life events showed decreased levels of NK cell activity when compared to a matched control group (Kiecolt-Glaser, 1984a).

There have been demonstrations of the effect of catecholamine treatments on HSV infections in animals. Schmidt and Rasmussen (1960) produced recurrent herpetic encephalitis in 60% of rabbits (within two weeks) by injecting epinephrine subcutaneously at distinct sites. Laibson and Kibrick (1966) reported reactivation of herpetic keratitis in rabbits following the intramuscular administration of epinephrine over a three day period. Virus was isolated between one to 15 days after the first epinephrine stimulation.

Several studies have demonstrated an association between stress and recurrent HSV skin disease in otherwise healthy adults. A survey of 3100 individuals with genital herpes revealed that 83% regarded stress as a factor contributing to recurrent infections (Help membership HSV, 1981). As well, anecdotal reports suggest that before academic examinations and other particularly stressful circumstances, cold sores are more frequent (Blank and Brody, 1950). Bierman (1983) reported that in their epidemiologic survey, 86% of patients claimed that emotional stress activated their genital herpes simplex infection. Another study found that general unhappiness among nursing students predicted the frequency of herpes labialis lesions (cold sores) (Luborsky et al, 1976). In this study, Luborsky et al (1976) posed two questions regarding the role of psychological factors in the production of recurrent herpes labialis, to young women entering nurses' training: 1) What psychological factors predict the frequency of later occurrence of recurrent herpes labialis? and 2) What psychological factors appear immediately before specific episodes of the illness? In response to the first question, it was found that those who judged themselves as more unhappy tended to have more recurrent herpes labialis outbreak during their first year of nurses' training (they also reported a history of more illnesses in general and more psychological complaints). In response to the second question, the results did not show systematic relationships between mood factors

and the onset of recurrent herpes labialis (or aphthous ulcers, or colds).

Silver et al (1986) reporting on the extent of genital herpes infections in the United States, commented that clinical observations and anecdotal reports indicated that herpes can have a profound psychological impact on victims as well as the fact that psychological factors may have an effect on recurrence and severity. However, he noted that there have been few systematic investigations of the role of stress and psychological factors in recurrent genital herpes infections.

They therefore evaluated the relationships between symptoms, recurrence, severity and a range of psychological factors in a sample of men and women with recurrent genital herpes infections. As well, they measured recent exposure to stressful life events, perceived social support and coping style. This survey indicated that persons suffering from severe cases of recurrent genital herpes infection showed high levels of emotional dysfunction. In discussing their findings, Silver et al (1986) cited numerous reports in which other investigators have observed similar results. These citations included: Goldmeier and Johnson (1982) who monitored the progress of 58 patients with first attacks of genital herpes for up to 30 weeks and found that patients with higher levels of non-psychotic psychiatric illness had higher recurrence rates, Duer (1982) who found higher levels of emotional distress in genital herpes patients versus

labial herpes patients and women with no herpes infections, and Taylor (1978) who found no higher frequency of recent stressful life events among women with genital herpes than among chronic vaginitis patients or normal controls but found that herpes patients with a high recurrence rate ($\geq$4 per year) reported a higher frequency of negative stressful events than those with a low recurrence rate ($\leq$3 per year).

Individuals experiencing 3-4 episodes of recurrent oral herpes infection were interviewed to determine if stressful life events occurred prior to the onset of recurrent infection. The major areas assessed included: stressful life events, global support, daily "hassles" and uplifts, anxiety and tension, coping ability, personality type, depression and well being (Schmidt et al, 1985). Subjects were asked to relate life events and how they were feeling one week prior to the recurrent infection. One week prior to the appearance of a recurrent lesion, 4 psychological state measures were identified in the study population that differed from the dormant stage. These included an increased level of stressful life events, elevated levels of anxiety measured by two different scales, and increased daily "hassles" and frustrations. The two most prominent types of stressful life events that occurred one week prior to the onset of recurrent disease were interpersonal problems and work-related difficulties. This report also suggested that, despite the presence of positive coping skills, support and moderate levels of positive reinforcement, the presence of negative, stress-

provoking circumstances continued to be associated with an increased likelihood of recurrent HSV lesions. Anxiety items found to be statistically elevated one week prior to lesion onset included tension, edginess, a panicky sensation, uneasiness, and restlessness. Nervousness and shakiness were not statistically different, but they too tended to be increased.

Given the information presented, it appears that stress alters homeostasis and that this alteration in homeostasis affects susceptibility to recurrent cutaneous HSV infection. It is therefore relevant to determine what stress alters and what the end result of this alteration is.

12. MECHANISM OF STRESS-INDUCED CHANGES IN IMMUNE FUNCTION: CATECHOLAMINES

It has been postulated by Schmidt et al (1985) that "certain psychosocial experiences" are capable of activating the neuroendocrine system. Among other results, there is an outpouring of catecholamines from the adrenal medulla. This, in turn, "modulates the cellular immune system and allows the dormant herpes virus to replicate, travel down the peripheral nerve and produce the vesicular eruption".

Glavin et al (1983) compared two forms of restraint stress for their ability to elevate plasma corticosterone (CS) in rats. Supine restraint stress for 2 hours at room temperature yielded plasma CS values of 64.8 ug/dL compared to 27.6 ug/dL in unrestrained controls, while rats restrained

in wire mesh had plasma CS levels of 55.6 ug/dL ($p<0.01$). Glavin (1985) showed that in rats subjected to cold restraint stress (3 hours in the supine position at 4-6°C), plasma CS concentrations were more than 2 times as high (68.3 ug/dL) as in unstressed controls (31.2 ug/dL) ($p<0.01$). Dugani and Glavin (1986) desensitized rats with chronic subcutaneous inoculations of capsaicin. They then subjected capsaicin-treated and control rats to either 0, 1, 2 or 3 hours of cold (4-6°C) restraint stress and measured plasma CS concentrations in each group. In all cases (including no stress), the capsaicin-treated rats had higher plasma CS concentrations than respective controls but stress also elevated further CS levels compared to controls. These results, taken together, indicate that restraint stress results in a significant increase in plasma CS in rats.

In regards to antibody response to foreign antigens, Shek and Sabiston (1983) used high and low immune responder mice to test the hypothesis that an antibody response, of a significant magnitude, may induce changes in circulating CS levels. Also tested was the effect antibody response had on modulating the CS circadian rhythm. When sheep red blood cells (SRBC) were used as antigen, it was found that induction of an anti-SRBC response led to a significant elevation of serum CS levels at 0800 hours on the day of peak antibody formation. This was observed in high responder mice but not low responder mice. It was also observed that antibody production appeared to disturb the normal circadian rhythm of

daily high (1600 hours) and low (0800 hours) CS levels. On the day of peak anti-SRBC response, the rhythm was reversed. Since CS is a biochemical index of stress in animals (Riley, 1981) and CS secretion is mediated by ACTH, Shek and Sabiston treated mice with diazepam to inhibit ACTH secretion. They found that this prevented the immunization-induced CS response (Shek and Sabiston, 1983). Their observations led them to hypothesize several potential points of regulation between the antibody response and CS level fluctuations. Thus, they suggested that the hypothalamic [corticotropin releasing hormone (CRH)], pituitary gland (ACTH) and adrenal gland (corticosterone) pathway may all be sites for endogenous immunological regulation. As to why such regulation is needed, it was suggested that corticosterone may play a dual role in regulating normal immune responses. First, CS secretion is enhanced to ensure the optimal proliferation of immunocompetent cells and secondly, it prevents excessive proliferation of antibody secretory cells (Shek and Sabiston, 1983). These inferences are supported by data showing that glucocorticoids promote immune processes (Manjan and Johnson, 1980) and suppress CS production (Crabtree et al, 1980).

The endogenous opioids (enkephalins and β-endorphin) have been shown to affect immune processes. Matheus et al (1983) tested the ability of β-endorphin and metenkephalin to enhance the activity of natural kill cells (NK). Using peripheral blood mononuclear cells (PBMNC) from healthy donors, it was demonstrated that β-endorphins and met-enkephalins

enhanced the NK activity of human PBMNC. β-endorphin appeared to be involved in the recruitment of effector cells from preNK cells as well being involved in increasing effector cell recycling (Matheus et al, 1983). Although the mechanism of opiate regulation of the immune system is unknown, the activities appear to be mediated through classical opiate receptors since naloxone, an opiate antagonist, inhibited NK augmentation by β-endorphin and met-enkephalin (Matheus et al, 1983). From these studies, one could postulate that suppression of endogenous opiates could suppress NK cell activity and thereby facilitate either the incidence and/or severity of recurrent HSV.

Recently, Brown and Van Epps (1986) reported that opioid peptides (β-endorphin and met-enkephalin) may enhance the production of gamma interferons (γ-IFN). Gamma interferon is a lymphokine that exerts a variety of effects on the immune response (Brown and Van Epps, 1986). In this study, concanavalin A (Con-A), a T-cell mitogen which is used to stimulate human mononuclear cells to produce IFN, was used to stimulate mononuclear cells to produce IFN in the presence of β-endorphin or met-enkephalin. It was found that both opiates enhanced IFN production. This effect was, however, variable between human blood donors and in some cases, opiates depressed IFN production. Indirect evidence suggested that classical opioid receptors did not mediate this response since it was found that IFN enhanced production was not inhibited by naloxone. As above, suppression of interferon

production could facilitate the incidence and/or severity of recurrent HSV infection.

Koff and Dunegan (1985) tested the ability of neurohormones and neuropeptides to modulate macrophage-mediated tumoricidal activity. Macrophages, when activated, developed a cytotoxic phenotype characterized by the selective binding and destruction of neoplastic cells without harm to normal cells. Peritoneal macrophages from pathogen-free male mice, were activated with recombinant γ -interferon and lipopolysaccharide (LPS). Activated macrophages were then tested against melanoma cell lines and cytotoxicity was assayed. It was found that ACTH and nonadrenaline blocked the ability of γ-IFN to activate murine macrophages to a cytolytic state. Since macrophages are important for surveillance against neoplastic disease, stress-induced suppression of macrophage activation to a cytolytic state may be an important finding, explaining, in part, reports of stress-induced enhancement of neoplastic disease (Riley, 1981).

Koff and Dunegan (1986) subsequently evaluated the ability of macrophages to lyse HSV infected cells in the presence and absence of norepinephrine, epinephrine or ACTH. Having demonstrated that macrophages will destroy HSV-infected cells following activation by γ-IFN, LPS or lymphokines, Koff and Dunegan demonstrated that norepinephrine and epinephrine blocked the capacity of macrophages to be activated by γ -IFN to a cytolytic state capable of recognizing and destroying HSV infected cells. Dopamine, serotonin and

ACTH did not block macrophage activation. These data suggested that selective stress-related neurohormones modify the cytolytic activity of macrophages against virus-infected cells and suggest a possible neuroendocrine-immunologic basis for the recurrence of HSV infection.

Rasmussen et al (1957) provided some earlier experimental evidence indicating an association between stress and susceptibility to HSV infection in mice. Their protocol for stressing a mouse was characterized by frequent, sustained stress with minimal physical stress and maintenance of normal nutritional status. They used an electric shock avoidance situation. From 50 to 80% of mice learned to avoid shock within 3-6 days. The number of stress days varied from 1-28 for different experiments. On the 29th day, all animals were inoculated with HSV (strain HF) intraperitoneally (100 LD_{50}) which resulted in a 50-60% fatality rate in control mice within 14 days compared to 74% in mice stressed by avoidance learning and 63% in mice stressed by confinement. A second stress procedure involving restraint stress was also employed in which mice were immobilized in fine mesh copper screen for 6 hours. It was observed that the more stress a mouse received (6 hours per day for 28 days of avoidance-learning stress), the more likely the mouse was to die and in a shorter period of time, following HSV inoculation. The results from restraint stress were similar in that more mice died with longer periods of stress, but in this experiment, there was no difference in survival time. Male mice seemed

to be more susceptible to the effect of stress than female mice, but this difference was not statistically significant. They concluded that stress induced greater susceptibiility to HSV infection as evidenced by increased death rate and a trend toward decreased survival times. The exact mechanism by which this occurred was not elucidated. However, it was suggested that the response was centrally mediated and may involve higher nervous centers.

13. RESTRAINT STRESS

In my experimental studies, I have utilized a well-characterized acute stress to attempt to increase activation of latent HSV DRG infection in rats. This stress uses restraint and has the following history and characteristics.

Restraint-induced stress was originally developed in 1936 by Selye to study peptic ulcer disease. He found that tying the limbs of rats together and wrapping them in towels generated signs of stress such as adrenal hypertrophy, thymicolymphatic involution and gastric ulcers. The model has been subsequently modified by Bonfils and Lambling (1963), Brodie et al (1961), Vincent et al (1977), and Glavin (1980).

The characteristics of the model described by Brodie et al (1961) included: starvation prior to restraint, which enhanced the production of gastric ulcers; rats and mice developed more lesions than did hamsters, guinea pigs, rabbits and monkeys; healing of gastric ulceration was com-

plete within 72 hours if free feeding was allowed but could take up to 196 hours if food was withheld during recovery. There was no adaptation to repeated periods of restraint and hypophysectomy did not affect lesion incidence, but vagotomy decreased it while adrenalectomy increased the number of gastric ulcerations. Vasodilatory agents reduced the incidence of ulcers while vasoconstrictors increased lesion incidence.

Further modifications led to the model of restraint-stress induced gastric ulceration I have used in Glavin's laboratory (Glavin, 1980). This model involves immobilization of the animals in a supine position with the limbs drawn out at 45°. The animals are kept at 4-6°C for 3-4 hours because it has been shown that a combination of stress and cold act synergistically to enhance gastric ulceration (Brodie et al, 1961; Glavin, 1980).

Based on work by Glavin (1980), two separate measurements are used to quantitate the degree of gastric damage in stressed rats. These include the number of individual ulcers present and the overall cumulative length of the ulcers with the latter being the more sensitive measure.

14. PUTATIVE FACTORS MEDIATING REACTIVATION OF LATENT HSV

The following review of nerve growth factor, epinephrine, norepinephine and hexamethylene-bisacetamide are provided to show why we chose them for in vitro manipulation of HSV infected tissues. Norepinephrine and epinephrine are endogenous compounds that may be released during stress and

modify HSV latency. We had hoped that these initial indirect studies might provide leads as to whether these endogenous compounds could have been associated with stress-induced recurrent HSV infection in rat ganglia.

A. Nerve Growth Factor

Nerve growth factor (NGF) is synthesized and released by target (the peripheral tissue innervated by the neuron) tissue. It binds specifically to nerve terminal receptors and is conveyed by retrograde transport to the neuronal soma (Hendry et al, 1974). Thoenen and Barde (1980) reported that sympathetic and neural crest-derived sensory neurons in vivo and in vitro require NGF for maintaining normal functions and depending on age and the cell type, for survival.

Wilcox and Johnson (1987) attempted to test the effects of NGF on reactivation of latent HSV in vitro based on a report by Price and Schmitz (1978) suggesting that reactivation of latent HSV is preceeded by the loss of trophic support provided to the neuron by the peripheral target. NGF is the only neurotrophic agent isolated to date (Thoenen and Barde, 1980). As well, previous attempts to induce reactivation by central rhizotomy, and axotomy produce a reduction or cessation in neurotrophic support transported retrograde from the neuronal targets (Wilcox and Johnson, 1987). Neurons that harbour latent HSV are NGF-dependent while the non-neuronal cells such as fibroblasts and Schwann cells are neither NGF-dependent nor responsive (Wilcox and Johnson, 1987).

To test NGF effects on neurons harbouring latent HSV, Wilcox and Johnson (1987) developed an in vitro latency system in which cultures of primary sympathetic neurons were inoculated with a low multiplicity of infection of HSV. Surviving cells showed no evidence of viral infection but a percentage of them harboured latent HSV as evidenced by plaque-forming assays, freeze thawing of cells, CPE and detection of virus specific thymidine kinase. To get the dissociated ganglionic cells to grow in culture, they were placed in medium supplemented with 50 ng/ml of NGF. The addition of anti-NGF to cultures resulted in reactivation of latent HSV depending on the conditions used to establish the latent infection (Wilcox and Johnson, 1987). Reactivation of latent HSV was also achieved by using medium lacking NGF. A difference was noted between the times for reactivation of latent HSV to occur, with the anti-NGF treatment leading to the appearance of HSV CPE sooner than treatments that simply involved washing off of NGF. This difference in time for HSV to appear was attributed to the report that NGF is a "sticky" molecule (Thoenen and Barde, 1980) and residual NGF probably stayed in culture even after washing. It was concluded that NGF antagonism or deprivation by washing resulted in the reactivation of latent HSV in sympathetic neuronal cultures. By corollary, perturbations that damage either the nerve terminal or the terminal field of the neuron may be expected to cause reactivation by producing a reduction or cessation in NGF normally transported in a retrograde direction to the neuronal cell body.

B. Hexamethylene-Bis-Acetamide

Bernstein (1987) suggested that demethylation may play a role in reactivation of latent HSV. To test this hypothesis, the demethylating agent hexamethylene-bis-acetamide (HEX) was added *in vitro* to guinea pig dorsal root ganglia harbouring latent HSV-2. HSV was recovered from 42% of dorsal root ganglia by seven days in the HEX group but from only 8% of untreated ganglia. As well, HSV was recovered from more vaginal and cervical explant cultures treated with HEX than from those without (24% vs 8%, respectively). HEX had no effect on acute HSV replication *per se* .

Based on this study and a suggestion from Dr. L.R. Stanberry (personal communication) that this agent might yield similar results in rats, we tested the ability of HEX to reactivate latent HSV from rat lumbar dorsal root ganglia.

C. Monensin

Monensin is a carboxylic polyether ionophore which is thought to catalyze the exchange of sodium and potassium ions across biological membranes. It is thought to disrupt ion gradients that appear to be crucial to the budding of vesicles from the Golgi compex (Johnson and Spear, 1982).

Johnson and Spear (1982) infected HEp-2 and Vero cells with HSV-1 and two hours following adsorption, added 0.2 uM monensin to the culture medium. They found that in monensin-treated cultures, yields of extracellular virus were reduced to 0.5% of control values. Viral protein synthesis was not inhibited by monensin but late stages in post-translational

processing of the viral glycoproteins were blocked. As well, the transport of viral glycoproteins to the cell surface was also blocked by monensin. From these results, they concluded that the Golgi apparatus was involved in the egress of herpes simplex virus from infected cells.

Based on the findings of this study, we hypothesized that rats inoculated with HSV-1 and subsequently inoculated with monensin might accumulate HSV (antigen) within acutely infected neurons and that this accumulation might permit definitive immunohistological identification of the neurons involved in primary infection. This hypothesis was based on the expectation that monensin would prevent the release of virus from infected cells and if the timing of infection were accurate, the only cells displaying antigen would be those initially infected following inoculation of HSV.

VIII. MATERIALS AND METHODS

1. RATS

Sprague-Dawley female rats (Charles River Canada Inc., St. Constant, Quebec) weighing 70-100 grams were used. These rats were from a strain known to be free of HSV antibody. Female sex was chosen by coin toss. For experiments, rats were assigned to experimental and control groups by use of a table of random numbers.

2. CELL CULTURE MEDIUM

Materials used for cell culture included minimal essential medium (MEM) (Flow Laboratories, McLean, Virginia, USA), L-glutamine (Gibco, Burlington, Ontario, Canada) used at 1%, sodium bicarbonate (Gibco, Burlington, Ontario, Canada) used to adjust the pH of MEM to 7.4, trypsin (Flow Laboratories, McLean, Virginia, USA), fetal bovine serum (FBS) (Gibco, Burlington, Ontario, Canada), and phosphate buffered saline (PBS) (Oxoid, Nepean, Ontario, Canada).

3. HUMAN NEONATAL FORESKIN FIBROBLAST (HUFF) CELL CULTURE

Human neonatal foreskin fibroblast (HUFF) cells were obtained from the Cadham Provincial Laboratory, Winnipeg, Manitoba, Canada. HUFF cells were propagated in flasks and used to prepare monolayers in tubes, employing standard methods: cells were propagated in 175 cm^2 tissue culture flasks (NUNC, Gibco Canada, Burlington, Ontario, Canada) to confluency at 37°C in growth medium consisting of MEM and 10%

FBS. To propagate cells, growth medium was discarded and the monolayer washed with 10 ml PBS. The PBS was drained, 3 ml of 0.25% trypsin added, and the flask returned to the 37°C incubator for 10-15 minutes. After this time, when the cells had detached from the flask and separated from each other, they were washed from the bottle with MEM containing 10% FBS and evenly distributed between two 175 cm^2 flasks. Growth medium (45 ml) was added and the flasks returned to the 37°C incubator.

To prepare cell culture tubes, the same procedure as above was used but instead of dividing cells into flasks, the trypsinized cells were transferred to growth medium (60 ml MEM containing 10% FBS) in an Erlenmeyer flask. A magnetic stirrer was added and the mixture stirred at room temperature for one minute to suspend the cells. To each tube, 1.2 ml of growth medium containing cells was added, approximately 50 cell culture tubes (Corning, Fisher Scientific, Fairlawn, New Jersey, USA) (125 mm x 17 mm) being made from the cells in each 175 cm^2 flask. The tubes were placed in a 5% CO_2 incubator at 37°C. After 48-72 hours, confluent adherent monolayers were visible. Growth medium was replaced with 1.2 ml of maintenance medium (MEM supplemented with 5% FBS and antibiotics which were penicillin 100 IU/ml, gentamicin 250 ug/ml and mycostatin 25 IU/ml).

4. VIRUS

HSV-76, type 1, was used for this study (Blondeau et al, 1986). This virus had been characterized and typed by restriction endonuclease analysis (Blondeau et al, 1988) and compared to prototype strain ATCC VR #539, HSV type 1, strain MacIntyre. Using the procedure of McFarlane and James (1984) and the restriction enzymes BamHI, BglIII, EcoRI, HindIII, KpnI, PstI, SalI, SstI, XbaI, XhoI, the banding profiles between HSV-76 and MacIntyre were identical with all enzymes except KpnI. This enzyme detailed a one band difference between the two isolates.

The virus was propagated in Vero cells obtained from the Cadham Provincial Laboratory, Winnipeg, Manitoba, Canada, in mycoplasma- and antibiotic-free MEM with 5-10% FBS inactivated for 30 min at 56°C. When infected monolayers showed 70-80% CPE, cell cultures were freeze-thawed twice, the suspension clarified by low speed centrifugation and passed through a 0.22 um filter (Nalgene®, Rochester, New York, USA). Aliquots were stored frozen at -70°C. The virus was titrated by calculating the median tissue culture infectious dose ($TCID_{50}$) on Vero cells using Costar® 24 well plates (Costar, Cambridge, Massachusetts, USA). The titer of the virus stocks were $10^{8.33}$ $TCID_{50}$ per ml and $10^{8.50}$ $TCID_{50}$ per ml. Virus was used after the fourth and fifth passage following isolation from the patient. For some experiments, stock virus was diluted in MEM or PBS.

5. INOCULATION OF RATS

Rats were anesthetized with 5 mg/100 g pentobarbitol sodium (Abbott Laboratories, Ltd., Montreal, Quebec, Canada) injected intraperitoneally. Rats were inoculated in the ear pinna according to the method of Hill et al (1975), in the sciatic nerve or dorsum of the hind paw. Unilateral or bilateral inoculations of 5 ul to 100 ul were performed using a Hamilton microliter syringe (Fisher Scientific, Fairlawn, New Jersey, USA) and a 30 gauge needle. For sciatic nerve inoculation, the nerve was exposed aseptically approximately 2.5 cm from the midline and secured between two loose loops of suture for injections. In some studies, the nerve was gently crushed using a small hemostat at the site of subsequent inoculation.

Control animals were inoculated with PBS or MEM. Rats were examined twice daily for 28 days for signs of neurological abnormality and to determine time to death.

6. REMOVAL OF PERIPHERAL TISSUES AND SENSORY GANGLIA AND RECOVERY OF HSV

The results of infection were evaluated either 4 or 28 days following inoculation except where otherwise noted.

Rats were killed with 15 mg/100 g pentobarbitol sodium injected intraperitoneally. Once anaesthetized, rats were bled by cardiac puncture for collection of plasma and serum samples which were stored at -20°C.

Ipsilateral and contralateral second, third and fourth cervical dorsal root ganglia or fourth, fifth, and sixth lumbar dorsal root ganglia were removed and analyzed for HSV in rats inoculated in the ear pinna and the sciatic nerve or dorsal hind paw skin, respectively, as follows.

