Chronic latent herpesvirus infections and their activation
by Patrick Herbert Cleveland

A thesis submitted to the Graduate Faculty In partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE in Microbiology
Montana State University
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Abstract:
The experiments reported in this paper were designed to demonstrate the nature of long term herpesvirus infections and to clarify their mode of activation.

Rabbits were inoculated intramuscularly and intradermally with several herpes simplex virus strains. Paralysis developed in about 70% of the rabbits. Some weeks or months later, injections of adrenalin increased the severity of paralysis in paralyzed rabbits and stimulated paralysis in symptomless rabbits that received the virus but had not developed a clinical reaction initially.

Virus could not be isolated from the central nervous systems of these rabbits. This experiment illustrates that herpes simplex strains inoculated extraneurally are almost always capable of establishing a clinical or subclinical long term infection of the central nervous system. The mode of adrenalin activation of paralysis is not known though an attempt was made to clarify the problem.

Prolonged treatment of 9 paralyzed rabbits several months after viral inoculation with predef 2X R precipitated a fatal herpes myelitis in 1 of the 9 rabbits. Virus was isolated from the central nervous system of this rabbit. The circumstances involving the activation of this long term infection strongly indicated that herpes simplex virus can cause chronic latent infections in rabbits.

Attempts to activate long term herpes simplex and equine herpesvirus type I infections in mice with adrenalin and cortisone injections were unsuccessful.

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ABSTRACT

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INTRODUCTION

The herpes virus group may be defined as the collection of viruses that possess an icosahedral shaped capsid, formed by 162 subunits or capsomeres. The capsids are usually surrounded by a loose outer envelope originating from one of the host cell membranes. Two further criteria that perhaps should be added are (1) the genome consists of deoxyribonucleic acid and (2) viral reproduction takes place within the host nucleus. The latter characteristic is morphologically defined by intranuclear inclusions in infected cells.

The viruses that are included in the herpes virus group are shown in Table I under the name of their natural host, although some of those listed have yet to be shown to fulfill all three criteria.

The clinical effects produced by the herpesvirus group vary from minor skin eruptions to fatal infections of the central nervous system, as shown on Table II. The most intriguing characteristic of the group is the ability of herpes simplex, varicella/ zoster, and the human cytomegaloviruses, to establish long term infections (chronic and/or latent* infections). The precise nature of the virus-host relationship during the infection is not known. Various investigators have speculated that the virus may enter into a provirus state with the host DNA (Plummer, 1967), others have predicted that the virus lies latent in some cytoplasmic organelle

* Latent - virus is present but is not reproducing

TABLE I. - Viruses composing the herpes group listed under the name of their natural host
(Plummer, 1967)

MAN	MONKEYS	HORSE	BOVINE	PIG
Herpes simplex	B-virus of rhesus and cynamolgus monkeys	Equine herpesvirus type 1 (rhinopneumonitis or equine abortion virus)	Infectious bovine rhinotracheitis virus (IBR)	Pseudorabies virus (Aujeszky's disease virus or mad itch virus)
Varicella/zoster (chickenpox/ shingles)	SA8 of vervet monkeys			
	Marmoset herpesvirus			
Cytomegalovirus (salivary gland virus)	Cytomegalovirus of vervet monkeys	Equine herpesvirus type 2 (LK virus)		

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CAT	DOG	GUINEA PIG	MOUSE	CHICKEN
Feline rhino-tracheitis virus	Canine herpesvirus	Cytomegalovirus	Cytomegalovirus	Infectious laryngo-tracheitis virus (ILT)

(J.

TABLE II. Comparison of the Clinical Properties of the Herpesviruses During Natural Infections of the Hosts. (Plummer 1967)

	Simplex	Vericella/zester	Cytomegalovirus	B-virus	SA8	Marmoset herpes	Vervet cytomegalovirus	Equine type 1	Equine type 2	IBR	Pseudorabies	Cat herpes	Dog herpes	Guinea pig cytomegalovirus	Mouse cytomegalovirus	IIT
Growth in respiratory tract	+	+	+	+	+?	+	+?	+	+	+	+	+	?	+	+	+
Respiratory disease	?	∓	?	?	?	?	?	+	?	+	∓	+	?	?	?	+
Inclusions in salivary glands	?	?	+	?	?	?	+	?	?	?	?	?	?	+	+	?
Vesicular skin eruption	+	+	-	+	?	?	?	-	?	+	-	-(?)	?	-	-	-(?)
Venereal disease	+	-	-	?	?	?	?	?	?	+	?	?	?	?	?	+(?)
Conjunctivitis	+	-	-	?	?	?	?	?	?	+	?	?	?	?	?	+
Invasion of foetus	?	-(?)	+	?	?	?	?	+	?	+	+	?	?	?	?	NA
Abortion	?	-(?)	±	?	?	?	?	+	?	+	+	?	?	?	?	NA
Permanent nonfatal foetal damage	?	-(?)	+	?	?	?	?	∓	?	∓(?)	∓(?)	?	?	?	?	NA
Infection of CNS	+	+	?	+	+	?	?	?	?	+	+	?	?	?	?	?
Encephalomyelitis	+	-	-(?)	±	?	?	?	?	?	+	+	?	?	?	?	?
Inflammation of dorsal nerve root	?	+	?	?	?	?	?	?	?	?	±	?	?	?	?	?
Chronic latent infection of:																
Nerve cells	+*	+	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Other cells		?	+?	?	?	?	?	?	?	?	?	?	?	?+	?+	?