Using a 100x magnification on a dissecting microscope (Olympus, Canlab, Mississauga, Ontario, Canada), the spinal cord was exposed by clean dissection and the appropriate cervical or lumbar ganglia identified and removed. To detect latent HSV, ganglia were placed individually in vials containing 1.5 ml MEM supplemented with 3% FBS and antibiotics (penicillin 100 IU/ml, gentamicin 250 ug/ml, and mycostatin 25 ug/ml) for 24-28 hours at 37°C in 5% CO_2 in humidified room air. This procedure is a modification of that described by Harbour et al (1981) and adapted by Blondeau et al (1986). Following this, ganglia were explanted individually onto monolayers of HUFF cells (time 0) (Blondeau et al, 1986) and left undisturbed for 48-50 hours at 37°C in 5% CO_2 in humidified air. Time 0 is defined as the time at which a ganglion was added to a HUFF monolayer. All tubes were coded prior to examination of CPE to control observer bias.

Monolayers were checked twice daily using a 40x inverted microscope for the appearance of HSV CPE. Cultures not showing CPE after 21 days were discarded as negative.

To detect productive HSV infection, samples of skin from inoculation sites, or from the sciatic nerve (mid-thigh, approximately 4 cm from spinal cord), were placed in indivi-

dual vials with supplemented MEM as described and frozen at -76°C until assayed. To culture HSV therein, the tissues were thawed and ground with a sterile mortar and pestle. Following grinding, 1 ml of MEM supplemented with 10% FBS and antibiotics (as described above) was added to the tissue. The tissue was centrifuged at low speed (1000 rpm) (4°C) for 10 minutes and 0.5 ml of supernatant inoculated into each of two tubes containing a monolayer of HUFF cells. These cultures were maintained for seven days and checked daily for the appearance of HSV CPE. Appearance of CPE characteristic for HSV (Embil and Faulkner, 1964) was interpreted as indicating presence of HSV.

Since virus appeared in contralateral ganglia in our initial experiments, we were concerned that virus might be reaching them by routes other than interneuronal spread. In an experiment to determine whether non-neuronal routes played a role in virus spread to these ganglia, we ligated the sciatic nerve on one side at two sites and resected the nerve between the ligatures. Control animals had sham operations including mobilization and isolation of the sciatic nerve of one leg. Virus was inoculated immediately thereafter subcutaneously in the dorsum of the hind paw of the leg with the transected sciatic nerve. The fourth, fifth and sixth lumbar ganglia from both sides were removed four days later and cultured for lytic HSV.

7. STATISTICAL ANALYSIS

Data were analyzed by Chi square test or Student's unpaired t-test with type I error probability controlled by Dunn's procedure (Kirk, 1982).

8. ASSAY OF TISSUE FOR VIRUS: $TCID_{50}$

To determine the natural history of HSV infection in rats following peripheral inoculation, rats were inoculated unilaterally in the dorsal skin of the hind paw with HSV-76, type 1. Minimal essential medium was inoculated into the opposite foot. At time 0 (prior to inoculation), 2, 4, 7, 14, 21 and 28 days, randomly selected rats were sacrificed and the following tissues collected, weighed and frozen at -70°C: left and right dorsal skin of hind paws, left and right 4th, 5th, 6th lumbar ganglia, left and right sciatic nerve, spinal cord (section between the 4th to 6th lumbar ganglia) and brain. Blood was collected and the serum removed and stored at -20°C. All tissue was collected, weighed and stored individually.

To assay and calculate the $TCID_{50}$, tissue was thawed and ground with a sterile mortar and pestle. Following grinding, 10-100 volumes by weight of MEM supplemented with 5% FBS, 100 IU/ml penicillin, 250 ug/ml gentamicin, and 25 IU/ml mycostatin was added to the tissue. This was then spun for five minutes at 4°C in a benchtop microfuge to sediment debris. Following centrifugation, the supernatant was refrozen at -70°C until assayed.

$TCID_{50}$ assays were carried out on NUNC® 96 well multi-well plates. Briefly, wells were seeded with 40,000 Vero cells per well in 0.2 ml of MEM supplemented with 5% FBS, 100 IU/ml penicillin and 250 ug/ml gentamicin. These plates were then incubated at 37°C for 24 hours in 5% CO_2 in humidified air to obtain a monolayer. At this time, tissue specimens were thawed and the supernatant assayed for infectious virus. To do this, medium was discarded from the plates. The virus was serially diluted in log_{10} steps to 10^{-5}. For inoculation, 0.2 ml was inoculated into each of four wells per dilution. The plates were then returned to 37°C in 5% CO_2 in humidified air and subsequently read every day for four days. Wells were scored as either positive or negative for HSV-like CPE and the $TCID_{50}$ calculated according to the method of Reed and Meunch (1938). The lower limit of detection for this assay was 10^3 virus particles.

9. DETECTION OF SERUM ANTIBODY

Serum collected from rats 0, 2, 4, 7, 14, 21, and 28 days after inoculation with HSV-1 was tested for the presence of complement fixing antibody by Dr. L. Sekla at the Cadham Provincial Laboratory, Winnipeg, Manitoba, Canada.

10. PREPARATION OF RATS FOR IMMUNOHISTOLOGIC STUDIES

Rats were anaesthetized with 25% chloralhydrate injected intraperitoneally. Immediately following anaesthesia and before death, rats were placed on ice, the heart exposed, and the right atrium severed. A 15 gauge needle was inserted

into the left ventricle and the rat was perfused with 50 ml of ice cold prefix containing 0.9% saline, 0.1% (w/v) sodium nitrite and 100 units of heparin. Following the prefix, the rats were perfused with ice cold 4% paraformaldehyde fixative consisting of 4% paraformaldehyde, 20% of 0.5 M sodium phosphate and 0.1-0.2 ml of 0.1 M sodium hydroxide (NaOH). Following fixing, the spinal cord was exposed by dissection and removed along with the left and right 4th, 5th and 6th lumbar ganglia. In some rats, the brain was also removed. The tissue was then placed in fixative overnight and the following day was transferred to 15% (w/v) sucrose consisting of 65% distilled H_2O, 20% of 0.5 M phosphate, 15% sucrose and 1 mg of sodium azide. The tissue remained in the 15% sucrose until analyzed immunohistologically.

11. IMMUNOHISTOLOGICAL EXAMINATION OF RAT TISSUE

Tissue to be examined immunohistologically was mounted in O.C.T. (optimal cutting temperature) mounting medium (Canlab, Mississauga, Ontario, Canada) on a metal block set on dry ice. The tissue was then placed at -24 to -26°C and allowed to equilibrate for 30 minutes. At this time, the tissue was cut in 15 um sections on a Kryostat (Wild Leitz Canada, Willowdale, Ontario, Canada) at -26°C and the sections were then melted onto a gelatin coated microscope slide (Fisher Scientific, Don Mills, Ontario, Canada). The slides containing tissue, were removed from -26°C and a layer of plastic cement was added as a border around the tissue to

prevent the antibody solution from running off the slide. The primary antibody was a polyclonal antibody to HSV that was raised in rabbits (Dimension Laboratories, Mississauga, Ontario, Canada). This antibody was diluted 1 ul in 1000 ul of PBS containing 0.3% Triton X-100 (Sigma Chemical Co., St. Louis, Missouri, USA) and 1% bovine serum albumin (BSA). The tissue section was covered with antibody solution and incubated for 48 hours at 4°C in a humidified chamber. After this time, the tissue was rinsed for two 30 minute intervals in PBS-containing 0.3% triton and goat antirabbit immunoglobulin (Miles Scientific, Canlab, Mississauga, Ontario, Canada) added at a dilution of 1:20 in PBS containing 0.3% Triton X-100 and 1% BSA. This was incubated for one hour at room temperature and rinsed with PBS-containing 0.3% Triton X-100 as previously described. After this, rabbit immunoglobulin conjugated to peroxidase antiperoxidase (PAP) (Miles Scientific, Canlab, Mississauga, Ontario, Canada) was added at a dilution of 1:250 in PBS containing 0.3% Triton X-100 and 1% BSA. The tissue was incubated for one hour at room temperature and rinsed according to the following protocol: 15 minutes with PBS containing 0.3% Triton X-100 and 1% BSA, followed by 2 rinses for 15 minutes each in 50 mM Tris, diaminobenzadine (DAB) (mixed as 20 mg DAB in 100 ml of 50 mM Tris) containing 15 ul of 30% H_2O_2 for 4-6 minutes, and 2 rinses for 15 minutes each in 50 mM Tris. At this time, the tissue was air dried overnight at room temperature.

The next day, the tissue was dehydrated according to the following protocol: H_2O for 2 minutes, 70, 80, 95% alcohol for 1.5 minutes each, 2 rinses in 100% alcohol for 1.5 minutes each, and 2 rinses in xylene for 3 minutes each. Following this, the slides were removed from the xylene, a coverslip affixed with Lipshaw coverglass mounting medium (Ingram and Bell Scientific, Don Mills, Ontario, Canada) and the preparation allowed to air dry. The tissue was then examined and photographed on a Leitz Dialax 20 microscope (Wild Leitz Canada, Willowdale, Ontario, Canada) at 10x, 40x and 100x.

Uninfected control tissue was stained in an identical fashion to that of infected tissue. As an additional control, infected control tissue was also stained as above with the exception of the primary HSV antibody. Control tissues from both procedures were uniformly negative.

12. SCIATIC NERVE WRAP WITH CAPSAICIN

Rats were anaesthetized and the sciatic nerve exposed by clean dissection as previously described (Inoculation of Rats). Cotton batting soaked in 1.5% capsaicin (Sigma Chemical Co., St. Louis, Missouri, USA) (Appendix 7) was wrapped around the nerve for 30 minutes. The exposed, unwrapped nerve and other tissues were kept moist by frequent irrigation with sterile saline. In one experiment, the saphenous nerve was transected. For controls, the nerve was wrapped in cotton batting soaked in vehicle alone. After

treatment, the cotton batting was removed and the incision closed by suturing. Animals were returned to cages and four days later, inoculated unilaterally in the dorsal skin of the hind paw of the treated leg with HSV-1 as previously described. Four days after HSV inoculation, the rats were sacrificed and the left and right 4th, 5th and 6th lumbar ganglia removed, as was a section of dorsal hind paw skin at the site of HSV inoculation and cultured for HSV as described (Removal of Peripheral Tissues and Sensory Ganglia and Recovery of HSV).

13. SCIATIC NERVE SECTION IN LATENTLY INFECTED RATS

Rats inoculated unilaterally with HSV-1 in the dorsal skin of the hind paw were selected for studies in which we attempted to reactivate latent virus by sciatic nerve sectioning. Thirty-six days post-inoculation, the rats were anaesthetized with sodium pentobarbitol and the nerve on the side of HSV inoculation exposed as previously described by clean dissection and sectioned approximately 4 cm from the spinal cord. The incision was closed by suturing and the rats returned to their cages. Control rats had the sciatic nerve exposed but not further manipulated. Two days later, the rats were sacrificed and the ipsilateral dorsal hind paw skin, sciatic nerve and ipsilateral and contralateral 4th, 5th and 6th lumbar ganglia removed and cultured for HSV.

14. INJECTION OF RATS WITH MONENSIN

Rats to be injected with monensin were first inoculated with HSV-1 in the dorsal skin of one hind paw. Monensin was prepared for injection as described in Appendix 3. Rats were randomly allocated to one of two groups. Rats in one group were injected with monensin, 0.1 ml intraperitoneally at 72 and 84 hours post-HSV inoculation, while the others received monensin, 0.1 ml intraperitoneally at 60, 72 and 84 hours post-HSV inoculation. Both groups of rats were sacrificed and perfused for immunohistologic studies approximately 12 hours following their final monensin injection.

15. RESTRAINT STRESS

Rats suspected of being latently infected ($\geq$28 days post-inoculation) were subjected to restraint stress as previously described. Rats were always restrained between 0800-0930 a.m. after food deprivation for 16-17 hours. Briefly, rats to be restrained had a short piece of adhesive tape wrapped around each paw such that a tab was produced. A safety pin was attached to each tab and the rat was placed in the supine position on an immobilization apparatus which held their limbs out at 45° angles. The rats were then placed at 4°C and, in the dark, left undisturbed for either 3 or 4 hours. Preliminary experiments indicated that 4 hours was more stressful than 3 hours. Control rats were treated in an identical fashion, but they remained unrestrained at room temperature. Following restraint, the tape was removed from

the limbs and the rats were returned to their home cages until sacrifice.

16. DETERMINATION OF STOMACH PATHOLOGY OF RESTRAINED RATS

At various intervals following restraint stress, control and restrained rats were analyzed for stomach ulceration. To do this, rats were sacrificed and their stomachs removed and fixed in 10% formalin. Stomachs were cleaned by swabbing to remove food and blood and coded by letter and number. The degree of stomach ulceration was calculated by counting the number of ulcers and measuring their length to determine cumulative length with a dissecting microscope with an ocular micrometer. All stomachs were evaluated by the same individual who was unaware of experimental conditions.

17. ASSAY OF PLASMA CORTICOSTERONE

The procedure for the assay of plasma CS is a modification of that which was previously reported by Van der Vies (1961). Plasma, frozen at -20°C, was thawed at room temperature and 0.5 ml of plasma was added to a 15 ml glass tube (with cover). To this was added 0.5 ml of distilled H_2O and 0.1 ml of 0.25 N sodium hydroxide (Fisher Scientific, Don Mills, Ontario, Canada). This mixture was shaken vigorously for 2-3 minutes by hand. To this was added 10 ml of carbon tetrachloride (CCL_4) (Fisher Scientific, Don Mills, Ontario, Canada) and methylene chloride (CH_2Cl_2) (Fisher Scientific, Don Mills, Ontario, Canada) at a ratio of 1:1. This mixture was then shaken mechanically in the dark for 30 minutes

(Reichert Scientific Instruments, New York, New York, USA). The mixture was filtered into a plain test tube using Whatman IPS phase separating paper (Whatman, Canlab, Mississauga, Ontario, Canada). The residue was discarded and 5 ml of the filtrate was pipetted into a centrifuge tube. To the filtrate was added 5 ml of 70% H_2SO_4 (Fisher Scientific, Don Mills, Ontario, Canada) mixed with 30% absolute ethanol (Canadian Industrial Alcohols and Chemicals, Ontario, Canada). This was shaken for 30 minutes in the dark again as described. At this stage, the tubes were removed every 10 minutes and shaken vigorously by hand. Following shaking, the mixtures were centrifuged for 5 minutes at 3000-3500 rpm in an International Centrifuge benchtop centrifuge (International Equipment, Massachusetts, USA). After this, the ethanol layer (usually top layer) was removed by aspiration and discarded. The acid layer (containing the CS) was then placed in the dark for 1.5 hours at room temperature. Following this, the CS concentration was measured by reading the mixture on a LS5 fluorescence spectrophotometer (Perkin-Elmer, Downsview, Ontario, Canada) set at 470 nM excitation, 522 nm emission and a slit width of 10.

An internal standard for this assay was prepared by dissolving 10 mg CS in 100 ml of 95% EtOH. From this, 1.0 ml (1.0 ug/ml) was added to 99.0 ml of 95% EtOH and 0.5 ml of this solution was placed into a test tube (see above) and dried with nitrogen gas for 20-30 minutes. After drying, 0.5

ml of distilled H_2O was added to the tube and this standard was treated the same as the other samples.

To prepare a tissue blank, 1.0 ml of distilled H_2O was added to a test tube and treated as the other samples.

18. TREATMENT OF LATENTLY INFECTED GANGLIA WITH EXOGENOUS COMPOUNDS

The protocol for testing the effect of exogenously added mediators on latently infected rat ganglia is, for the most, as described under "Removal of Peripheral Tissues and Sensory Ganglia and Recovery of HSV" with the exception that the following chemicals were added to the transport and maintenance media. The chemicals, hexamethylene-bis-acetamide, nerve growth factor (mouse, 2.5S), epinephrine (bitartrate salt), and norephinephrine (bitartrate salt) were purchased from Sigma (Sigma Chemical Co., St. Louis, Missouri, USA). These chemicals were used at the following concentrations: hexamethylene-bis-acetamide, 5 mM (concentration recommended by Dr. L.R. Stanberry, Children's Hospital, Cincinatti, Ohio: personal communication); NGF, 100 ng/ml and 50 ng/ml (50 ng/ml based on studies from Wilcox and Johnson [1987] and 100 ng/ml recommended by Dr. J.I. Nagy, University of Manitoba, Winnipeg, Canada: personal communication); epinephrine, 10^{-4}, 10^{-5}, 10^{-6} M; norepinephrine, 10^{-4}, 10^{-5}, 10^{-6} M. The medium containing the identical concentration of compound prepared fresh was replaced weekly. Cultures were screened as previously described and discarded as negative after 21 days.

Control cultures (without chemicals added) were treated in an identical manner.

IX. RESULTS

1. PARALYSIS AND DEATH OF RATS INOCULATED WITH HSV-1

Two to 3 days following unilateral pinna inoculation, 75% of ears appeared erythematous. Erythema persisted for 3-5 days. No other signs of local HSV infection were seen in these rats. In rats inoculated bilaterally in the pinnae (2.0×10^7 $TCID_{50}$) and sacrificed 4 days post-inoculation, 12/12 rats had erythema in at least one ear and 6/12 had cutaneous lesions which consisted of erythema and scab formation. Of rats given 1.0×10^8 $TCID_{50}$ in each pinna, 4/4 had erythema in both ears at day 4 and 4/4 had cutaneous lesions on one or both ears. Lesions were defined as erythema and ulcer/scab formation.

The proportions of animals developing hind limb paralysis and subsequently dying after inoculation at different sites with varying amounts of HSV are presented in Table 1. In 2 of 4 rats inoculated with 2.0×10^6 or 10^7 $TCID_{50}$ in one pinna, paralysis of the ipsilateral hindpaw was noted 6-7 days after inoculation (mean = 6.8; SE = 0.25; range = 6 to 7 days). It resolved in one surviving animal (inoculated with 2.0×10^6 $TCID_{50}$) approximately 2 days later. One to 2 of 4 animals died. Time to death was not different and data were therefore combined. Overall, the mean time to death was 8.3 days (SE = 0.88; range 8 to 10 days) after virus inoculation into the pinna.

Paralysis and death were also observed in rats inoculated in the sciatic nerve. Exposure of the sciatic nerve

TABLE 1. Paralysis and death of rats following peripheral inoculation with HSV-1 in the pinna, sciatic nerve or hind paw.

Site	Inoculum ($TCID_{50}$)	N	Limb Paralysis (%)	Death (%)
Pinna				
Unilateral	2.0×10^6	4	2/4 (50)	1/4 (25)
Unilateral	2.0×10^7	4	2/4 (50)	2/4 (50)
Sciatic				
Unilateral	1.0×10^3	3	1/3 (33)	1/3 (33)
Unilateral	1.0×10^4	3	2/3 (67)	2/3 (67)
Unilateral	1.0×10^5	3	1/3 (33)	3/3 (100)
Unilateral	1.0×10^6	3	3/3 (100)	3/3 (100)
Unilateral	Control	4	0/4 (0)	0/4 (0)
Bilateral	1.0×10^6	6	2/6 (33)	6/6 (100)
Bilateral	Control	6	0/6 (0)	0/6 (0)
Hind Paw				
Unilateral	6.4×10^6	20	5/20 (25)	6/20 (30)
Unilateral	3.2×10^7	8	6/8 (75)	5/8 (63)
Unilateral	Control	8	0/8 (0)	0/8 (0)
Bilateral	3.2×10^6	7	3/7 (43)	2/7 (29)
Bilateral	6.4×10^6	92	47/92 (51)	33/92 (36)
Bilateral	2.0×10^7	8	4/7 (57)	4/7 (57)
Bilateral	Control	4	0/4 (0)	0/4 (0)

and naked eye examination did not reveal any abnormality in the nerve. Rats developed paralysis between 4 and 7 days after inoculation (mean = 5.1; SE = 0.39; range = 4 to 7 days); all such rats died.

In part, due to the small numbers of rats inoculated with each dose of HSV, it was not possible to demonstrate any direct relationship between virus dose and mortality or paralysis. Death occurred on average 5.7 days (SE = 0.37; range = 3 to 8 days) after inoculation in the sciatic nerve. Due to the small number of rats that died following pinna inoculation, we could not compare death rates between rats inoculated in the pinna and sciatic nerve. Three rats survived the infection following sciatic nerve inoculation and were sacrificed 28 days after inoculation.

Following HSV injection under the dorsal skin of the hind paw, no skin lesions were seen. Since time to paralysis and death was not different between the groups, all data were combined. Paralysis was observed an average of 5.7 days (SE = 0.09; range = 4 to 7 days) following inoculation and resolved in survivors by 14 days after inoculation. Deaths occurred on average, 7.7 days (SE = 0.23; range = 5 to 11 days) after inoculation. The mean time to die was longer than after sciatic nerve injection ($p<0.0001$). Death rates between pinna and dorsal hind paw inoculated rats could not be compared statistically due to the small number of rats in the pinna inoculated groups.

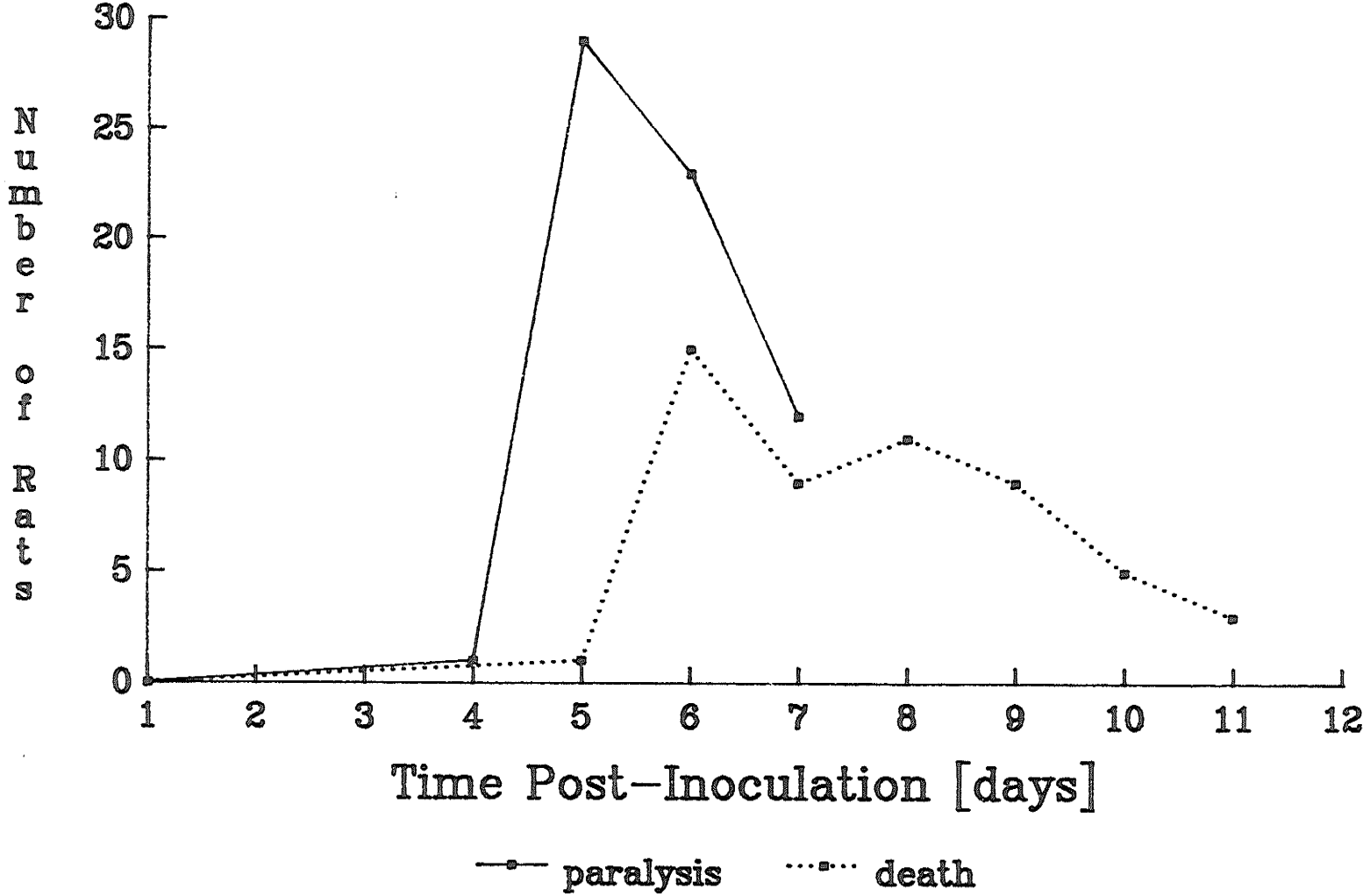
In these experiments, paralysis was associated with a fatal outcome when rats were inoculated in the pinna or sciatic nerve, since only one of 11 rats with paralysis survived (9.1%) (inoculated unilaterally in the pinna). Six rats inoculated in the sciatic nerve died without showing paralysis. Paralysis was not as reliable a predictor of death when rats were inoculated in the dorsal skin of the hind paw since 1/11 (9.1%) ($p<0.001$) rats that were inoculated unilaterally (3.2×10^7) and developed paralysis survived, and 15/54 (27.8%) ($p<0.001$) rats inoculated bilaterally (6.4×10^6) with subsequent paralysis survived. The proportions of paralyzed animals dying after unilateral (10/11) or bilateral (39/54) dorsal hind paw skin inoculation was not less than after unilateral (9/9) or bilateral (2/2) sciatic nerve injection.

Figure 1 shows the association of paralysis and death in rats following virus inoculation in the pinna and dorsal hind paw. Inoculation of HSV-1 into the pinna or under the skin of the dorsal hind paw was occasionally fatal in these rats. Direct injection of virus into the sciatic nerve increased the lethality and shortened the time to death.

2. EFFECTS OF VOLUME

Virus was inoculated in volumes ranging from 5 ul to 100 ul. Experiments were not carried out to test the effects of volume on a fixed amount of virus. Rather, the eventual choice of 20 ul as the standard amount for inoculation was based on technical criteria such as ease of inoculation,

Figure 1.Paralysis and Death of Rats Inoculated With Varying Amounts of HSV-1 in the Pinna and Dorsal Skin of the Hind Paw and Observed for 28 Days.



minimal fluid leakage and adequate apparent diffusion after inoculation. Smaller and larger volumes did not meet all of these criteria.

3. HSV RECOVERY FROM GANGLIA FOLLOWING INOCULATION IN THE PINNA

Results of HSV cultures of pinnae and ipsilateral cervical second (C2), third (C3), and fourth (C4) dorsal root ganglia are shown in Table 2. There was only a change in infection rates with unilateral versus bilateral inoculation at 2.0×10^7 $TCID_{50}$ ($p<0.01$). There were differences in the proportions of C_2, C_3 or C_4 dorsal root ganglia from which HSV was recovered following unilateral or bilateral injection of 2.0×10^7 $TCID_{50}$ of virus. After unilateral injection, 3/8 C2 ganglia were infected compared to 23/24 ($p\leq0.001$) after bilateral inoculation. The proportions of C3 ganglia infected after unilateral and bilateral inoculation were 3/7 and 23/24, respectively ($p\leq0.001$). The proportion of C4 ganglia infected after unilateral and bilateral inoculation were 3/8 and 22/24, respectively ($p\leq0.001$). Following injection of 1.0×10^8 $TCID_{50}$ unilaterally or bilaterally, there was a difference between the proportion of virus recovered from C2 dorsal root ganglia but not from C3 or C4. The proportion of C2 ganglia infected after unilateral and bilateral inoculation were 2/4 and 8/8, respectively ($p\leq0.05$). There was a difference in the proportions of ipsilateral versus contralateral ganglia from which HSV could be recovered when

TABLE 2. Herpes simplex virus cultures 4 days after subcutaneous inoculation of HSV in the pinna.

Inoculum ($TCID_{50}$)	Site	N	HSV Positive/Total Tested			
					Ganglia (%)	
			Rats	Ears	Ipsilateral	Contralateral
2.0×10^6	Unilateral	3	2/3	4/6	5/9 (56)	2/9 (22)
2.0×10^7	Unilateral	4	2/4	4/8	6/12 (50)†	3/11 (27)
1.0×10^8	Unilateral	2	2/2	2/4	6/6 (100)*	0/6 (0)*
2.0×10^7	Bilateral	12	12/12	20/22	68/72 (94)†	
1.0×10^8	Bilateral	4	4/4	8/8	20/24 (83)	
Control	Bilateral	3	0/3	0/2	0/15 (0)	

† $p<.01$.

* $p<.05$.

No other comparisons statistically different.

rats were inoculated with 1.0×10^8 $TCID_{50}$ ($p<0.05$) but not with any other dose. After unilateral injection, there was a nonlinear increase in the proportion of ipsilateral ganglia infected as a function of dose. This was not observed with bilateral injection.

All rats inoculated in the right ear pinna and analyzed at 21 days post-inoculation were infected, but 0% of ear tissue and only 28 to 33% of all ganglia were positive for HSV (Table 3). In rats inoculated with 2×10^7 $TCID_{50}$, there was a difference in the proportions of ipsilateral (4/6) versus contralateral (0/6) ganglia from which HSV could be recovered ($p<0.05$).

Figure 2 illustrates the time for appearance of HSV CPE for ganglia excised 4 or 21 days after pinna inoculation. CPE was observed between 2 (48 hours) and 16 days after the start of explantation. The mean time for detection of CPE in tubes containing ganglia removed 4 days after inoculation was 5.8 days after explantation (SE = 0.28; range = 2 to 16 days) (Figure 2). For ganglia removed 21 days after infection of the pinna, CPE was detected an average of 8.8 days (SE = 1.51; range = 3 to 17 days) after explantation ($p<0.006$).

4. HSV RECOVERY FROM GANGLIA REMOVED 4 DAYS FOLLOWING HSV INOCULATION IN THE SCIATIC NERVE (TABLE 4)

There was no difference between the proportions of fourth lumbar (L4), fifth lumbar (L5), and sixth lumbar (L6) dorsal root ganglia from which HSV could be recovered. There

TABLE 3. Herpes simplex virus cultures 21 days after subcutaneous inoculation in the pinna.

Inoculum ($TCID_{50}$)	Site	N	HSV Positive/Total Tested			
			Rats	Ears	Ganglia (%)	
					Ipsilateral	Contralateral
2.0×10^6	Unilateral	4	3/3	0/6	4/9 (44)	1/9 (11)
2.0×10^7	Unilateral	4	2/2	0/4	4/6 (67)†	0/6 (0)†

† $p<.05$.

No other comparisons statistically different.

TABLE 4. Herpes simplex virus cultures of tissues removed 4 days after bilateral inoculation into the sciatic nerve.

Inoculum ($TCID_{50}$)	N	HSV Positive/Total Tested			
		Rats	Nerve	Ganglia	(%)
1×10^3	3	1/3	3/6	6/18*†	(33)
1×10^4	3	1/3	2/6	6/18¶§	(33)
1×10^5	3	2/2	4/4	12/12*¶	(100)
1×10^6	13	10/11	18/22	56/66†§	(85)
Control	2	0/2	0/4	0/12	(0)

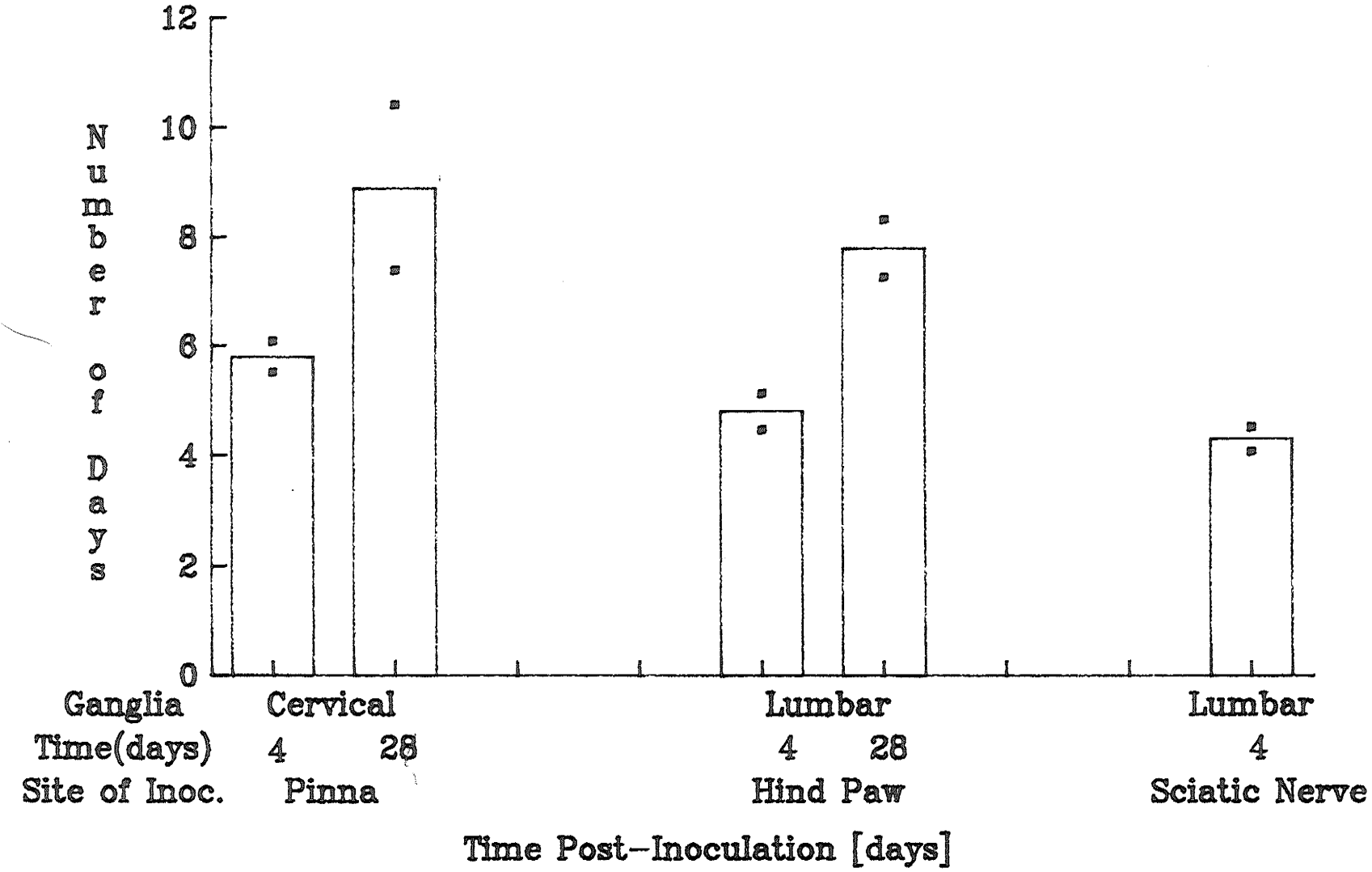
*p<0.01;

†p<0.01;

¶p<0.01;

§p<0.01

Figure 2. Time to Appearance of HSV-CPE in Monolayers on which were Cultivated Ganglia of Rats Inoculated 4 and 28 days previously in the Pinna, Sciatic Nerve or Dorsal Hind Paw. Data represent mean ± SE.



was not a direct relationship between inoculum size and percent positive ganglia.

The mean time to detect CPE was 4.4 days after explantation (SE = 0.22; range = 2 to 13 days). This was less than for detection of CPE in cervical ganglia following pinna injection, studied by identical methods ($p<0.0001$) (Figure 2). One interpretation of this difference is that direct nerve inoculation results in a higher concentration of virus in ganglia.

5. HSV RECOVERY FROM GANGLIA FOLLOWING INOCULATION OF RATS SUBCUTANEOUSLY IN THE DORSUM OF THE HIND PAW

The proportions of ipsilateral ganglia from which HSV was recovered were greater than the proportions of contralateral ganglia ($p<0.05$) infected when rats were inoculated unilaterally with 3.2×10^6 $TCID_{50}$ but not with any other amount tested (Table 5). There were no differences between the proportions of rats with contralateral ganglia containing HSV, as a function of dose. The proportion of ipsilateral ganglia positive following inoculation of 3.2×10^6 $TCID_{50}$'s was less than following inoculation of 6.4×10^6 ($p<0.05$) and different between inoculation of 6.4×10^6 and 2.0×10^7 $TCID_{50}$'s ($p<0.05$).

After unilateral and bilateral inoculation of dorsal hind paw skin, CPE characteristic of HSV infection was observed in monolayers on which ganglia were cultured, an average of 4.8 days after explantation (SE = 0.33; range = 2

TABLE 5. Herpes simplex virus cultures 4 days after subcutaneous inoculation in the dorsum of the rat hind paw.

Inoculum ($TCID_{50}$)	Site	N	HSV Positive/Total Tested			
			Rats	Skin	Ganglia (%)	
					Ipsilateral	Contralateral
3.2×10^6	Unilateral	8	8/8	5/16	15/24 (63)†,¶	7/23 (30)¶
6.4×10^6	Unilateral	7	7/7	5/14	19/21 (91)†,*	14/21 (67)
2.0×10^7	Unilateral	8	6/8	5/16	16/24 (67)*	11/24 (46)
2.0×10^7	Bilateral	7	7/7	6/14	34/42 (76)	
Control	Bilateral	2	0/2	0/4	0/2 (0)	

Ipsilateral ganglia: †$p<0.05$;

*$p<0.05$.

Ipsilateral vs contralateral ganglia: ¶$p<0.05$.

No other comparisons statistically different.

to 19 days). This was not different than the time to detect CPE in tubes containing cervical or lumbar ganglia following pinna or sciatic nerve inoculation, respectively ($p>0.05$; $p>0.05$). There was a difference in the proportions of L4, L5, L6 dorsal root ganglia from which HSV could be recovered for rats inoculated unilaterally with 3.2×10^6 $TCID_{50}$. Of 8 L4 ganglia, 4 were positive compared to 8/8 L5 ganglia ($p \leq 0.05$). Of 8 L5 ganglia, 8 were positive compared to 3/8 L6 ganglia ($p \leq 0.05$). There were no differences in proportions within any other group (Table 5). Hence, at inoculates less than 6.4×10^6 $TCID_{50}$, ganglia were not uniformly infected.

Seventy-five to 100% of rats inoculated unilaterally with HSV and killed 28 days post-inoculation were infected whereas no skin samples, 57 to 78% of ipsilateral ganglia, and 10% of contralateral ganglia were infected (Table 6). There was a difference in the proportion of ipsilateral compared to contralateral ganglia from which HSV was recovered following unilateral injection ($p<0.05$ for both inocula).

Culture of tissues from rats inoculated bilaterally with virus demonstrated that 100% of these animals were infected whereas no skin samples and 43 to 72% of ipsilateral ganglia were infected (Table 6). There was no difference in the proportion of rats infected after inoculation with 6.4×10^6 or 3.2×10^7 $TCID_{50}$ HSV unilaterally or bilaterally. Also,

TABLE 6. Herpes simplex virus cultures 28 days after subcutaneous inoculation in the dorsum of the rat hind paw.

Inoculum ($TCID_{50}$)	Site	N	HSV Positive/Total Tested			
					Ganglia (%)	
			Rats	Skin	Ipsilateral	Contralateral
6.4×10^6	Unilateral	8	6/8	0/16	13/23 (57)†	2/21 (10)†
3.2×10^7	Unilateral	8	3/3	0/4	7/9 (78)*	0/9 (0)*
Control	Unilateral	8	0/8	0/16	0/24 (0)	0/24 (0)
3.2×10^6	Bilateral	8	5/5	0/10	13/30 (43)	
2.0×10^7	Bilateral	8	3/3	0/6	13/18 (72)	
Control	Bilateral	10	0/7	0/14	0/42 (0)	

† $p<0.05$

* $p<0.05$

No other comparisons statistically different.

there were no differences in the proportion of L4, L5, L6 dorsal root ganglia from which HSV could be recovered at each inoculum.

Twenty-eight days after unilateral and bilateral dorsal skin of rear paw inoculation, virus could be recovered, an average of 7.8 days after explantation (SE = 0.53; range = 4 to 19 days) ($p<0.0001$ when comparing time to CPE at 4 vs 28 days post-inoculation). This was not different than the time to CPE from the ganglia of rats inoculated in the pinna and analyzed at 21 days ($p>0.05$) (Figure 2). Regardless of the site of inoculation or inoculum size, explant culture of ganglia removed 21 to 28 days after inoculation resulted in the appearance of HSV CPE in the monolayer after similar intervals.

6. EFFECTS OF LIGATION AND RESECTION OF THE SCIATIC NERVE ON LUMBAR GANGLION INFECTION FOLLOWING SUBCUTANEOUS INJECTION OF HSV IN THE HIND PAW

Sciatic nerve transection and ligation reduced the percentage of infected rats from 50 to 100% to 0 to 50% ($p<0.03$) (Table 7). The proportion of ipsilateral ganglia infected after inoculation of 3.2 or 6.4 x 10^6 $TCID_{50}$ (0 and 15 vs 30 or 100% respectively) was greater in the sham operated controls than in rats with transected sciatic nerve. These data indicate that the principle route of transmission of HSV to dorsal root ganglia was via intraaxonal transport.

TABLE 7. Recovery of herpes simplex virus 4 days after subcutaneous inoculation in the dorsum of the rat hind paw 2 days after ligation and transection of the ipsilateral sciatic nerve.

Inoculum ($TCID_{50}$)	Site	N	HSV Positive/Total Tested			
					Ganglia (%)	
			Rats	Skin	Ipsilateral	Contralateral
3.2×10^6	Unilateral	4	0/4	1/8	0/12 (0)†	0/12 (0)
3.2×10^6*	Unilateral	2	1/2	1/4	2/6 (33)†	0/6 (0)
6.4×10^6	Unilateral	7	1/7	1/4	3/20 (15)¶	0/19 (0)
6.4×10^6*	Unilateral	2	2/2	1/4	6/6 (100)¶	4/5 (80)
3.2×10^7	Unilateral	4	2/4	2/8	6/12 (50)	5/11 (46)
3.2×10^7*	Unilateral	1	1/1	1/2	3/3 (100)	0/3 (0)

* Controls: Sham operation including sciatic nerve mobilization but no sciatic ligation or section prior to ipsilateral subcutaneous inoculation of HSV in the dorsum of the hind paw.

† $p<0.05$.

¶ $p<0.01$.

No other comparisons statistically different.

7. CHARACTERISTICS OF THE RAT MODEL SELECTED FOR DEFINITIVE STUDIES

Based on the aforementioned data, for our model, we selected inoculation of 6.4×10^6 $TCID_{50}$ of HSV-1 in 20 ul subcutaneously in the dorsal hind paw. To study the time course of the clinical, virological and serological characteristics of this model, rats were sacrificed on days 0, 2, 4, 7, 14, 21 and 28. Dorsal hind paw skin, sciatic nerve, lumbar dorsal root ganglia, spinal cord and brain were tested for virus and HSV-1 antigen. Blood was also collected to determine serum antibody.

A. Serum Antibody

HSV antibody was first detected in the serum of rats 2 days after inoculation in 1 of 8 rats and in 2 of 5 rats by day 4 (Figure 3). The titer increased until day 7 until after which it remained unchanged. The proportion of rats with detectable antibody 7, 14, 21 and 28 days after inoculation were 5/5, 4/4, 4/4 and 4/4. This response is consistent with what is expected following primary infection with HSV.

B. Virus Isolation

To detect productive HSV infection in rat tissues, they were homogenized and the supernatant cultured on HUFF monolayers (as described in Materials and Methods) (Figure 4). HSV was not recovered from contralateral hind paw skin, sciatic nerve, and dorsal root ganglia nor from lumbar spinal cord segments or brain (data not shown). Virus was recovered

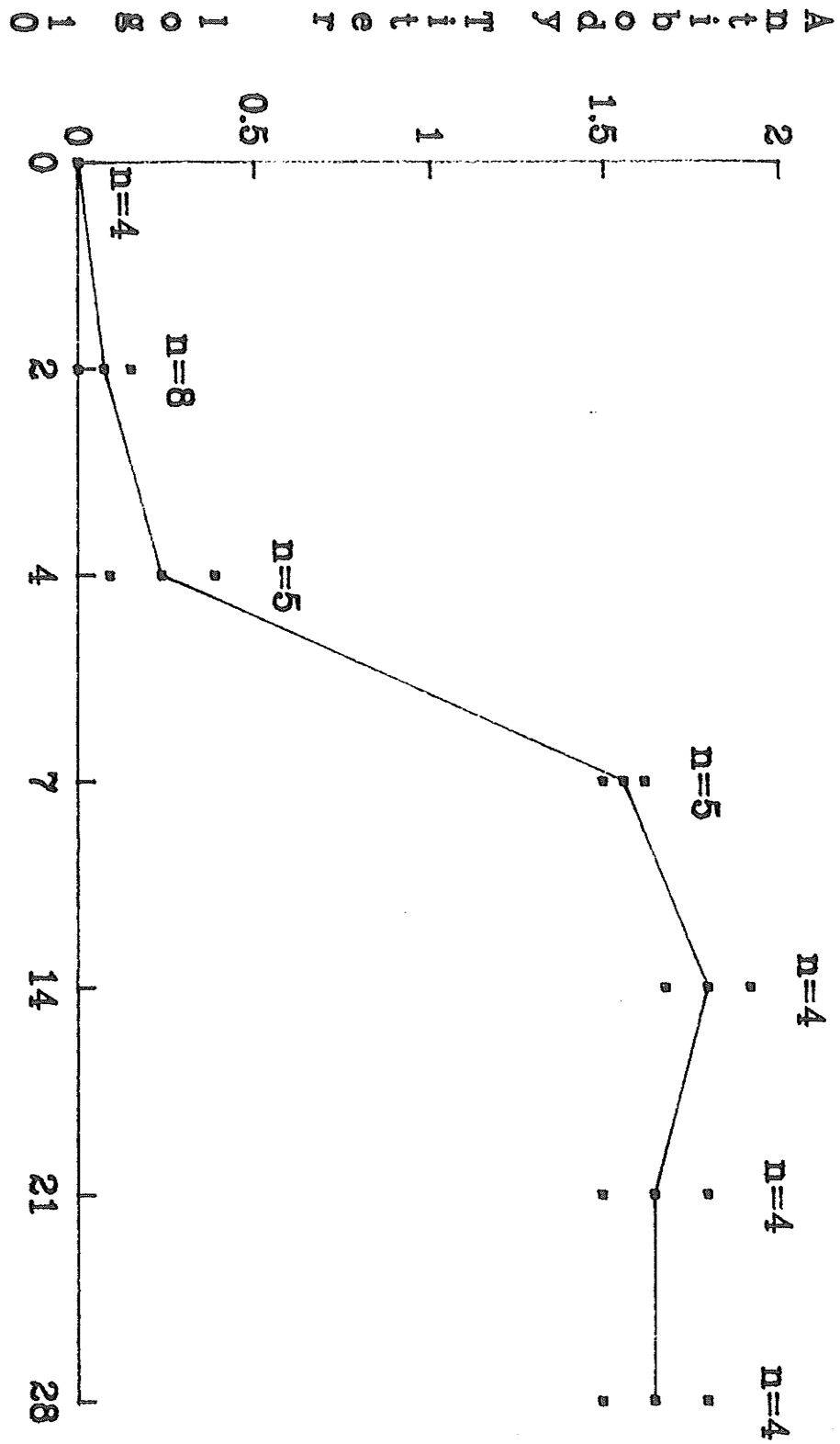
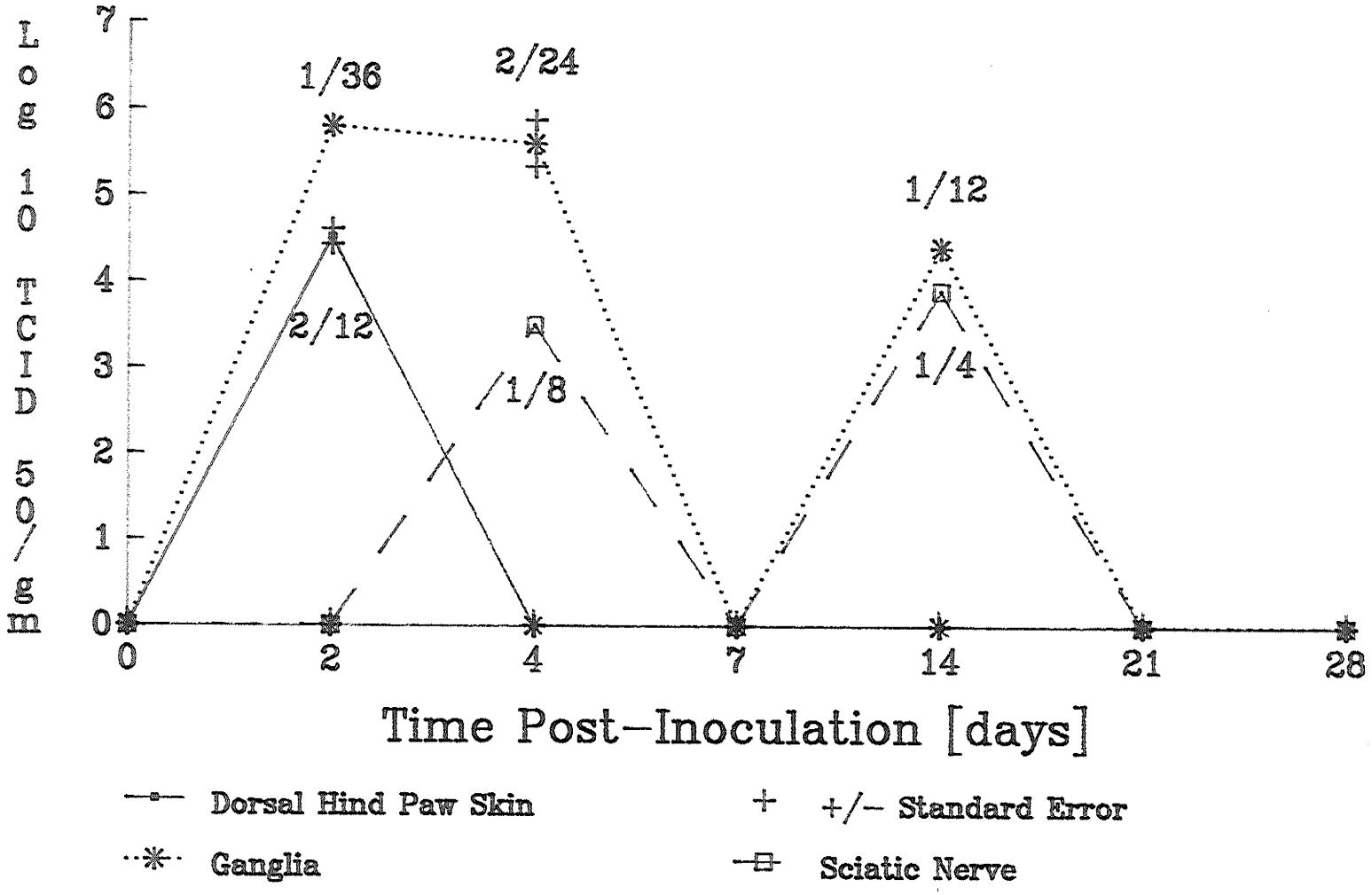


Figure 3. HSV Antibody Response in Serum of Rats to Primary Infection. Data represent mean ± SE.

Figure 4. Concentrations of HSV in Ipsilateral Homogenized Rat Tissues Following Inoculation of HSV-1 in the Dorsum of the Hind Paw. Data represent the mean of the positive samples ± SE.



from ipsilateral hind paw skin, sciatic nerve and dorsal root ganglia. In dorsal hind paw skin, virus was first detected on day 2, reaching a maximum concentration on day 4 and not subsequently detected thereafter. In dorsal root ganglia, virus was first detected on day 2 at a concentration that did not change until day 7 at which time it was no longer detected. The mean concentration at 14 days represented virus in 1 of 12 ganglia tested.

After 14 days, there was no productive infection and this supported our conclusion that, after 21 days, virus exists in a latent form.

C. Immunohistology of Acute Infection

Immunohistological staining of rat lumbar ganglia during the primary infection showed that viral antigen could not be detected one day post-inoculation (PI). By 2 days PI, nerve fibers were positive for virus antigen in at least 1 sampled ganglion (Table 8, Figure 5). Between 58 hours PI and 4 days PI, viral antigen was detected in numerous small type neurons (Figures 6, 7, 8, respectively). Lysis of neuronal cells was evident by day 4 with cellular debris identifiable at this time (Figure 8). By day 5 PI, only 1 ganglion stained positively and at day 6 and 9, no HSV antigen was detectable (Figures 9, 10 and 11, respectively). Ganglia from control animals that were not inoculated with virus or that were not reacted with the primary antibody were all negative (data not shown).

TABLE 8. Immunohistologic staining of lumbar (L) L4, L5 and L6 ganglia for HSV antigen following subcutaneous inoculation of dorsal hind paw skin with HSV-1.

Days Post-Inoculation	N*	Positive†/ Total #	Description
1	2	0/2	No evidence of virus
2	3	1/3	Nerve fibres positive
2.4	2	2/2	1-2 cells; large and small?; destruction
3	5	4/5	1-20 cells; mainly small; fibers positive
3 (control)	1	0/1	No evidence of virus
4	6	4/6	1-20 cells; mainly small; destruction
4 (control)	2	0/2	No evidence of virus
5	4	1/4	1 cell; poor sections
6	2	0/2	No evidence of virus
9	2	0/2	No evidence of virus

Rats inoculated with 6.4×10^6 $TCID_{50}$.

* N indicates the number of rats.

† Positive indicates rats with at least 1 ganglion positive.

FIGURE 5. Immunoperoxidase staining of rat sciatic nerve fibers 2 days following inoculation of 6.4×10^6 $TCID_{50}$ HSV-1 subcutaneously in the ipsilateral hind paw. The arrow points to an area which stains positive for HSV antigens. Magnification = 400x.

FIGURE 6. Immunoperoxidase staining of rat lumbar ganglion 58 hours following inoculation of 6.4 x 10^6 $TCID_{50}$ HSV-1 subcutaneously in the ipsilateral dorsal hind paw. The small arrow (⬅) points to a small type B unmyelinated neuron which stains positive for HSV antigen. The large arrow ⟷ points to an unstained large type myelinated neuron. Magnification = 400x.

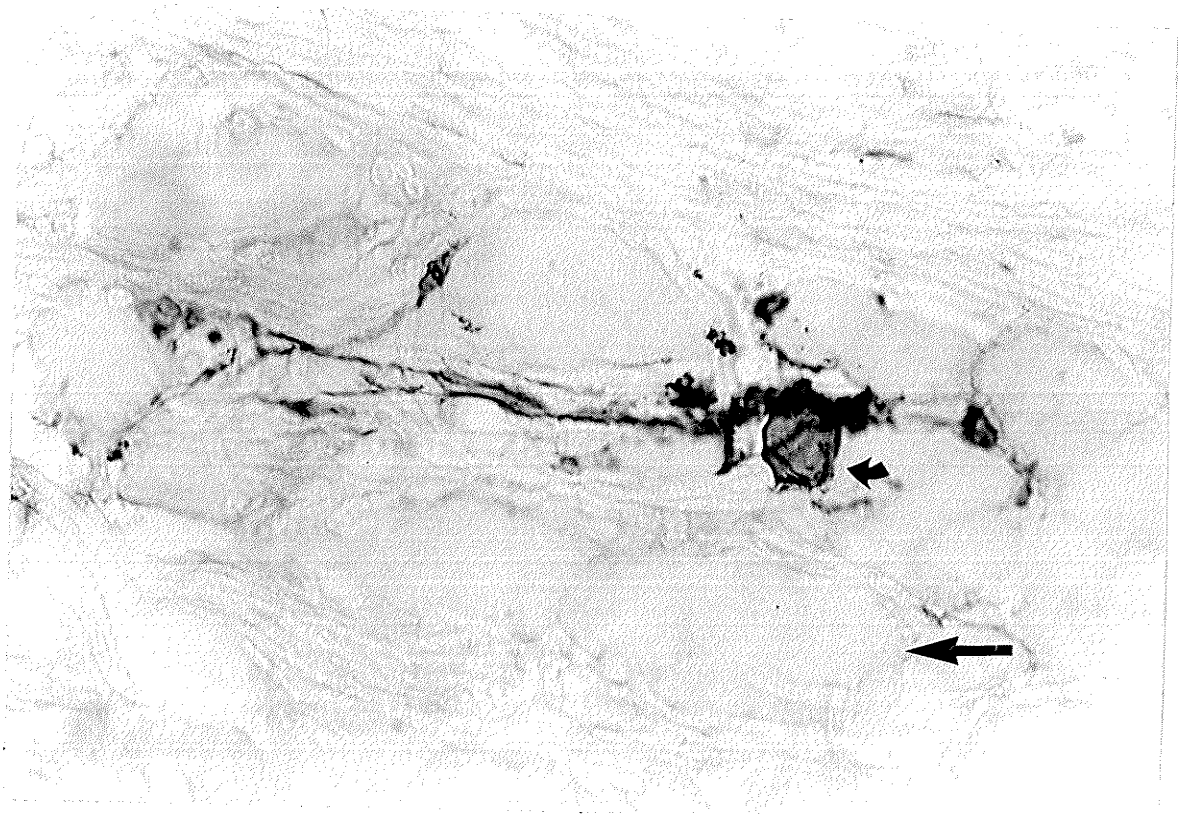


FIGURE 7. Immunoperoxidase staining of rat lumbar ganglion 3 days following inoculation of 6.4×10^6 $TCID_{50}$ HSV-1 subcutaneously in the ipsilateral dorsal hind paw. The small arrow ( ) points to a small type B unmyelinated neuron which stains positive for HSV antigen. The large arrow points to an unstained large type myelinated neuron. Magnification = 400x.

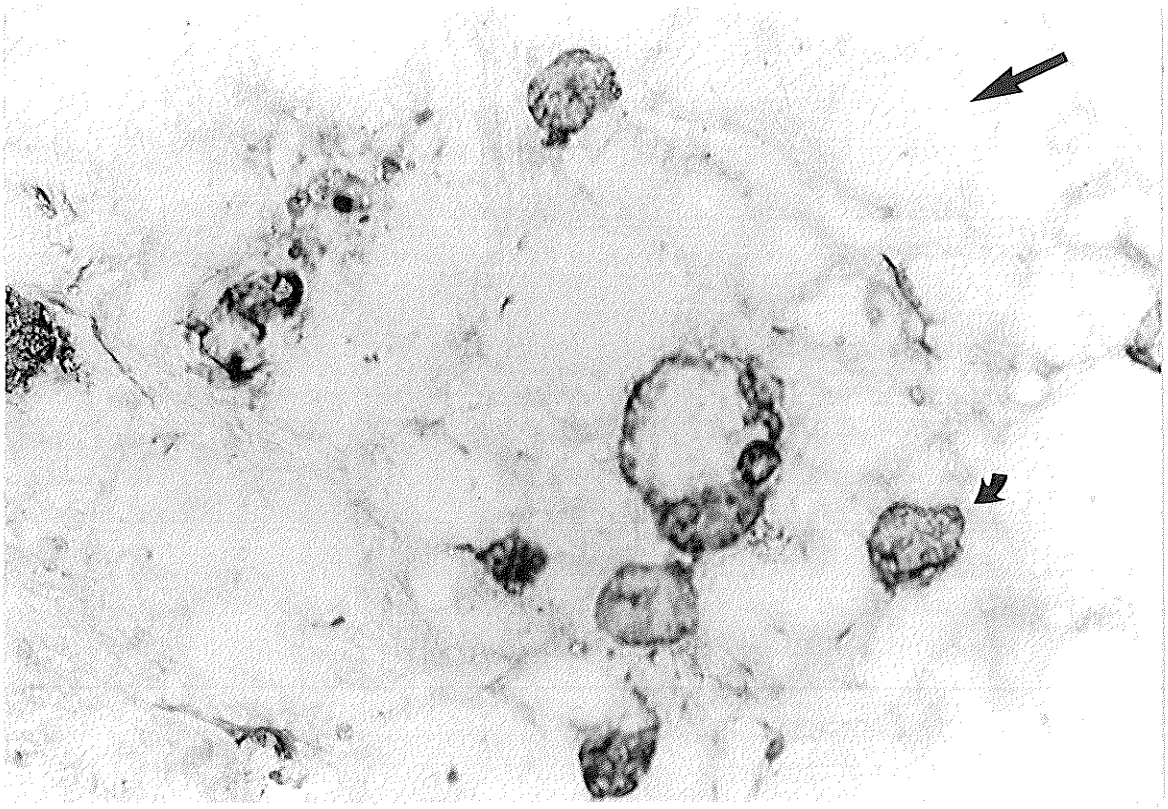


FIGURE 8. Immunoperoxidase staining of rat lumbar ganglion 4 days following inoculation of 6.4×10^6 $TCID_{50}$ HSV-1 subcutaneously in the ipsilateral dorsal hind paw. The small arrow ( ) points to a small type B unmyelinated neuron which stains positive for HSV antigen. The large arrow points to an unstained large type myelinated neuron. Stained cellular debris is noted around the small type neuron. Magnification = 400x.

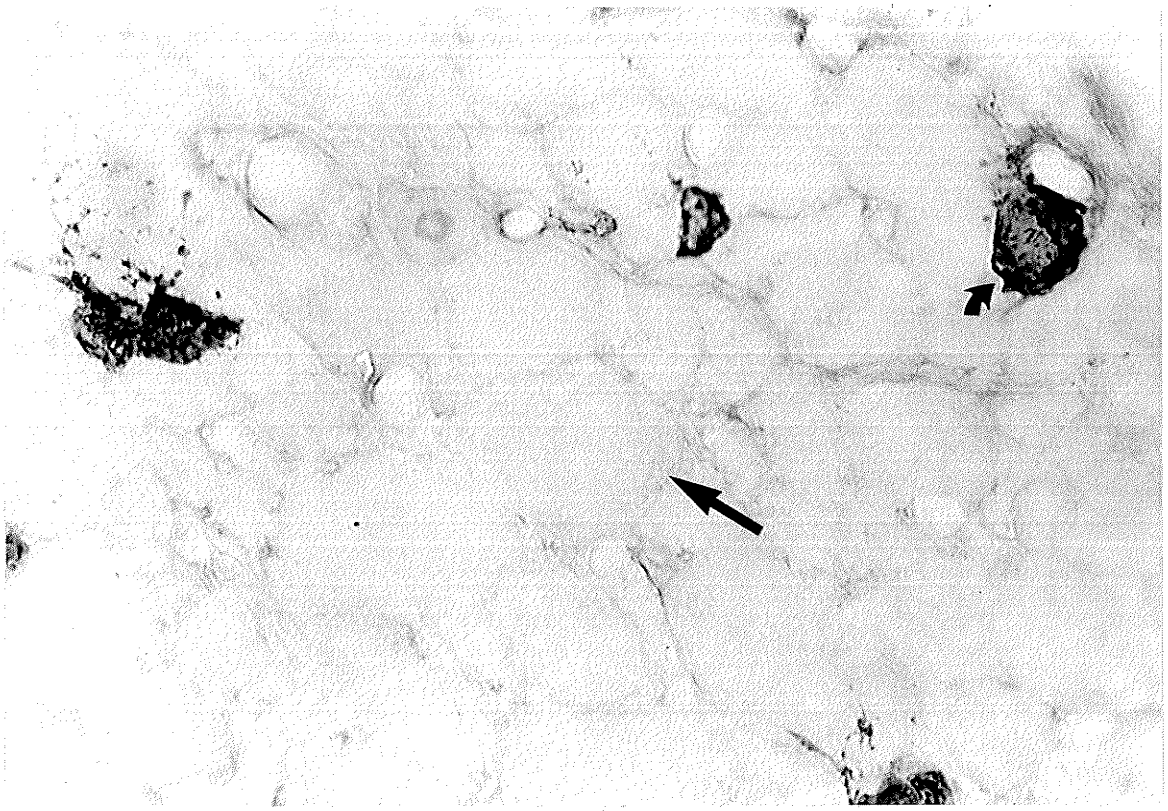


FIGURE 9. Immunoperoxidase staining of rat lumbar ganglion 5 days following inoculation of 6.4×10^6 $TCID_{50}$ HSV-1 subcutaneously in the ipsilateral dorsal hind paw. The arrow points to a small type B unmyelinated neuron which stains positive for HSV antigen. Magnification = 400x.

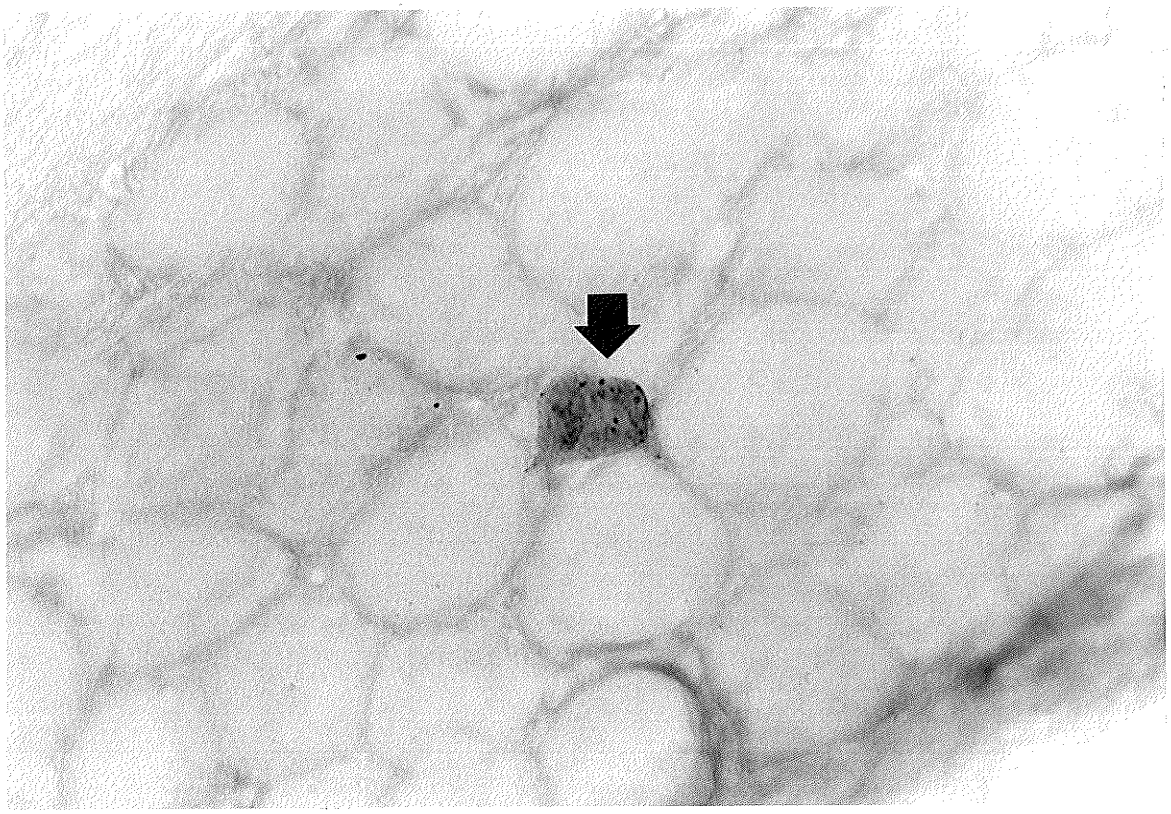


FIGURE 10. Immunoperoxidase staining of rat lumbar ganglion 6 days following inoculation of 6.4×10^6 $TCID_{50}$ HSV-1 subcutaneously in the ipsilateral dorsal hind paw. No evidence of HSV antigen was detected. Magnification = 400x.

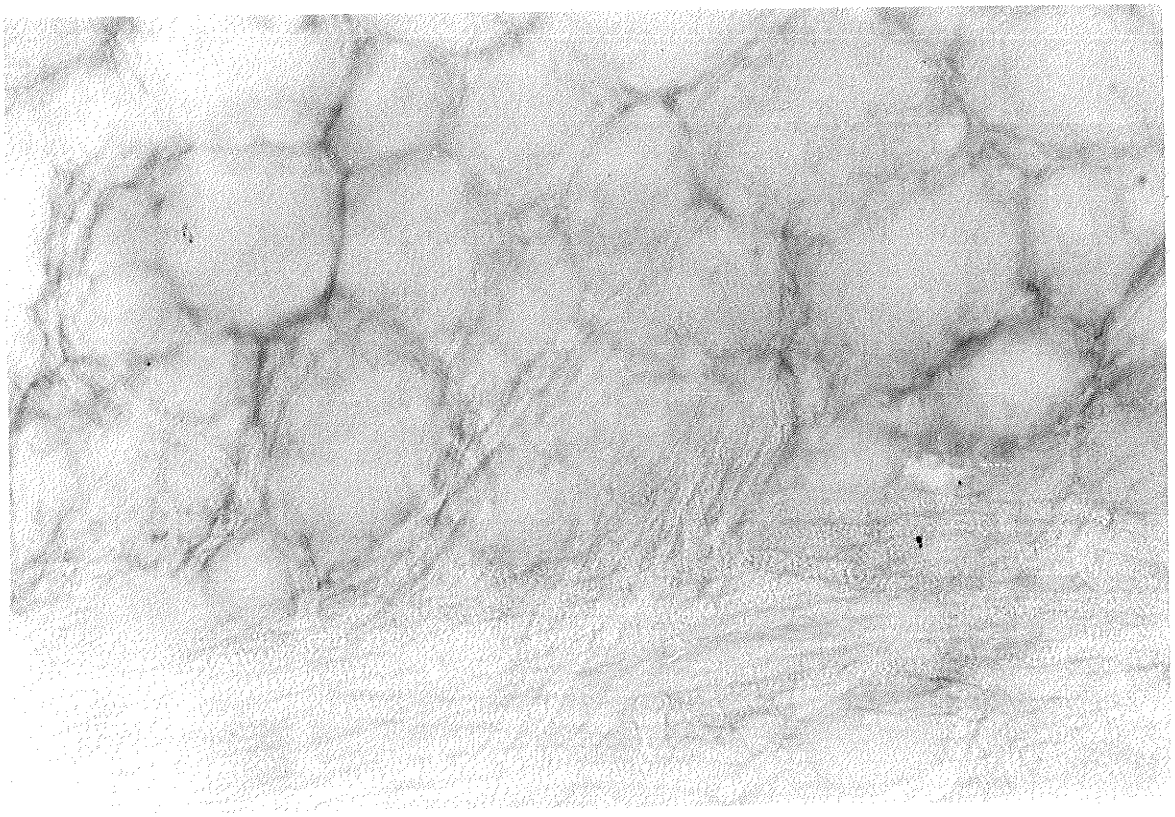
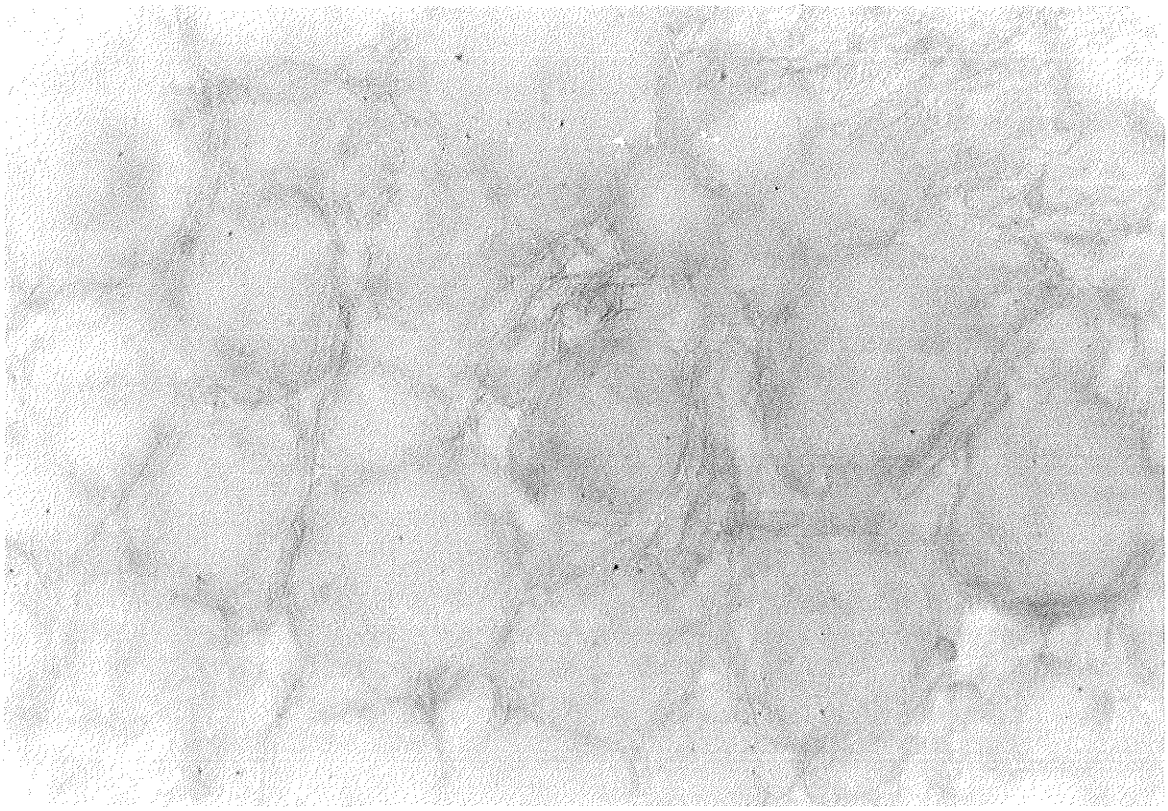


FIGURE 11. Immunoperoxidase staining of rat lumbar ganglion 9 days following inoculation of 6.4×10^6 $TCID_{50}$ HSV-1 subcutaneously in the ipsilateral hind paw skin. No cells were positive for HSV. Magnification = 400x.



D. Immunohistology of Rat Lumbar Ganglia 28 to 45 Days After HSV-1 Injection Subcutaneously in the Dorsum of the Hind Paw

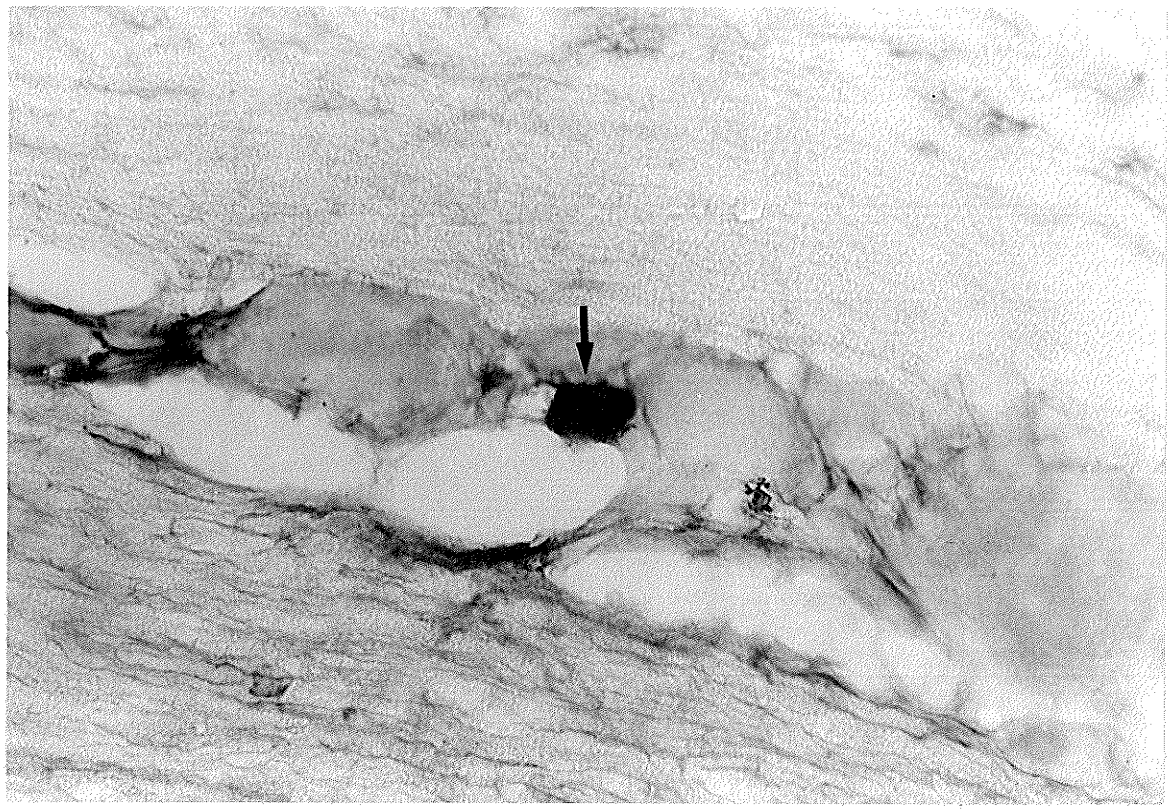
Immunoperoxidase staining of 12 lumbar ganglia 2 days after manipulation and transection of the ipsilateral sciatic nerve to activate ganglionic infection 28 days following injection of HSV-1 in the dorsal hind paw revealed HSV antigen in 1 ganglion (Figure 12). The staining was detected in a small type neuronal cell body. It was not possible to conclude that this manipulation had activated HSV latent infection in dorsal root ganglia.

E. Immunohistology of In Vitro Reactivation

To determine whether the cell types staining for HSV antigen during the primary infection were the same or different than the cell types in which HSV activation occurred *in vitro*, ganglia from rats infected 28 days previously were cocultivated and stained for antigen after CPE was detected.

Fifty-four ganglia from 8 rats were removed 28 days after injection of 6.4×10^6 $TCID_{50}$ subcutaneously in the dorsal hind paws and cocultured with HUFF cells. Of the 12 positive ganglia that were analyzed immunohistologically, the mean time to HSV CPE by cocultivation of 9/12 of these ganglia was 6.6 ± 0.40 (range 5 to 9 days) (times not accurately recorded for 3/12). At various times after the identification of HSV-like CPE, ganglia were removed from the monolayers, fixed in formalin and stained as described. Regardless of the time at which the ganglia were removed from the

FIGURE 12. Immunoperoxidase staining of rat lumbar ganglion 40 days following injection of 6.4×10^6 $TCID_{50}$ HSV-1 subcutaneously in the ipsilateral dorsum of the hind paw. Rats had sciatic nerve neurectomy 48 hours prior to sacrifice. The arrow (⟹) points to a small type B unmyelinated neuron which stains positive for HSV antigen. Magnification = 400x.



infected monolayers, primarily small type neuronal cells stained positive for virus antigen (Figures 13, 14). Six ganglia from monolayers not showing HSV-like CPE were negative for HSV antigens. It was concluded that antigen was detectable in this one cell type but we could not exclude that some apparent large cells staining for antigen were, in fact, large cells rather than swollen infected small cells.

F. Recovery of HSV from Lumbar Ganglia Following Sciatic Nerve Transection

Rats injected 36 days previously subcutaneously in 1 dorsal hind paw had their sciatic nerves exposed and in 1 group, transected (Table 9). There were no difference between the number of rats or the number of ganglia positive for HSV between the groups. The times for HSV CPE to appear in the experimental and control groups were 9.0 and 11.0 days, respectively. No virus was recovered from skin samples. It was concluded that sciatic nerve transection did not reduce the time for HSV CPE to appear in coculture.

G. Effects of Topical Capsaicin on Recovery of HSV from Lumbar Ganglia of Rats

Table 10 summarizes data from rats treated with topical capsaicin (as described in the footnote) and controls. Capsaicin treatment had no effect on the proportions of rats, skin and ganglia from which HSV could be recovered. The interval for CPE to appear in each group were 5.1 ± 0.32 for rats treated bilaterally, 6.1 ± 1.7 for rats treated unilaterally, and 5.7 ± 1.3 for untreated rats. These times

TABLE 9. Effects of sectioning the sciatic nerve 36 days following inoculation of 6.4×10^6 $TCID_{50}$ HSV-1 subcutaneously in the ipsilateral dorsal hind paw skin, on recovery of HSV by cocultivation.

Treatment	Time After Nerve Sectioning or Not	N	HSV Positive/Total Tested		
			Rats	Skin	Ganglia (%)
Nerve transected	48 hours	5	1/5	0/10	1/30 (3)
Nerve not transected	48 hours	4	1/4	0/8	1/24 (4)

No comparisons statistically different.

TABLE 10. Effects of topical capsaicin on recovery of HSV from tissues of rats sacrificed 4 days after inoculation with HSV-1 subcutaneously in the dorsum of the hind paw.

Inoculum/Foot ($TCID_{50}$)	Site	HSV Positive/Total Tested			
		N	Rats	Skin	Ganglia (%)
6.4×10^6 (treated)*	LRDS†	4	3/4	6/8	15/24 (62.5)
6.4×10^6 (treated)*	RDS†	3	2/3	2/6	7/18 (38.9)
6.4×10^6 (untreated)	LRDS	2	1/2	2/4	6/12 (50.0)

* Rats were pretreated topically with 30 ul of 1% capsaicin in 100% alcohol applied to the skin of the dorsal hind paw twice daily for 2 days preceeding virus inoculation.

† LRDS = Left and right dorsal skin of hind paw; RDS = right dorsal skin of hind paw.

FIGURE 13. Immunoperoxidase staining of rat lumbar ganglion for HSV-1 antigen 1 day following observation of HSV-CPE in HUFF monolayers. The small arrow (↙) points to a small type B unmyelinated neuron that stains positive for HSV antigen. The large arrow ⟷ points to an unstained large type myelinated neuron. Magnification = 400x.

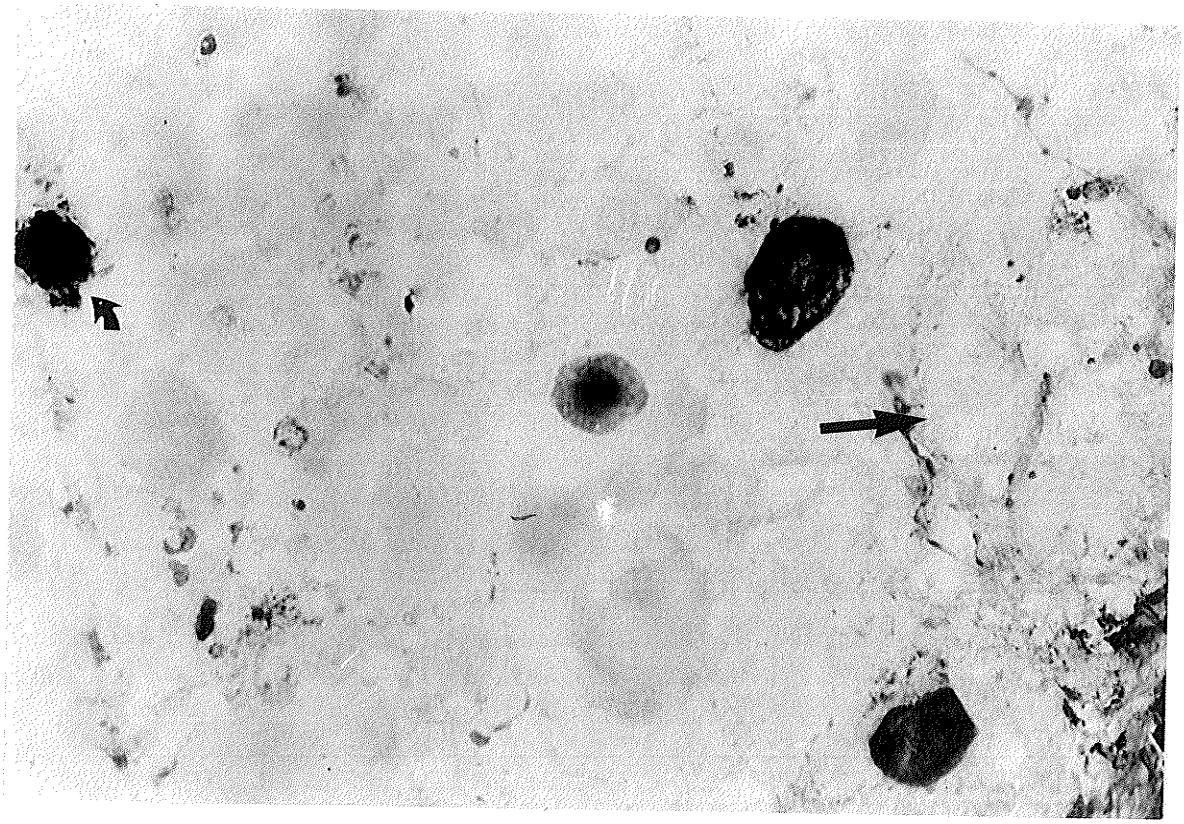
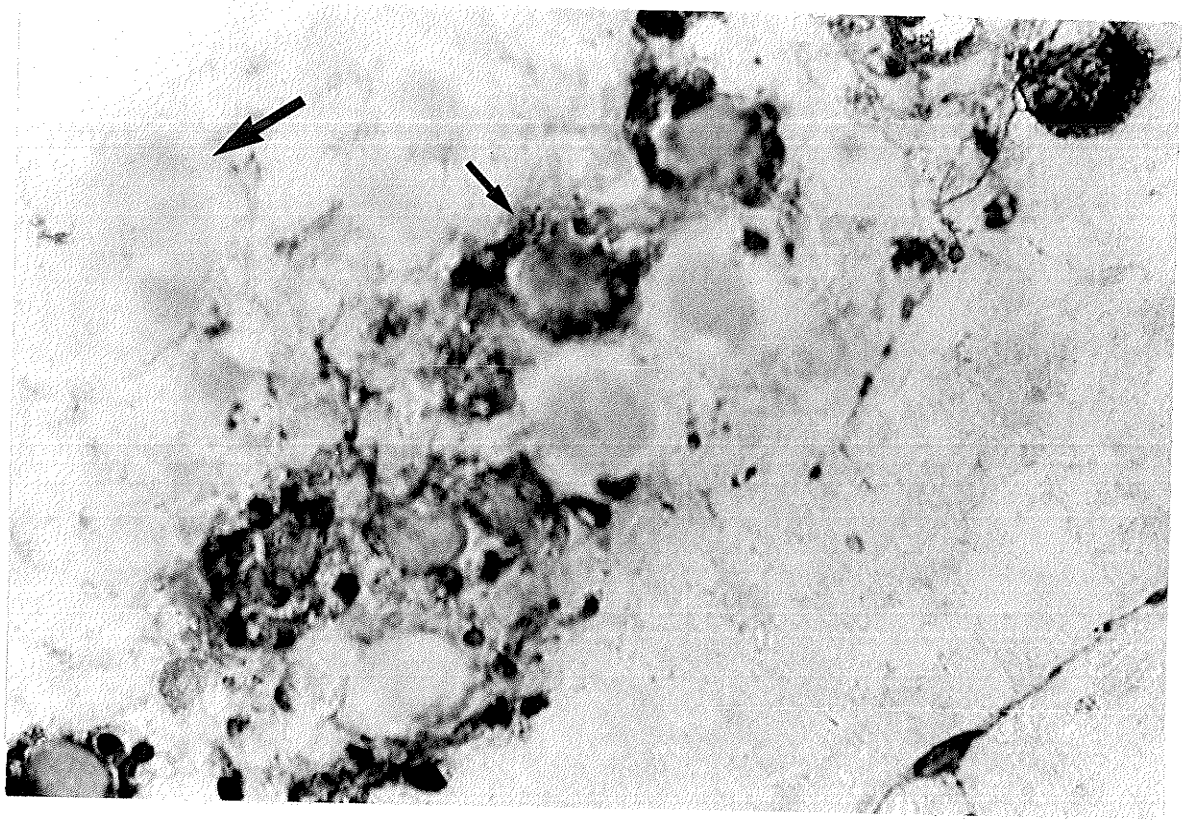


FIGURE 14. Immunoperoxidase staining of rat lumbar ganglion for HSV-1 antigen 2 days following observation of HSV-CPE in HUFF monolayers. The small arrow (⟶) points to a small type B unmyelinated neuron which stains positive for HSV antigen. The large arrow ( ) points to an unstained large type myelinated neuron. Magnification = 400x.



were not statistically different. It was concluded that topical capsaicin did not prevent ganglionic infection, suggesting that topical application did not affect intra-axonal migration of HSV.

H. Effects of Direct Application of Capsaicin to the Sciatic Nerve and Saphenous Nerve Section on Recovery of HSV from Lumbar Ganglia of Rats

In view of the lack of an effect of topical capsaicin of intraaxonal migration of HSV, we evaluated the effect of direct application of capsaicin to the nerve fibres.

Direct application of capsaicin to the sciatic nerve was not more effective than control in preventing lumbar dorsal root ganglia infection in rats with the saphenous nerve transected to prevent intraaxonal migration of HSV from the hind paw to ganglia via that nerve. Direct application of capsaicin without saphenous nerve transection was less effective at preventing lumbar dorsal root ganglia infection than was direct application of capsaicin with saphenous nerve transection (Table 11). It was concluded that saphenous nerve transection was more effective than capsaicin in preventing HSV migration from the hind paw to the dorsal root ganglia.

Virus was only isolated from ipsilateral dorsal hind paw skin specimens. The proportion of positive cultures was inversely related to the proportion of ganglia that contained HSV. Thus, rats whose saphenous nerve had been transected had lower proportions of ganglia positive for HSV than rats

TABLE 11. Recovery of HSV from rat tissues 4 days following direct application of capsaicin to the sciatic nerve with the saphenous nerve transected.

Inoculum/Foot ($TCID_{50}$)	Site●	HSV Positive/Total Tested N	Rats	Skin	Ganglia (%)
6.4×10^6*	LDS	7	2/7	6/14§	2/40§ (5)
6.4×10^6 (control)	LDS	4	3/4	3/8†	0/22† (0)
6.4×10^6**	LDS	5	2/5	0/10†§	7/30†§(23)

● Left dorsal skin (unilateral).

* Rats were pretreated 4 days prior to HSV inoculation by application of either 1.5% capsaicin in Tween 80-sterile saline-alcohol applied to the left sciatic nerve with the ipsilateral saphenous nerve transected, or Tween 80-sterile saline-alcohol as control. Inoculation was immediately at the end of last capsaicin treatment and rats sacrificed 4 days later.

** Rats were pretreated with capsaicin as described without transection of the saphenous nerve.

§ $p<0.05$.

† $p<0.05$.

whose saphenous nerve had not been transected. The converse was true with respect to the probability of demonstrating HSV in skin samples.

It was concluded that direct application of capsaicin to the sciatic nerve did not appear to prevent HSV migration from the rear hind paw to the contiguous dorsal root ganglia.

The times required for CPE to appear in the HUFF monolayers were 8 and 14 days for ganglia from rats treated with capsaicin and saphenous nerve transection. The mean time for CPE to appear in monolayers on which ganglia from rats treated with capsaicin alone was 5.3 ± 1.2 days. All these times are not inconsistent with data from other rats whose tissues were cultured 4 days after HSV injection.

I. Effects of Monensin on Recovery of HSV from Lumbar Dorsal Root Ganglia of Rats Infected 4 Days Previously

To test the hypothesis that large type myelinated neurons may be infected with HSV in amounts that were too small to detect with immunoperoxidase staining as previously described, rats were treated with monensin, a compound that inhibits packaging of protein. It was postulated that such an effect on HSV packaging would result in the intracellular accummulation of virus to levels that would be detectable by our staining protocol.

Three rats were given either 2 or 3 injections with monensin as described in Materials and Methods. One rat from each group died. Immunohistological examination of the 4th and 5th lumbar dorsal root ganglia showed that the majority

of cells containing HSV antigen were small type unmyelinated neurons (data not shown); in some sections, evidence of cell destruction (debris) was found (data not shown). Occasionally, large cells were observed to contain HSV antigen but the numbers of cells were not different than in studies where monensin was not used.

J. Recovery of HSV from Tissues of Rats Sacrificed 31 and 52 Days Following Injection of Virus

Rats infected for either 31 or 52 days were examined to determine the percentage of rats, skin and ganglia positive for HSV (Table 12). HSV was not recovered from any skin samples. There was no difference between the proportion of rats positive from each group. Virus was recovered from both ipsilateral and contralateral rat ganglia within each group. There were no differences either within or between groups in regards to the number of ipsilateral, contralateral or total ganglia positive, and no difference in the proportion of L4, L5 or L6 ganglia positive for HSV. The interval after explantation for CPE to appear was not different: 6.8 ± 0.93 days at 31 days PI compared to 8.0 ± 0.78 days ($p>0.05$) at 52 days PI. It was concluded that rats harboured HSV in the dorsal root ganglia up to 52 days after inoculation and the proportion of ganglia harbouring virus were within the range previously observed in rats inoculated with the same amount of virus at the same site and sacrificed 21 days (Table 3) or 28 days (Table 6) after inoculation. The interval between initiation of explantation and the appearance of CPE was not

TABLE 12. Recovery of HSV from tissues of rats infected with HSV-1 31 or 52 days previously by subcutaneous injection in the dorsal skin of the right hind paw.

Inoculum ($TCID_{50}$)	Site	N	HSV Positive/Total Tested			
					Ganglia (%)	
			Rats	Skin	Ipsilateral	Contralateral
6.4×10^6 (31 days PI)*	RDS**	6	2/6	0/12	6/18 (33)	5/18 (20)
6.4×10^6 (52 days PI)	RDS	5	3/5	0/10	6/15 (40)	3/15 (20)

* PI = Post-infection.

** RDS = Right dorsal hind paw skin.

different than that previously observed for rats sacrificed 21 or 28 days after inoculation (vide supra).

K. Recovery of HSV from Rat Ganglia Removed at 4 or 29 Days PI and Cultured in the Presence of Hexamethylene-Bis-Acetamide

Hexamethylene-bis-acetamide has previously been postulated to be a demethylating agent and thereby may modify latency if methylation is important for that phenomenon. We therefore treated ganglia from rats 4 and 29 days following inoculation of HSV-1 in the dorsal skin of the hind paw to test this hypothesis.

Rats infected unilaterally in the dorsum of the hind paw were sacrificed 4 or 29 days PI (Table 13) and dorsal ganglia incubated in hexamethylene-bis-acetamide as described in Materials and Methods prior to cocultivation.

In rats sacrificed 4 days PI, there were no differences between the 2 groups with regard to the proportion of rats positive or skin samples positive for HSV. No virus was recovered from contralateral skin samples. There was no difference between the proportion of ipsilateral ganglia positive from each group. The fraction of ipsilateral ganglia (7/18) positive was greater than the fraction of contralateral ganglia (2/18) ($p<0.05$) within the hexamethylene-bis-acetamide group, but not within the control group. Also, the interval between initiation of cocultivation and the appearance of HSV CPE was not different (3.6 ± 0.38 vs 3.1 ± 0.46) ($p>0.05$). We concluded that hexamethylene-bis-

TABLE 13. Effects of hexamethylene-bis-acetamide on recovery of HSV from lumbar ganglia of rats 4 days post-inoculation.

		HSV Positive/Total Tested				
					Ganglia	
Inoculum/Foot ($TCID_{50}$)	Site	N	Rats	Skin	Ipsi-lateral	Contra-lateral
6.4×10^6	RDS*	6	3/6	6/12	7/18†	2/18†
6.4×10^6**	RDS	6	3/6	4/12	9/18	5/18

* RDS = Right dorsal hind paw skin.

** Controls.

† $p<0.05$

acetamide did not affect HSV infection of dorsal root ganglia.

In rats sacrificed 29 days PI (Table 14), there was no difference between the two groups in regards to the number of rats positive for HSV. Virus was not isolated from any skin samples. There was also no difference between the numbers of ipsilateral versus contralateral ganglia positive within each group or between each group. There was no difference between the proportion of (L4, L5, L6) ganglia from which HSV could be isolated in either the treated or control group. There was no difference in the total number of ganglia positive between each group. There was, however, a significant difference in the intervals between initiation of cocultivation and the appearance of HSV CPE of each group. CPE was detected in the hexamethylene-bis-acetamide group 4.5 ± 0.72 days compared to 8.92 ± 1.42 days ($p<0.01$) for the control group. This implies that demethylation converted latent HSV infection to a productive one.

8. EFFECTS OF RESTRAINT STRESS

Four effects of stress were measured: death, stomach ulcers, plasma CS, and time to appearance of HSV-CPE in HUFF monolayers on which lumbar dorsal root ganglia were cocultured.

A. Death

Of 55 HSV-1 inoculated rats that were cold restraint stressed for 4 hours, 13 died compared to 1/15 uninoculated

TABLE 14. Effects of hexamethylene-bis-acetamide on recovery of HSV from lumbar ganglia of rats 29 days post-inoculation.

			HSV Positive/Total Tested			
					Ganglia	
Inoculum/Foot ($TCID_{50}$)	Site	N	Rats	Skin	Ipsi-lateral	Contra-lateral
6.4×10^6	RDS*	5	3/5	0/10	7/14	4/13
6.4×10^6**	RDS	4	2/4	0/8	6/12	6/12

* RDS = Right dorsal hind paw skin.

** Controls.

No comparisons statistically different.

stressed rats and 0/29 inoculated unstressed rats ($p<0.01$ for 13/55 vs 0/29). None of 15 infected rats stressed for 3 hours died compared to 13/55 rats stressed for 4 hours ($p<0.02$). It was concluded that there was a difference between inoculated and uninoculated stressed rats in their mortality following 4 hours of stress and that 4 hours of stress killed more infected animals than 3 hours exposure.

B. Ulcers

Rats exposed to cold-restraint stress for 3 hours at 4°C did not develop stomach ulcers. Ulcers developed after 3 hours of stress as evidenced in rats that died between 3 and 4 hours of the stress protocol. We concluded that 3 hours of stress did not induce stomach ulcers and therefore stressed rats for 4 hours.

The number of ulcers and their mean cumulative length per rat exposed to 4 hours cold-restraint stress are presented in Figure 15 and Table 15. Infected and uninfected unstressed rats did not develop any stomach ulceration. There was no difference between the mean number of ulcers in each of the 12 stressed infected rats (16.3 ± 0.85) and the 10 stressed uninfected (20.0 ± 2.3) ($p>0.16$) rats sacrificed at the end of the period of stress. Mean cumulative ulcer length (mm) per rat was also not different between stressed groups (38.6 ± 2.4 and 44.0 ± 3.9 mm, respectively; $p>0.26$). Scars indicative of recovery and healing were detected from 24 hours post-stress onward (Figure 16). By 96 hours post-stress, ulceration was not detected and scars were all but

TABLE 15. Gastric ulcers and scars in rats exposed to cold restraint stress for 4 hours at 4°C, 31 days after inoculation of HSV-1, 6.4 x 10^6 $TCID_{50}$, into the hind paws subcutaneously.

	Stressed				Unstressed		
Interval (hrs) Between End of Stress and Sacrifice	Number of Rats with Ulcers or Scars/Total		Number of Ulcers or Scars/Rat Mean ± SE	Cumulative Length (mm) of Ulcers/ Rat Mean±SE	Number of Rats with Ulcers or Scars/Total	Number of Ulcers or Scars/Rat Mean ± SE	Cumulative Length (mm) of Ulcers/ Rat Mean±SE
Uninoculated							
0	10/10	U	20.0 ± 2.3	44.0 ± 3.9	0/10	NA	NA
Inoculated							
0	12/12	U	16.3 ± 0.85	38.6 ± 2.4	0/5	0	0
6	3/3	U	9.0 ± 0.59	29.0 ± 5.3			
12	4/4	U	7.5 ± 0.9	20.3 ± 2.7	0/4	0	0
24	4/4	U	3.8 ± 1.5	6.3 ± 1.4	0/3	0	0
	1/4	S	0.5 ± 0.5	5.0 ± 5.0			
48	1/6	U	1.2 ± 1.1	1.5 ± 1.5	0/6	0	0
	4/6	S	1.2 ± 0.4	4.3 ± 1.5			
72	1/8	U	0.4 ± 0.2	1.1 ± 0.6	0/5	0	0
	7/8	S	1.3 ± 0.3	5.9 ± 1.1			
96	5/6	S	1.5 ± 0.4	10.8 ± 3.7	0/5	0	0
144	3/5	S	0.6 ± 0.3	3.2 ± 1.6	0/4	0	0

† U = ulcer; S = scar.

Figure 15. Mean number of Stomach Ulcers and Mean Cumulative Ulcer Length in Rats Inoculated 31 days previously with HSV-1, $6.4 \times 10^6 TCID_{50}$ into the Hind Paws Subcutaneously, and Cold Restraint Stressed for 4 Hours at 4° C.

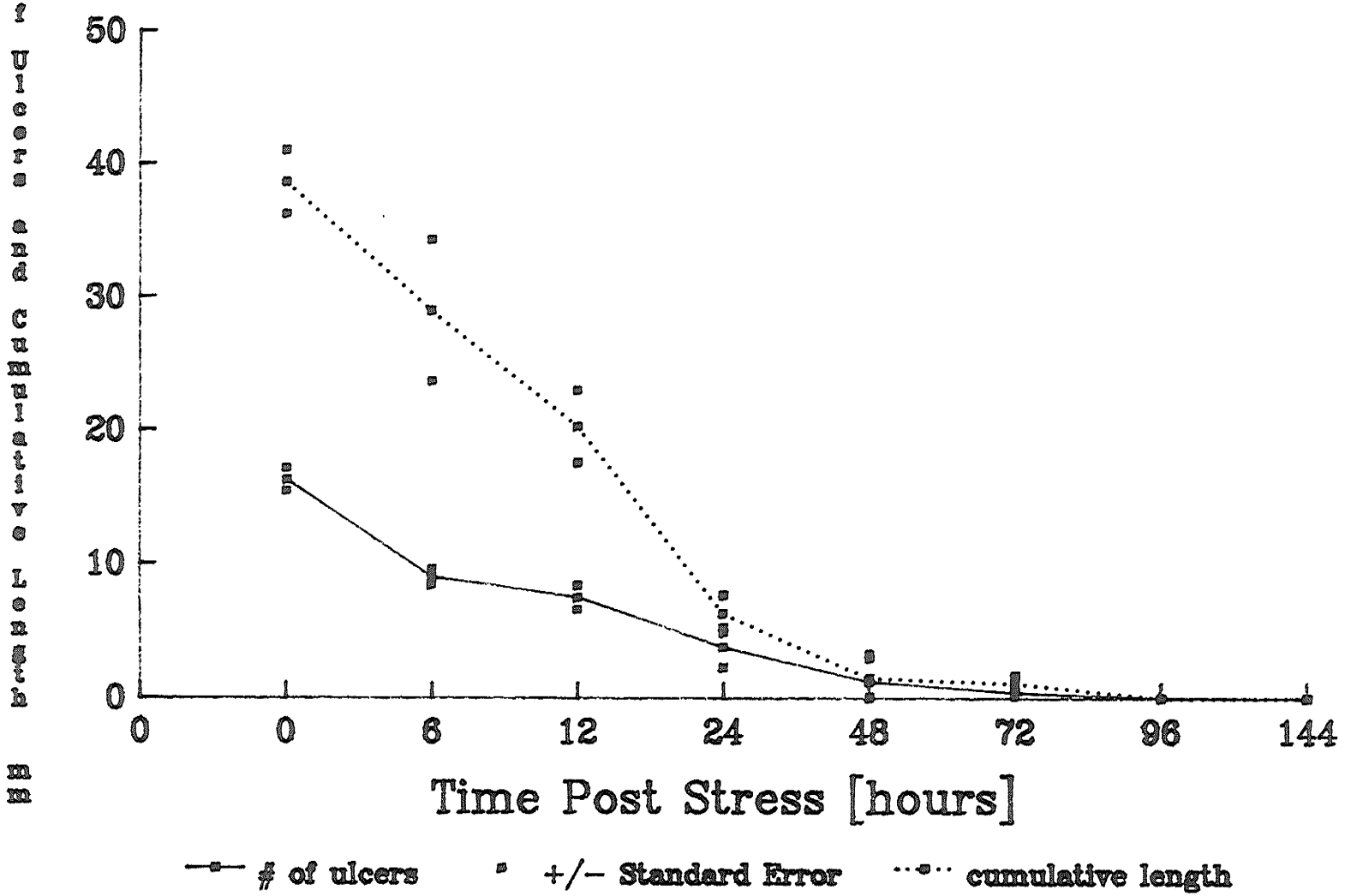
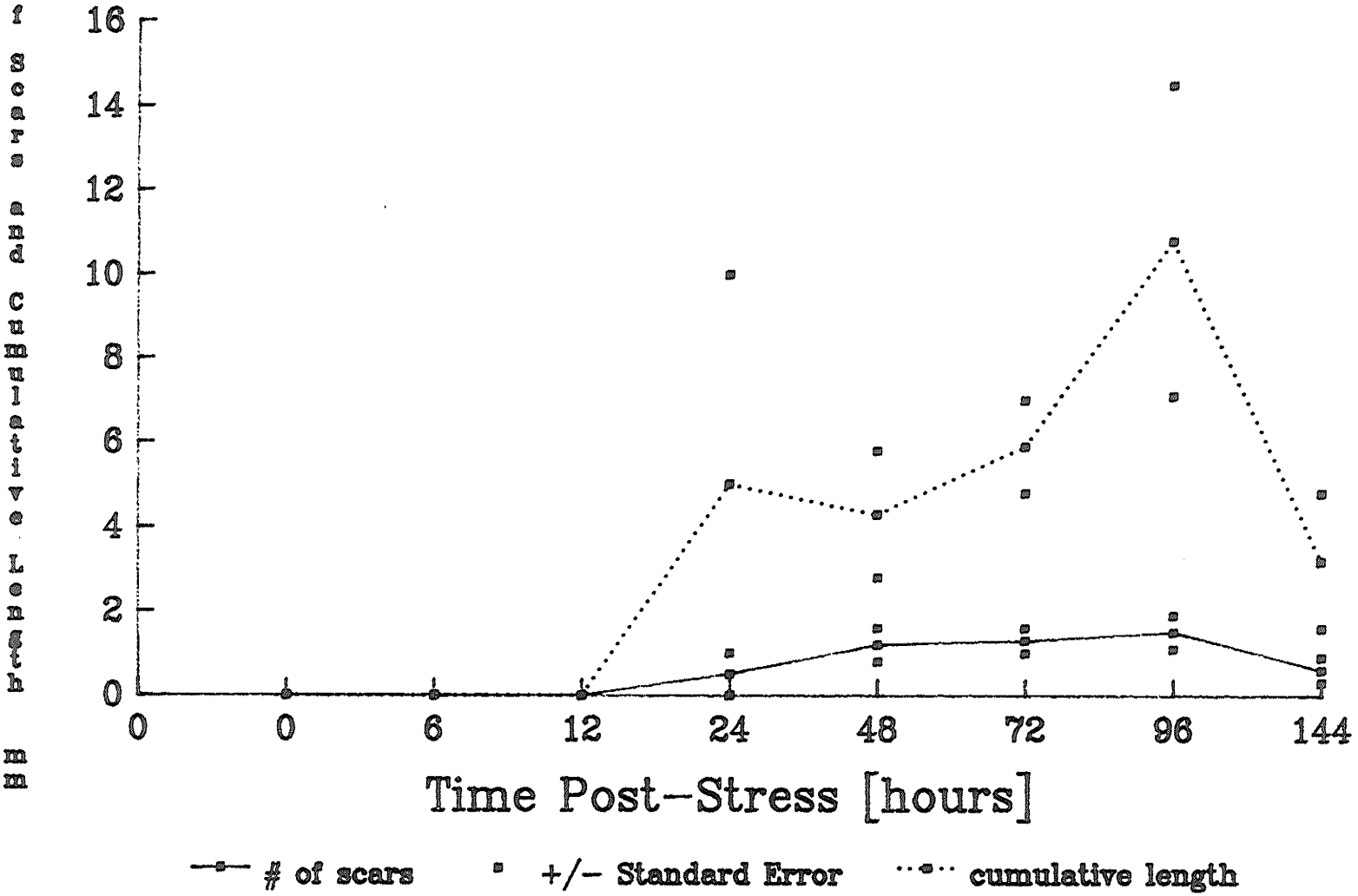


Figure 16. Mean number of Stomach Scars and Mean Cumulative Scar Length in Rats Inoculated 31 days previously with HSV-1 6.4×10^6 $TCID_{50}$, into the Hind Paws Subcutaneously, and Cold Restraint Stressed for 4 Hours at 4°C.



gone by 144 hours post-stress. No scar tissue was ever detected in the unstressed groups. Figure 17 shows typical stomach ulcerations in a rat cold restraint stressed for 4 hours.

In the 13 infected stressed rats that died during exposure to stress, there was no difference between the mean number of ulcers (11.1 ± 1.4) versus infected stressed rats killed at the end of the stress period (16.3 ± 0.85; $p>0.59$). However, the mean number of ulcers was less in the dead animals (11.1 ± 1.4) compared to uninoculated stressed rats killed at the end of the stress period (20.0 ± 2.3; $p<0.01$). Mean cumulative ulcer length was not different between dead (31.8 ± 3.9) and inoculated stressed (38.6 ± 2.4) rats ($p>0.15$) but was significantly less in dead (31.8 ± 3.9) than uninoculated stressed (44.0 ± 3.9) ($p<0.04$) rats. There was no apparent difference in ulcers between the 1 dead uninoculated stressed rat and the others that died.

We conclude that 4 hours of stress caused severe stomach ulceration and that the degree of severity was not different between infected rats and uninfected control rats exposed to stress.

C. Corticosterone (CS)

Plasma CS (ug%) was measured in inoculated and control rats sacrificed 4 days PI. Figure 18 shows the mean CS levels between rats injected with HSV-1 in different amounts subcutaneously in the dorsal hind paw. The CS concentration in rats injected with 20 or 100 ul of HSV-1 were not differ-

FIGURE 17. Stomach ulcers in a rat with latent HSV-1 infection exposed to cold restraint stress for 4 hours. The stomach from an unstressed infected control rat is shown on the left.

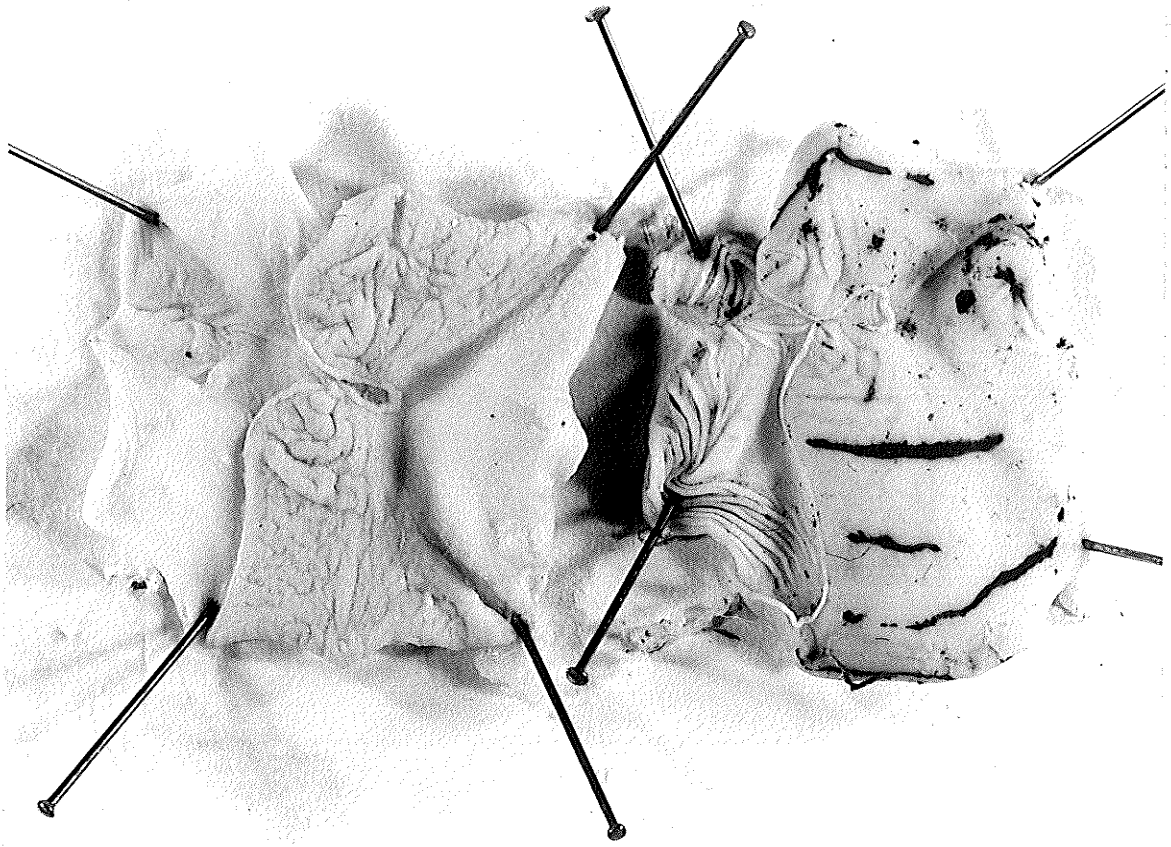
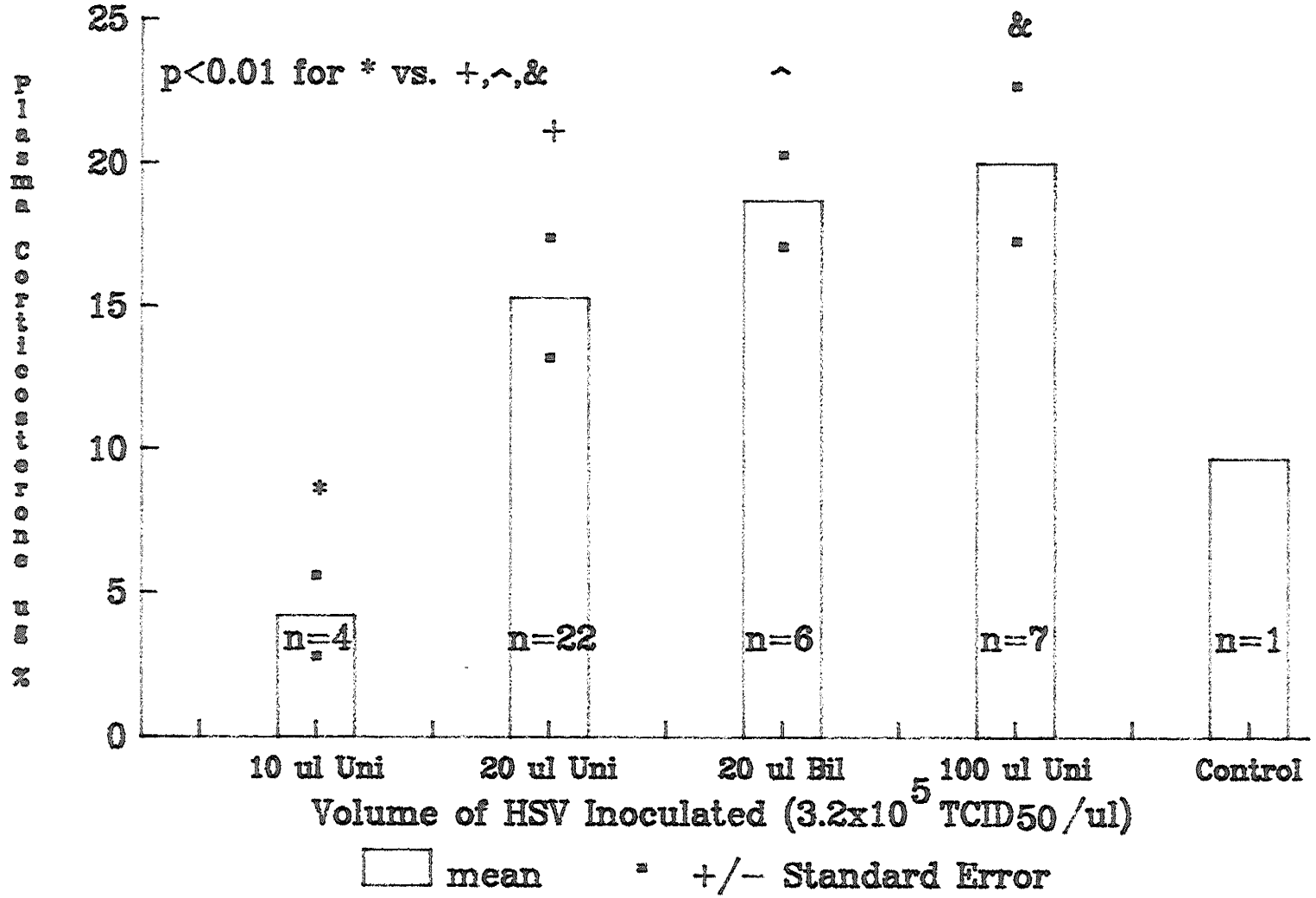


Figure 18. Plasma Corticosterone Concentrations in Rats 4 days after Inoculation with Different Amounts of HSV-1 Subcutaneously into the Hind Paw(s).



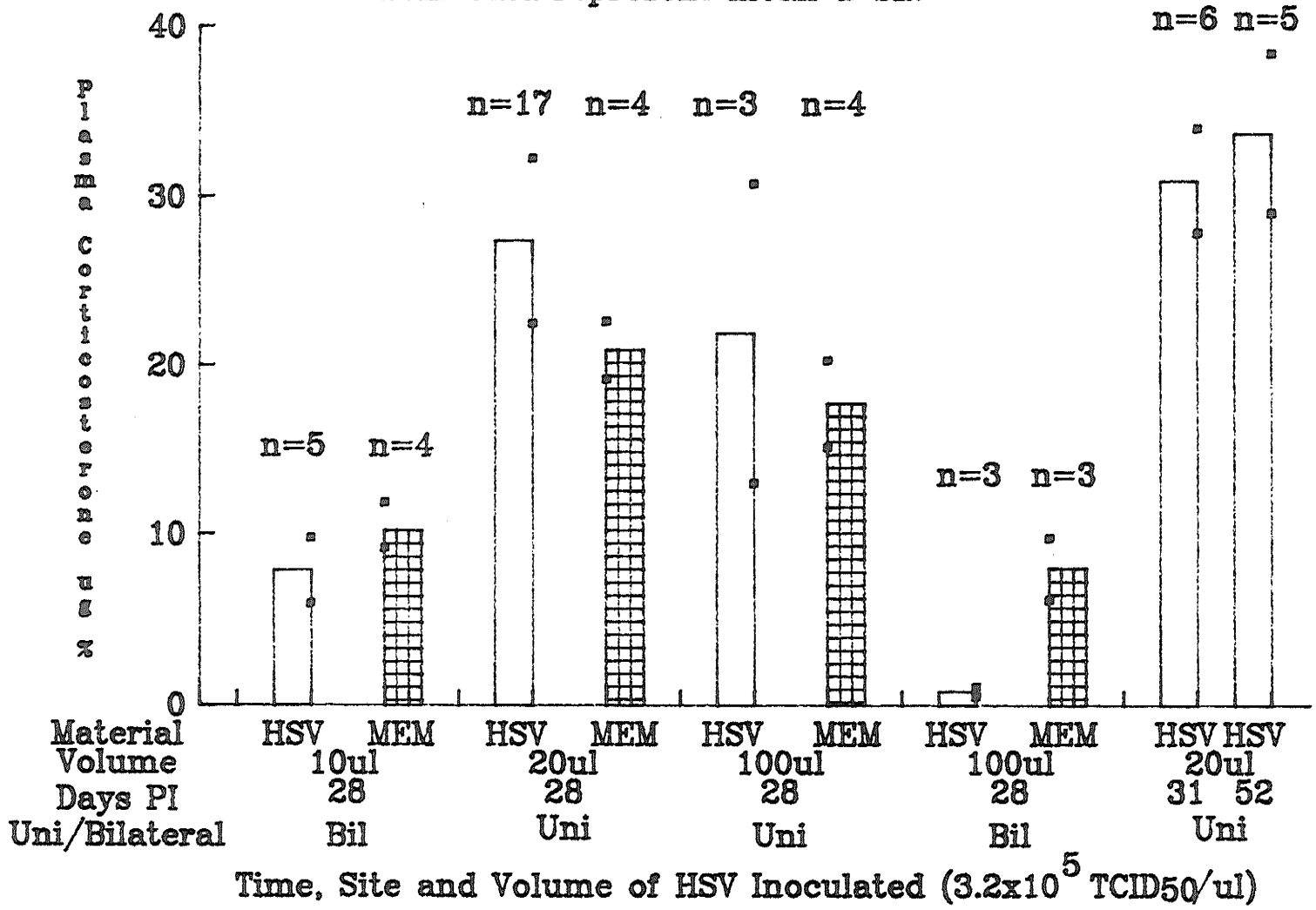
ent from each other but were greater than the concentration in uninoculated controls and rats injected with 10 ul ($p \leq 0.01$ in all 3 comparisons). The CS concentration was not different between rats injected with 10 ul of HSV-1 and in uninoculated control (n=1). These data show that infection of rats with more than 6.4×10^6 $TCID_{50}$ of HSV-1 caused a significant elevation of plasma CS that was independent of inoculum size, thereafter.

Figure 19 illustrates CS concentrations in inoculated and uninoculated control rats that were infected with HSV-1, 28, 31 and 52 days previously. Since assays for CS in animals investigated in the different experiments were run at different times, comparisons between the results of different experiments is not possible. Rather, comparisons are made between results from experimental and control rats whose CS levels were measured concurrently.

As is evident from Figure 19, in animals studied 28 days after infection with different volumes (inocula) of HSV-1, significant differences were not noted between any of the experimental and control groups except a trend towards a difference ($p>0.05$) between rats given HSV-1 in 100 ul bilaterally (3.2×10^7 $TCID_{50}$) and controls.

Rats killed 31 and 52 days after HSV-1 inoculation were given 6.4×10^6 $TCID_{50}$ in 20 ul subcutaneously in the hind paws. However, the numbers here are too small for statistical comparison. No difference was seen between infected rats sacrificed on days 31 or 52 PI.

Figure 19. Plasma Corticosterone Concentrations in Rats Latently Infected with HSV-1 Injected in Different Volumes Subcutaneously in the Dorsal Hind Paw(s), 28 to 52 days after Infection. Data represent mean ± SE.



Time, Site and Volume of HSV Inoculated (3.2×10^5 TCID50/ul)

We conclude that there was no difference between rats sacrificed at 28, 31 or 52 days after infection and their respective controls in regards to the concentration of plasma CS.

i) CS Following Sciatic Nerve Manipulation. CS concentrations in rats whose sciatic nerve had been transected two days prior to removal of ganglia are summarized in Figure 20. Comparisons between concurrent experimental and control groups showed no differences. These results indicate that manipulation of the sciatic nerve is not in itself sufficient to significantly elevate plasma CS in comparison with controls.

ii) CS Following 3 Hours of Cold Restraint Stress. Figure 21 shows data on CS in our model of latent infection exposed to 3 hours of cold restraint stress. Differences in mean CS concentration were noted between stressed (53.8 ± 5.9) and unstressed (20.3 ± 6.3) infected rats ($p<0.01$) immediately at the end of the stress (time 0) and between stressed (16.8 ± 2.4) and unstressed (24.5 ± 2.2) infected rats ($p<0.05$) at 24 hours after the end of the stress. No difference was detectable between groups 48 hours after the end of stress (21.1 ± 3.3 vs 21.6 ± 3.5 respectively, $p>0.05$).

We conclude that the CS concentration in the experimental groups was different at time 0 but not at any time thereafter. In control rats killed at the end of the stress period or at 24 and 48 hours, there were

Figure 20. Plasma Corticosterone Concentrations in Rats whose Sciatic Nerve was Transected 4 or 28 days following Inoculation of Different Amounts of HSV-1 Subcutaneously in the Ipsilateral Hind Paw. Data represent mean ± SE.

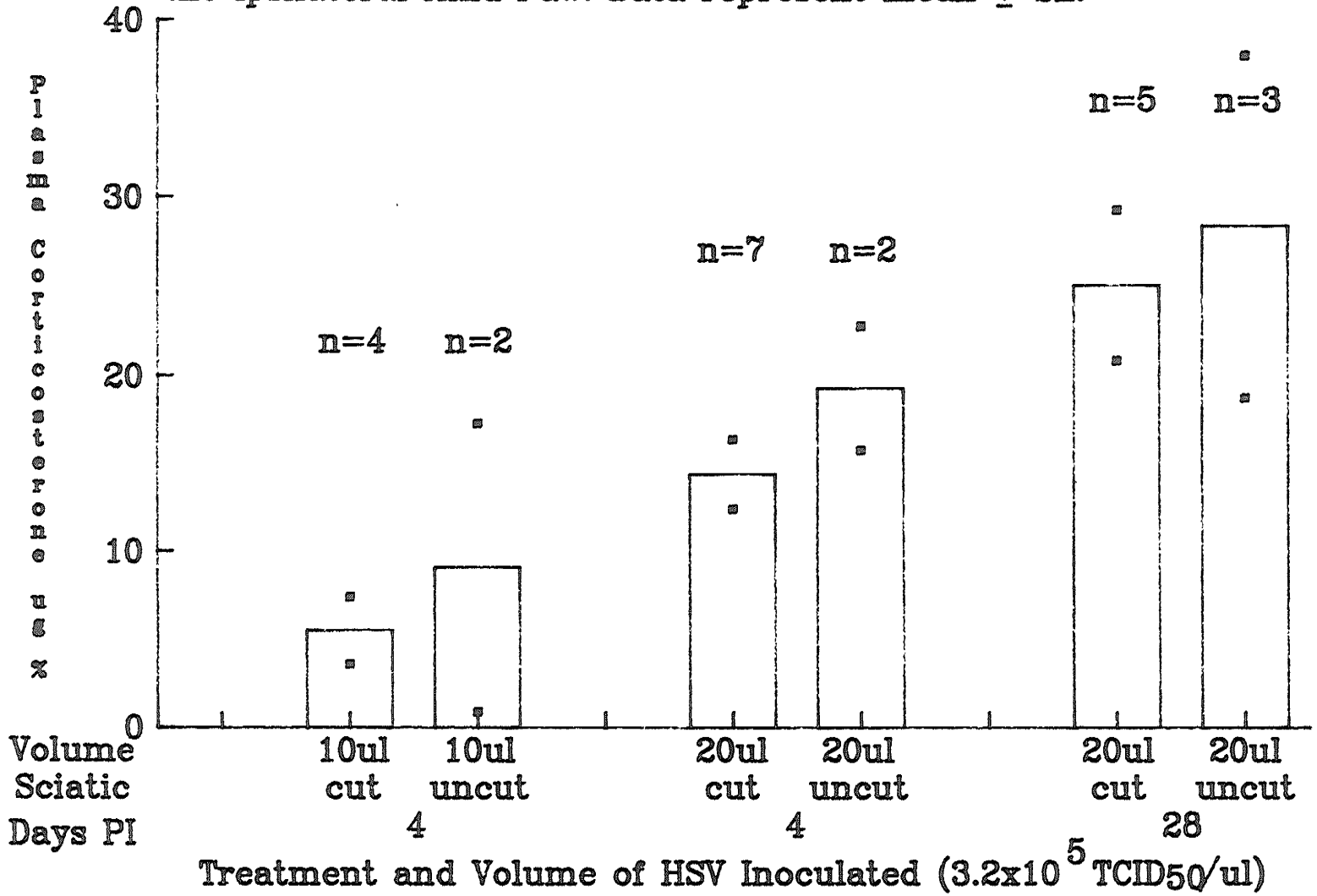
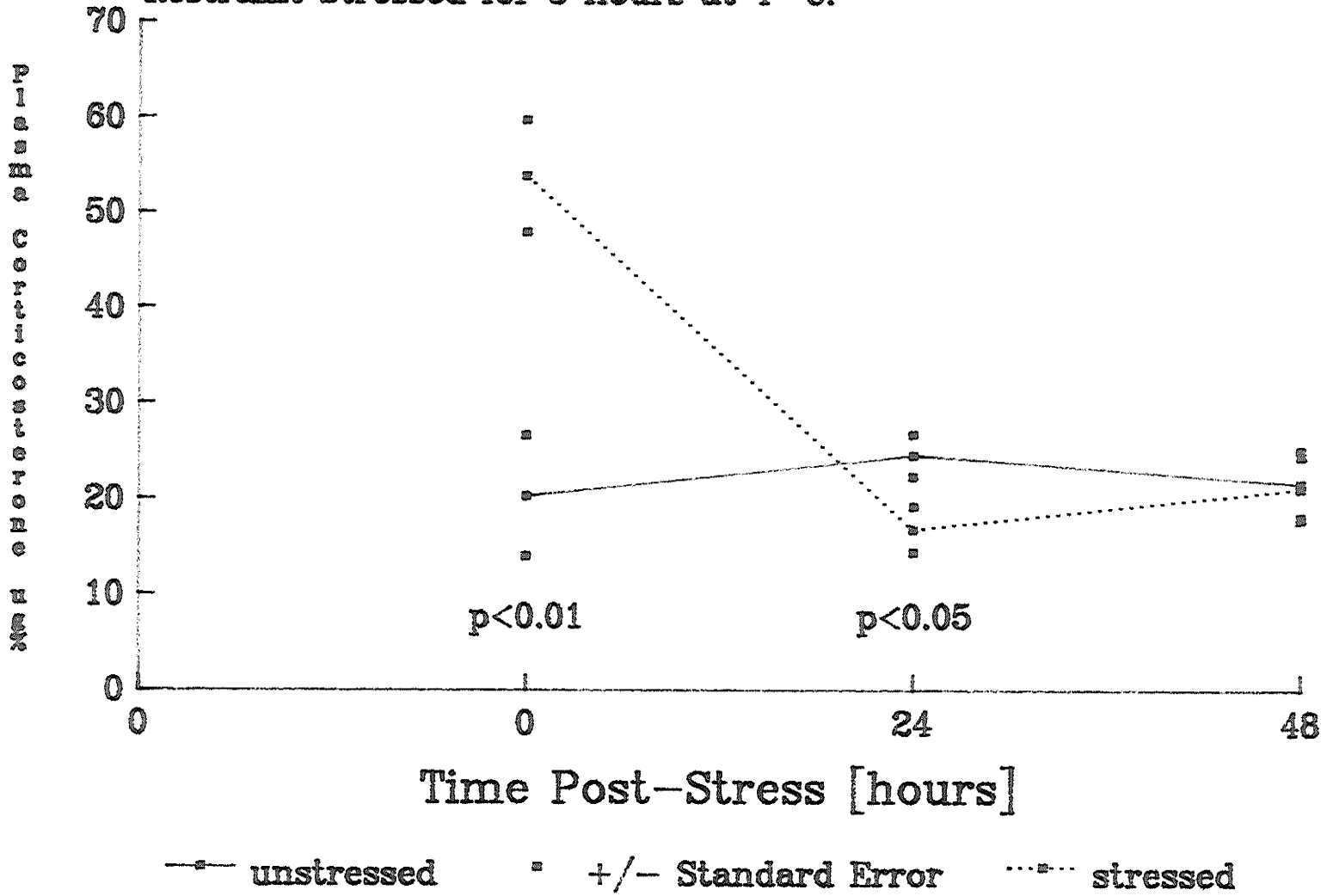


Figure 21. Plasma Corticosterone Concentrations in Rats Infected 31 days previously with 6.4×10^6 $TCID_{50}$ of HSV-1 Subcutaneously in the Dorsum of the Hind Paws and Cold Restraint Stressed for 3 Hours at 4 °C.



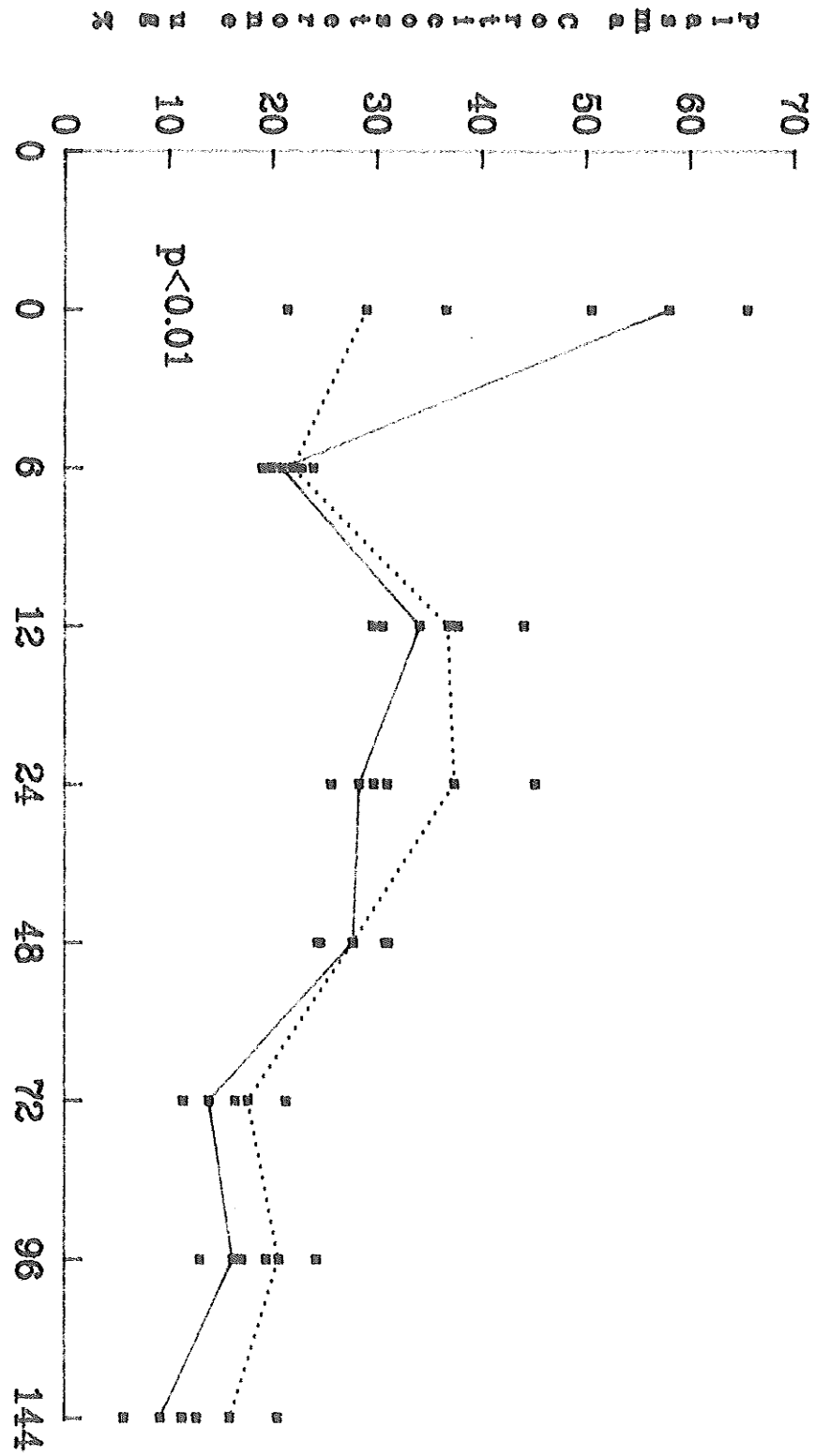
no differences in the mean CS concentrations. Plasma CS was only elevated at the end of the stress.

iii) CS Following 4 Hours of Cold Restraint Stress.

Figure 22 shows CS concentration in rats infected 31 days previously with 6.4×10^6 $TCID_{50}$ subcutaneously in the dorsum of the hind paws, subjected to 4 hours of cold restraint stress, and sacrificed over the next 144 hours. The CS concentrations of uninoculated stressed (74.3 ± 5.5) rats were greater than those of uninoculated unstressed animals (42.3 ± 3.3) ($p<0.01$) immediately at the end of the stress (time 0) (data not shown). CS concentrations of inoculated stressed (58.0 ± 7.5) and inoculated unstressed (29.0 ± 7.6) rats at time 0 ($p<0.01$) were different. Differences were not seen between inoculated stressed and inoculated unstressed rats at 6 hours (20.9 ± 1.9 vs 21.9 ± 2.0; $p>0.05$), 12 hours (34.1 ± 3.6 vs 36.8 ± 7.2; $p>0.05$), 24 hours (28.28 ± 2.7 vs 37.37 ± 7.7; $p>0.05$), 48 hours (27.73 ± 3.4 vs 27.69 ± 3.1; $p>0.05$), 72 hours (13.91 ± 2.5 vs 17.6 ± 3.7; $p>0.05$), 96 hours (16.18 ± 3.2 vs 20.58 ± 3.6; $p>0.05$), or 144 hours (9.24 ± 3.5 vs 15.98 ± 4.6; $p>0.05$), respectively. CS concentrations were not different between uninoculated stressed (74.3 ± 5.5) (data not shown) and inoculated stressed (58.0 ± 7.5) rats ($p<0.09$).

We conclude that CS concentrations in the experimental groups was different at time 0 but not at any time thereafter indicating that the effects of stress

Figure 22. Plasma Corticosterone Concentrations in Rats Infected 31 days previously with $6.4x10^6$ TCID50 of HSV-1 Subcutaneously in the Dorsum of the Hind Paws and Cold Restraint Stressed for 4 Hours at 4 °C.



were maximal immediately at the end of the stress procedure.

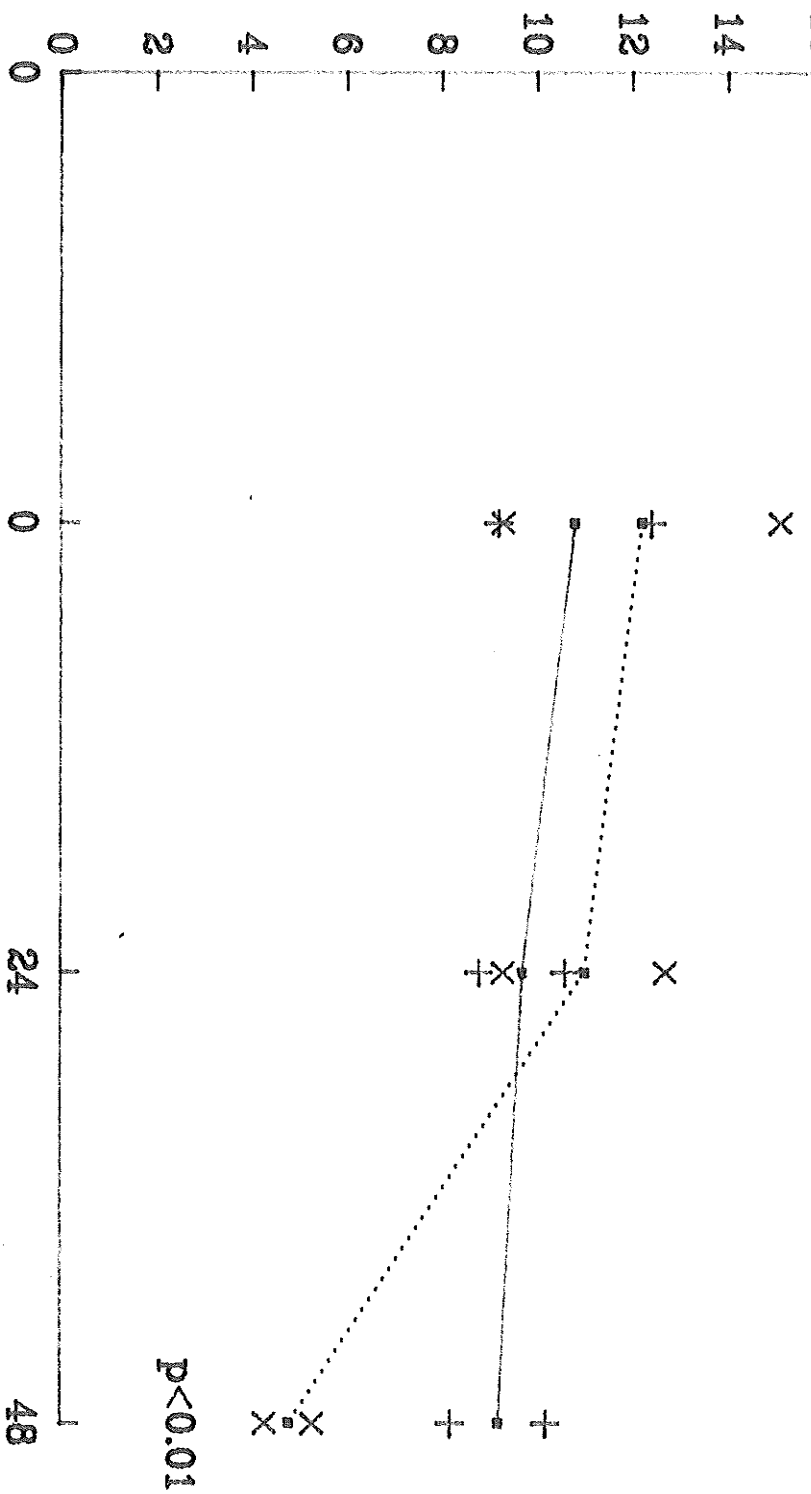
D. Time for HSV-CPE to Appear in HUFF Cells Cocultivated with Lumbar Ganglia of Rats Subjected to Cold Restraint Stress

i) For 3 Hours. Rats inoculated with 6.4×10^6 $TCID_{50}$ HSV-1 subcutaneously in 1 hind paw 31 days previously were subjected to 3 hours cold restraint stress. The times from explantation for HSV-CPE to appear in HUFF cells cocultivated with the ganglia are shown in Figure 23. There was no difference between the time (days) between inoculated stressed (10.8 ± 1.6) and inoculated unstressed (12.2 ± 2.9) rats ($p>0.05$) sacrificed at the end of the stress period (time 0) or 24 hours later (9.7 ± 0.9 vs 11.0 ± 1.7; $p>0.05$) respectively. However, a difference was seen at 48 hours post-stress (9.2 ± 1.0 vs 4.8 ± 0.50, respectively; $p<0.01$).

We conclude that 3 hours of cold restraint stress was not intense enough to reactivate latent virus immediately following stress or at 24 hours later. At 48 hours, control unstressed rats had a shorter time to HSV-CPE than did stressed rats. The positive ganglia in the control group all came from 1 rat indicating that this rat was different than those of our model.

ii) For 4 Hours. Rats inoculated with 6.4×10^6 $TCID_{50}$ HSV-1 subcutaneously in the hind paws 31 days previously were subjected to 4 hours of cold restraint stress. The times from explantation for HSV-CPE to appear in HUFF

Figure 23. Time for HSV-CPE to appear in Huff cells Cocultivated with Lumbar Ganglia of Rats Infected 31 days previously with 6.4×10^6 $TCID_{50}$ Subcutaneously in the Dorsal Hind Paws and Cold Restraint Stressed for 3 Hours at 4 °C.



cells cocultivated with the ganglia are shown in Figure 24. There were no differences between the times of inoculated stressed and inoculated unstressed rats 0, 6, 12, 24, 72, 96, 144 hours following the end of the stress period: 7.5 ± 0.77 vs 7.4 ± 0.43, $p>0.05$; 9.67 ± 1.20 vs 10.0 ± 0.41, $p>0.05$; 8.4 ± 1.51 vs 12.5 ± 2.53, $p>0.05$; 6.8 ± 0.70 vs 7.8 ± 1.83, $p>0.05$; 7.5 ± 0.80 vs 7.4 ± 0.65, $p>0.05$; 7.0 ± 1.0 vs 7.0 ± 1.1, $p>0.05$; 11.3 ± 2.3 vs 9.2 ± 2.1, $p>0.05$, respectively. A difference was noted between the times for CPE between inoculated stressed (8.3 ± 0.60) and inoculated unstressed (10.2 ± 1.0) rats ($p<0.05$) 48 hours post-stress but both means are within the range of means for the other 14 groups.

We conclude that 4 hours of cold restraint stress did not reactivate latent virus immediately at the end of the stress procedure nor at times up to 48 hours after stress. Virus was reactivated faster in stressed rats at 48 hours after stress and this observation may relate to the time necessary for reactivation of HSV to occur.

E. Recovery of HSV from Rats Infected 31 Days Previously and Cold Restraint Stressed

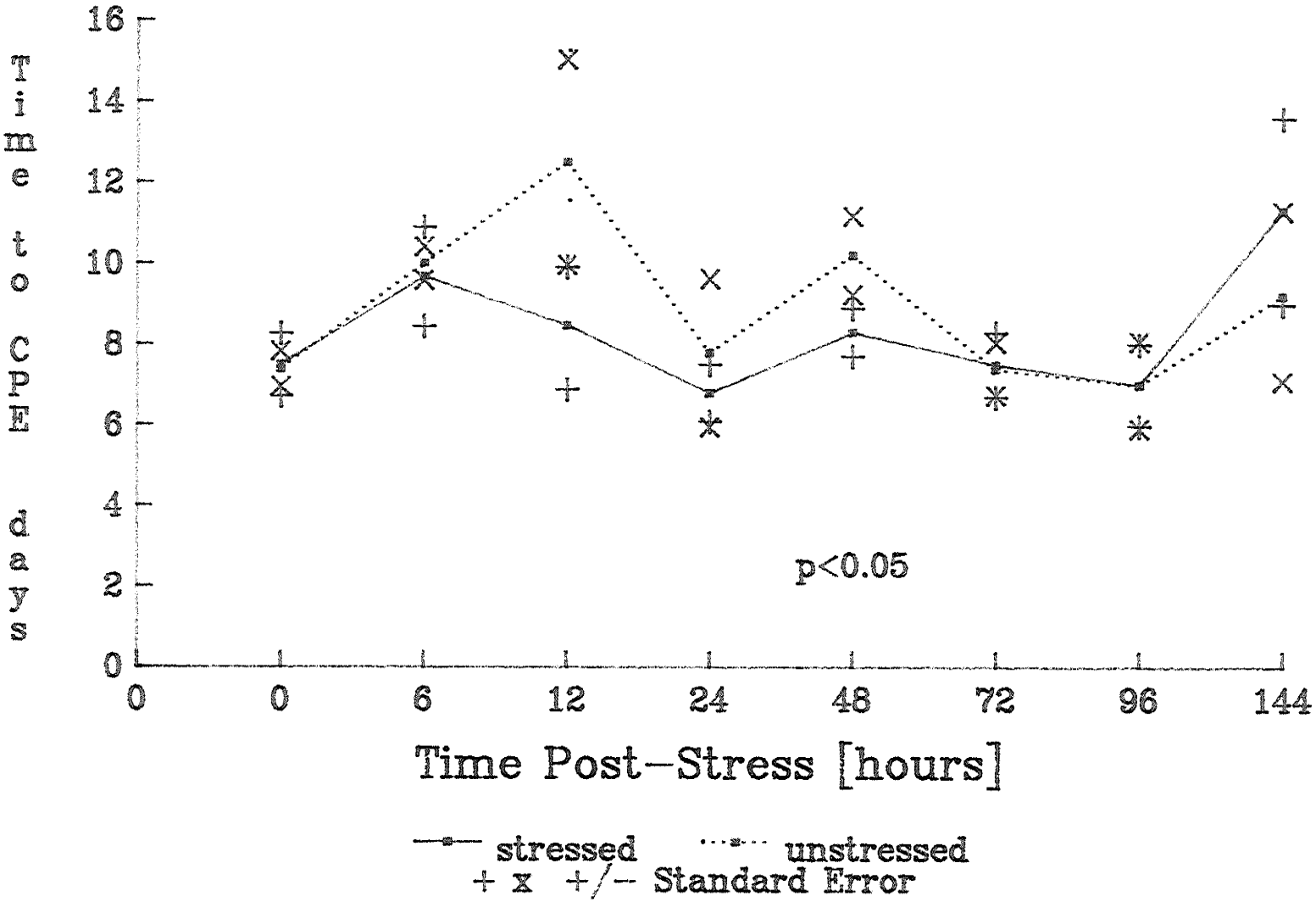
i) For 3 Hours. Table 16 summarizes data obtained from rats infected 31 days previously by subcutaneous inoculation of 6.4×10^6 $TCID_{50}$ in the hind paws and subjected to 3 hours of cold restraint stress. No difference was seen between the proportion of inoculated

TABLE 16. Recovery of HSV from lumbar ganglia and dorsal hind paw skin of rats infected with 6.4×10^6 $TCID_{50}$ HSV-1 by subcutaneous injection in the dorsum of hind paws 31 days previously and cold restraint stressed for 3 hours.

Interval Between the End of Stress and Sacrifice (hrs)	N	HSV Positive/Total Tested		
		Rats	Skin	Ganglia (%)
0 hours	5	4/5	0/10	8/30 (27)
0 hours*	5	3/5	0/10	5/29 (17)
24 hours	5	4/5	0/10	14/30 (47)
24 hours*	5	4/5	0/10	12/29 (41)
48 hours	5	4/5	0/10	12/30 (40)
48 hours*	6	2/6	0/12	6/34 (18)

* Unstressed Controls.

Figure 24. Time for HSV-CPE to appear in Huff cells Cocultivated with Lumbar Ganglia of Rats Infected 31 days previously with 6.4×10^6 $TCID_{50}$ Subcutaneously in the Dorsal Hind Paws and Cold Restraint Stressed for 4 Hours at 4 °C.



stressed rats (4/5) positive for HSV and inoculated unstressed (3/5) rats ($p>0.05$) 0 hours post-stress. A difference is observed between the proportion of ganglia positive for HSV from inoculated stressed (12/30) rats and inoculated unstressed (6/34) rats 48 hours post-stress ($p<0.05$). Virus was not isolated from any skin tissue sampled. No other comparisons were statistically different.

We conclude that 3 hours of stress did not affect the percentage of ganglia positive for HSV between stressed and unstressed rats.

ii) For 4 Hours. Rats were injected 31 days previously with 6.4×10^6 $TCID_{50}$ HSV-1 subcutaneously in the dorsum of the hind paws. Table 17 shows data obtained from these rats subjected to 4 hours of cold restraint stress and unstressed infected control animals. Differences were not seen when the proportions of rats positive for HSV were compared between infected stressed and infected unstressed rats sacrificed 0, 24, 48, 72, 96 and 144 hours after the end of the stress. A difference was not seen between the number of stressed (3/4) rats positive for HSV compared to unstressed (1/4) rats ($p>0.05$). Virus was not recovered from any skin tissue sampled. Differences were not seen between the number of ganglia positive between infected stressed and infected unstressed rats at 0, 6, 24, 96, 144 hours post-stress. A significant difference was seen between the number of

TABLE 17. HSV-CPE in HUFF cells cocultured with lumbar ganglia of rats infected with 6.4×10^6 $TCID_{50}$ HSV-1 31 days previously and exposed to cold restraint stress for 4 hours at 4°C.

Interval Between the End of Stress and Sacrifice (hrs)	N	HSV Positive/Total Tested		
		Rats	Skin	Ganglia (%)
0 hours	8	4/8	0/16	29/69 (42)
0 hours*	5	2/5	0/10	22/53 (42)
6 hours	3	1/3	0/6	3/18 (17)
6 hours*	3	2/3	0/6	6/18 (33)
12 hours	4	3/4	0/8	11/24 (46)§
12 hours*	4	1/4	0/8	2/23 (9)§
24 hours	4	3/4	0/8	10/24 (42)
24 hours*	3	2/3	0/6	6/18 (33)
48 hours	9	6/9	0/18	26/54 (48)
48 hours*	14	9/14	0/28	22/82 (27)
72 hours	8	3/8	0/16	17/48 (35)
72 hours*	5	4/5	0/10	18/29 (62)
96 hours	6	2/6	0/12	8/36 (22)
96 hours*	5	3/5	0/10	10/30 (33)
144 hours	5	2/5	0/10	7/30 (23)
144 hours*	4	1/4	0/8	6/24 (25)

* Unstressed Controls

§ $p<0.01$

ganglia positive from infected stressed and infected unstressed rats at 12 hours (11/24 vs 2/23, $p<0.01$), 48 hours (26/54 vs 22/82, $p<0.05$) and 72 hours (17/48 vs 18/29, $p<0.05$), respectively. We conclude that 4 hours of stress did not consistently result in differences in the proportion of stressed and unstressed rats and ganglia from which HSV could be recovered.

F. Effects of Putative Mediators of Stress-Induced Activation of Latent HSV Infection

i) Nerve Growth Factor. Lumbar ganglia from rats infected with 6.4×10^6 $TCID_{50}$ HSV-1 by subcutaneous injection in the dorsum of the hind paws 28 days previously were cultured in the presence of nerve growth factor (NGF) (Table 18). There was a difference in the number of rats positive for HSV between the 50 ng NGF group (0/2) and the control group (2/2), however these numbers are too small to attain statistical significance. Skin tissue was not cultured in the presence of NGF. There was no virus isolated from skin samples from any of the groups. There was a difference between the number of ganglia positive in the 50 ng NGF group (0/12) and the control group (3/12) ($p<0.05$). No other statistical comparisons were significantly different. The time to CPE within each group was as follows: 50 ng NGF, no cultures positive; 100 ng NGF, 9.5 ± 3.5 days; control, 10.3 ± 2.1 days. These values were insufficient to allow for statistical comparison. We concluded that

TABLE 18. Effects of nerve growth factor on recovery of HSV from lumbar ganglia of rats 28 days after inoculation subcutaneously in the dorsum of the hind paw.

Inoculum ($TCID_{50}$)	NGF Conc. (ng/ml)	N	HSV Positive/Total Tested		
			Rats	Skin¶	Ganglia (%)
6.4×10^6	50	2	0/2†	0/4	0/12 (0)*
6.4×10^6	100	2	1/2	0/4	2/12 (17)
6.4×10^6	(Control)	2	2/2†	0/4	5/12 (25)*

¶ Skin not cultured in presence of NGF.

† $p<0.05$

* $p<0.05$

No other statistical comparisons significant.

Ganglia were maintained in transport medium containing 50 or 100 ng/ml NGF for 24 hours prior to intiation of explant culture on HUFF cell monolayers.

nerve growth factor (over the dosages tested) did not result in a higher percentage of rats or ganglia positive for HSV between experimental and control groups.

ii) Norepinephrine (NE). Lumbar ganglia from rats infected with 6.4×10^6 $TCID_{50}$ HSV-1 by subcutaneous injection in the dorsum of the hind paws 28 days previously were cultured in medium containing norepinephrine (NE) (Table 19). There was no difference when the proportion of rats positive for HSV was compared between the four groups. The skin samples were not assayed in the presence of NE. Virus was not detected in any of the skin samples assayed. There was no difference when the number of ganglia positive for HSV in the 10^{-5} M NE (2/12), 10^{-4} M NE (2/12) and control (no NE) (1/11) were compared to each other. A large proportion of ganglia treated with 10^{-6} M NE (5/11) were positive for HSV-CPE compared to control (1/11) ganglia ($p<0.05$). No other statistical comparisons were significantly different. The time (days) for CPE to appear in the various groups were as follows: 10^{-6} M NE, 9.4 ± 1.5; 10^{-5} M NE, 12.5 ± 2.5; 10^{-4} M NE, 7.5 ± 1.5; control, 7. These data were insufficient to compare statistically. Comparison of pooled data from control ganglia from the NGF, NE and E (epinephrine) experiments (below) with pooled data from the three concentrations of NE used was not statistically different (9.3 ± 1.3 vs 9.7 ± 1.1, $p>0.05$, respectively). We concluded that over the concentrations

TABLE 19. Effects of norepinephrine on recovery of HSV from lumbar ganglia of rats 28 days after bilateral inoculation subcutaneously in the dorsum of the hind paw.

Inoculum ($TCID_{50}$)	Norepinephine conc. (M)	N	HSV Positive/Total Tested Rats	Skin†	Ganglia (%)
6.4×10^6	10^{-6}	2	1/2	0/4	5/11 (46)*
6.4×10^6	10^{-5}	2	1/2	0/4	2/12 (17)
6.4×10^6	10^{-4}	2	2/2	0/4	2/12 (17)
6.4×10^6	(Control)	2	1/2	0/4	1/11 (9)*

† Skin not cultured in presence of norepinephrine.

* $p<0.05$

No other statistical comparisons significant.

Ganglia were maintained in transport medium containing 10^{-4}, 10^{-5}, 10^{-6} M norepinephrine for 24 hours prior to initiation of explant culture on Huff cell monolayers.

tested, norepinephrine did not result in a difference in the proportions of rats or ganglia positive for HSV between the experimental and control groups.

iii) Epinephrine (E). Lumbar ganglia from rats infected with 6.4×10^6 $TCID_{50}$ HSV-1 subcutaneously in the dorsum of the hind paws 28 days previously were cultured in the presence of epinephrine (E) (Table 20). There was no difference in the number of rats positive for HSV between the groups. Skin samples were not assayed in the presence of E. No virus was isolated from cultured skin samples. There was no difference in the proportion of ganglia positive for HSV between the groups. The times (days) for CPE to appear in each of the groups were: 10^{-6} M E, 13.7 ± 0.34; 10^{-5} M E, 10.7 ± 2.2; 10^{-4} M E, 8.8 ± 1.7; control, 9 ± 3.0. These data were insufficient to compare statistically. Comparison of pooled data from control ganglia from the NGF, NE and E experiments with pooled data from the three concentrations of E used were not statistically different (9.3 ± 1.3 vs 10.6 ± 1.1, $p>0.05$, respectively). We concluded that over the concentrations tested, epinephrine did not result in a difference in the proportions of rats or ganglia positive for HSV between the experimental and control groups.

TABLE 20. Effects of epinephrine on recovery of HSV from lumbar ganglia of rats 28 days after bilateral inoculation subcutaneously in the dorsum of the hind paws.

Inoculum ($TCID_{50}$)	Epinephrine Conc. (M)	N	HSV Positive/Total Tested		
			Rats	Skin†	Ganglia (%)
6.4×10^6	(10^{-6})	2	1/2	0/4	3/12 (24)
6.4×10^6	(10^{-5})	2	2/2	0/4	3/12 (25)
6.4×10^6	(10^{-4})	2	2/2	0/4	5/12 (42)
6.4×10^6	Control	2	2/2	0/4	2/12 (17)

† Skin not cultured in presence of epinephrine.

No comparisons statistically significant.

Ganglia were maintained in transport medium containing 10^{-4}, 10^{-5}, 10^{-6} M epinephrine for 24 hours prior to initiation of explant culture on Huff cell monolayers.

DISCUSSION

The noteworthy observations from these experiments were that latent HSV infection of lumbar ganglia was produced in otherwise healthy outbred rats by injection of virus in the dorsal skin of the hind paw and that latent infection could be activated by subjecting the animals to stress.

In these rats, we developed a reliable, predictable and reproducible animal model of acute and latent HSV dorsal root ganglion infection that conforms in several aspects with our current concepts of this infection in healthy human subjects.

According to Klein (1982), the acute phase of herpes simplex virus infection in experimental animals spans 1-14 days while the latent stage begins after 14 days following inoculation. Acutely infected rats may be described as having HSV recovered from dorsal root ganglia less than 6 days following the start of cocultivation, while HSV is recovered greater than 6 days following the start of cocultivation from latently infected rats. This definition was selected based on a previous report (Knott et al, 1973) showing the isolation of latent HSV 7 to 19 days following cocultivation of brain stem tissue of rabbit and 6 to 18 days following cocultivation of spinal cord and sacro-sciatic spinal ganglia of mice. As well, Hill et al (1975) reported the isolation of virus from the cervical ganglia of latently infected mice 7 to 21 days following cocultivation. Kristensson et al (1979) reported that the time elapsing between the onset of cultivation of ganglionic tissue and

appearance of CPE was 7-16 days with a mean of 11 days. By this definition, it is clear that rat ganglia can be either acutely or latently infected.

We observed a difference in the interval required for CPE to appear after cocultivation of dorsal root ganglia removed 4 and 28 (and in one study, 21 days) days after subcutaneous inoculation in the dorsum of the hind paws. CPE appeared in the HUFF cell monolayers 5.8 ± 0.28 (mean ± SE) days after the start of cocultivation and 8.8 ± 1.51 days, respectively. The difference was interpreted as indicating that HSV was present in lytic and latent states, respectively. The time for CPE to appear in rats inoculated 28 days previously is in agreement with results obtained in other animals and man, infected with HSV. Thus, Lewis et al (1984) reported the isolation of latent virus from the trigeminal and vagus ganglia of human cadavers 8-30 days after the start of explantation of tissue. As well, others have observed that the time to recovery of HSV from ganglia of infected rabbits (Gerdes and Smith, 1983), and mice (Hill et al, 1975) after HSV inoculation in eye (rabbit latent virus recovered from trigeminal ganglia 7-21 days following the start of cocultivation; Gerdes and Smith, 1983), mouse ear (cervical ganglia were removed on day 14, 21 and 28 after inoculation and virus was recovered 7-21 days after the start of cocultivation; Hill et al, 1975), and guinea pig vagina (viral thymidine kinase activity negative up to 7 days in ganglia cell cultures; infectious virus not detectable in

ganglia until second week of culture) (Fong and Scriba, 1980; Scriba, 1981) was similar to that which we report. Stanberry et al (1982) reported that HSV type-2 was recovered 20-28 days following the start of cocultivation from the dorsal root ganglia of guinea pigs sacrificed 95 days post-inoculation. Failure to eliminate the difference in time to appearance of CPE by homogenization of ganglia immediately after removal at 4 and 28 days after infection, was interpreted as indicating that dorsal root ganglia removed at 4 days contained HSV in a different state than ganglia removed at day 28. It was proposed that the different states were of productive infection and latency, respectively. Our data, demonstrating the difference in time to appearance of CPE in ganglia removed at 4 and 28 days strongly suggested that we too were examining productive ganglionic and latent ganglionic, HSV infection, respectively.

The observation that the intervals after cocultivation required for HSV CPE to appear overlapped for ganglia removed 4 and 28 days after injection suggested that, at least at these two times, HSV may exist in the ganglia in state(s) that form a spectrum between productive and latent states. In molecular terms, HSV in these ganglia must have been spontaneously activated yet not have yielded productive or lytic infection at the time the rats were sacrificed. Scriba (1981) has made similar observations. She reported that HSV may persistently infect ganglia of guinea pigs, defined as the frequent isolation of HSV from the skin at the site of

inoculation. In our experiments, it was not possible to exclude the possibility that persistent infection may have also existed in rats. The second possibility was that the apparent productive infection in "latently" infected rat ganglia was due to spontaneous reactivation of virus within some ganglia. The observation of spontaneous reactivation in mice has been previously reported by Hill et al (1975 and 1980) who described intervals up to 18 days for CPE to appear in monolayers on which ganglia removed 28 days after initiation of infection were cocultivated.

This rat model of human cutaneous and contiguous sensory ganglion infection was characterized by occasional fatalities preceded by paralysis probably due to encephalitis, consistent but not completely uniform, contiguous ipsilateral sensory ganglia infection and occasional contralateral ganglion infection. Immunohistologic staining demonstrated HSV antigen, primarily in small type neurons but not also Schwann cells of nonmyelinated axons as reported by Cook and Stevens (1973), by the third day after peripheral inoculation, consistent with intervals of 2 (Kristensson et al, 1978) up to 8 days previously reported (Itoyoma et al, 1984; Cook and Stevens, 1973). Latent ganglion infection was demonstrated after intervals as long as 52 days post-infection. Immunohistologic staining of latent infected ganglia immediately after removal did not demonstrate HSV antigen sufficiently to enable us to demonstrate which cells harboured latent HSV. However, studies of ganglia stained at intervals after initi-

ation of cocultivation consistently demonstrated HSV antigen in small type neurons.

A conservative interpretation of these results would be that the virus recovered in cell culture originally came from the cocultivated ganglia. However, we could not, at this time, be certain whether neurons staining for HSV antigen represented cells containing endogenous reactivated virus or cells that had been reinfected from the infected monolayer. Since few neurons contained antigen and others have reported that 0.1% of neurons in the ganglia of mice are infected with HSV (Walz et al, 1976; Klein, 1982), we suspect the former explanation to be more correct. In support of the former explanation was the observation that ganglia removed from infected monolayers as early as possible after recognition of CPE (hours after initiation of cocultivation) stained positive for HSV antigens.

In our infected rats, topical capsaicin applied to the dorsal hind paw skin did not affect the establishment of lumbar ganglion infection after subsequent injection of HSV into the same skin site. This was not unexpected since the ability of capsaicin to destroy small type neurons is observed only in neonatal animals (Jansco et al, 1977; Nagy et al, 1981) and only such a neuropathic effect altered the course of HSV infection in mice (Ljungdahl et al, 1986). That capsaicin applied directly to the sciatic nerve in our animals also was without effect was surprising since transient neuronal dysfunction has been observed in such situations.

However, since no positive control was included, it is uncertain whether our inability to demonstrate an effect was due to the treatment regimen used or an inability of capsaicin to affect HSV transport.

In rats with latent HSV infection, manipulation and sectioning of the ipsilateral sciatic nerve did not shorten the time for CPE to appear around cocultivated ganglia as has been described in mice (McLennan and Darby, 1980; Klein, 1982). Since HSV reactivation occurs in patients after trigeminal ganglion manipulation for treatment of tic doloureux, in this regard, mice may be a better model of HSV infection than rats. By contrast, incubation of latently infected ganglia with hexamethylene-bis-acetamide reduced the interval required for CPE to appear in the substrate monolayer and indirectly supported the theory that methylation, which this compound interferes with, may be important in the maintenance of HSV latency.

Restraint stress at 4°C for 4 hours activated latent HSV lumbar ganglion infection, caused gastric ulcers and elevated plasma CS concentrations. This observation provides direct support to the concept of stress-induced reactivation of cutaneous genital or orolabial HSV infection derived from clinical studies (Luborsky et al, 1976; Silver et al, 1986; Duer, 1982; Taylor, 1978; Goldmeier and Johnson, 1982). Whether control of stress in patients would obviate or reduce recurrences of cutaneous HSV infection has not been tested but should theoretically be effective therapeutically.

The association between stress and activation of latent HSV infection was strengthened by the demonstration that exposure to 4 hours cold restraint stress produced parallel, greater effects than 3 hours exposure in all parameters: proportion dying ($p<0.01$), time to CPE and number of ulcers (16.3 versus 0), cumulative length of ulcers per rat (38.6 versus 0) and CS concentration (58.0 versus 53.8), respectively.

Since stress, in some way, modified latency in rats, the question then becomes, how did it do so? Previously, Shek and Sabisdon (1983) proposed that stress affecting the anterior hypothalamus would result in the release of corticotropin-releasing hormone (CRH) that would act on the anterior pituitary to cause release of adrenocorticotropin (ACTH). The ATCH in turn stimulated the adrenal cortex and resulted in the synthesis and release of corticosterone. The corticosterone could then bind via the glucocorticoid receptor on cells of the immune system and negatively affect them; ie., suppression, since immune surveillance has been suggested as a mechanism for controlling the latent state (Klein, 1982; Blyth and Hill, 1984). Suppression of immune function may allow, not for reactivation per se of latent virus, but for expression of virus already reactivated or, if immune surveillance were responsible for latency, suppression of immune function would permit reactivation of latent virus. This theory may have some merit if animals were studied for expression of recurrent infection following stress. However,

in rats, we have not been able to demonstrate recurrent infection and because of this, this theory may best be studied in other animal models. This hypothesis is supported by Kiecott-Glaser and colleagues' observation that the stress of writing exams and loneliness reduced natural killer cell activity in medical students (1984a and 1984b).

However, another possibile mechanism of reactivation may involve neurotransmitters. Specifically, neurotransmitters, neuropeptides or neurohormones may act on the latent virus to modify latency and result in reactivation of latent HSV.

As indicated in Tables 19, 20 and 21, we tested 3 compounds (nerve growth factor, norepinephrine and epinephrine) for their ability to modify reactivation of latent virus from rat dorsal root ganglia. We were not able to demonstrate any suppressive or stimulatory effect of these compounds, in the ranges tested, on the proportion of rats or ganglia positive for HSV or the time to CPE between treated and control groups. This finding was limited by the sample size tested and the small range of dosages studied. It may also be that these compounds are not sufficient individually to modify latency but that in combination with each other and/or other compounds, may act synergistically to modify latency.

SUMMARY

We have developed a new rat model for studying acute and latent HSV infection in dorsal root ganglia. This model appeared to be as useful and as dependable as other models currently being used for HSV research. Because of the established use of rats in laboratory research, anatomical characterization and value in pharmacological research, this model has considerable potential in helping elucidate the pathogenesis of acute and latent HSV infections, and the testing of potentially new antiviral compounds directed against HSV infection.

Using the rat model, we have identified a population of neurons that appear to be selective substrate cells for HSV infections during both the acute stage of virus infection and during reactivation of latent HSV in vitro. These cells are identified as small type B unmyelinated neurons. HSV antigen was detected in these neurons as early as 58 hours post-inoculation using the technique of PAP, whereas control neurons did not have any antigen.

In addition, it was possible to demonstrate that stress could reactivate latent HSV infection in ganglia of animals cold restraint stressed for 4°C evidenced by a reduction in the time for CPE to appear in monolayers on which ganglia were cocultivated. This effect of stress on latent HSV infection was paralleled by other known effects of stress: increased number and severity of stomach ulcerations, and levels of corticosterone.

Initial attempts to demonstrate a role for a limited number of endogenous neuropeptides and neurohormones in accelerating the time for CPE to appear when applied individually to ganglia *in vitro* did not suggest a role for any of them.

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XIII. APPENDIX 1

Capsaicin (1 ml)

15 mg capsaicin

0.1 ml ethanol

0.1 ml Tween 80

0.8 ml Sterile saline (0.90%)

15% Sucrose (100 ml)

15 g sucrose

20 ml phosphate 0.5 M (PO_4)

1 mg sodium azide

65 ml distilled H_2O

PBS - 10% Triton (100 ml)

10 ml saline (0.9%)

20 ml 0.5 M PO_4

70 ml distilled H_2O

1 ml triton

4% Paraformaldehyde (100 ml)

4 g paraformaldehyde

80 ml distilled H_2O

20 ml 0.5 M PO_4

0.1-0.2 ml NaOH

APPENDIX 1 (continued)

Prefix (100 ml)

10 ml saline (9%)

10 ml 0.5 M PO_4

80 ml distilled H_2O

0.1 g $NaNO_2$

10 ul heparin

PBS (100 ml)

10 ml saline (9%)

20 ml 0.5 M PO_4

70 ml distilled H_2O

APPENDIX 2

Media

5% FBS

1% pen/gent

Epinephrine (M.W. = 183.2)

1 g into 100 ml media = 54.6 mM

1 ml of 54.6 mM + 53.6 ml media = 1 mM

10 ml of 1 mM + 90 ml media = 100 uM

10 ml of 100 uM + 90 ml media = 10 uM

10 ml of 10 uM + 90 ml media = 1 uM

Norepinephrine (M.W. = 169.18)

1 g into 100 ml media = 59.1 mM

1 ml of 59.1 mM + 58.1 ml media = 1 mM

10 ml of 1 mM + 90 ml media = 100 uM

10 ml of 100 uM + 90 ml media = 10 uM

10 ml of 10 uM + 90 ml media = 1 uM

Nerve Growth Factor (from Mouse Submaxillary Gland - 2.55)

0.01 mg + 100 ml media = 100 ng/ml

30 ml of 100 ng/ml + 30 ml media = 50 ng/ml