* but which is uncertain

NA = not applicable

(Paine, 1964) or that the virus is continually reproducing in certain organs (Kaufman, 1967). The nature of the stimulation of virus back into rapid reproduction is unknown. Is the virus indeed being stimulated into activity and subsequently producing the paralysis or is it a stimulation of an autoimmune response that causes the paralysis?

Due to the many similarities that exist among viruses of the herpesvirus group (Table II) it would appear that, if long term infections can be established by herpes simplex virus, varicella/zoster virus and human cytomegalovirus, then other members of the group may also be capable of doing the same.

This thesis will attempt to clarify 3 facets of long term herpesvirus-host relationships; (1) whether the infections are chronic, latent or pseudolatent, (2) whether symptom development is due to a chemical activation of the virus or to the suppression of the host immune mechanism or to the stimulation of an autoimmune response, and (3) whether equine herpesvirus type I can establish a long term infection and, if so, whether the infection can be chemically activated.

A. Clinical effects

The nature of long term infections can best be understood after reviewing the clinical effects of several herpesviruses. Table II may be used as a helpful guide in comparing the clinical effects of these viruses.

1. Herpes simplex

Herpes simplex virus infections are found more frequently in children than adults. The primary infection is usually clinical. After the disappearance of the initial symptoms the virus may persist in many, if not all, of the subjects. In most cases this long term infection is symptomless, but in some, lesions reappear due to the activation of virus by fever, sunlight and a variety of other stimuli (Ormsby and Montgomery, 1948; Warren et al., 1940; Keddie et al., 1941; Abraham, 1934; Blum, 1926; Van Rooyen, et al., 1941; Dunbar, 1938; Schmidt and Rasmussen, 1960; and Good and Campbell, 1948).

The mouth of young children is the most common site for the primary infection, wherein the virus causes an acute stomatitis with a vesicular eruption on the mucous membranes (Scott et al., 1941). Fever frequently accompanies such primary infections of the mouth. Hale et al. (1963) have reported that extreme irritability may accompany the stomatitis. This observation may be interpreted as a frequent sub-clinical involvement of the central nervous system.

The circulating antibody that arises and persists after the stomatitis does not prevent the reoccurance of cold sores near the primary lesion, but it does confer some degree of protection against re-infection of other sites on the body. In addition to the mouth, the virus can also establish a keratoconjunctivitis of the eye, (Ormsky, 1957); a generalized infection of the newborn with marked involvement of the liver, terminating fatally (eg. Zuelzer and Stulberg, 1957; Bird and

Gardner, 1959; Tucker and Scofield, 1961: Bird, et al., 1963); infection of the genital organs (Slavin and Gavett, 1946; Esteves and Pinto, 1952); and, not uncommonly, vesicular eruptions on the epidermis almost anywhere on the body (Stern et al., 1959; Hambrick et al., 1962; Selling and Kibrick, 1964; Dyke et al., 1965; Wheeler and Cabaness, 1965).

The strains of herpes simplex virus have been divided into two subtypes by comparison of cross neutralization reactions (Schneweis, 1962; Plummer, 1964). Recently Dowdle and Pauls (in press) have shown that this division also has a clinical significance in that the subtype 2 strains are more commonly associated with the veneral form of the infection and that subtype 1 strains are more commonly associated with cold sores in the mouth region.

Experimental inoculation into the skin or muscle of rabbits of subtype 1 and subtype 2 strains of herpes simplex revealed that MS strain (subtype 2) was more neurovirulent than the L_2 strain (subtype 1) (Plummer and Hackett, 1966). The paralysis produced by the neurovirulent MS strain was due to inflammation and damage of the corresponding dorsal (sensory) nerve roots, ganglia, and horns. The affinity for the dorsal nerve roots, ganglia and horns is very similar to that of varicella/zoster during shingles and of pseudorabies infections of cats and dogs.

2. Varicella/zoster virus

Varicella/zoster virus infections are characterized by extensive vesicular lesions of the skin in the primary infection (chickenpox).

A more generalized infection occurs with the newborn, and, as with
herpes simplex, it terminates fatally. A common complication of
varicella in adults is pneumonitis (Weinstein and Meade, 1956; Krugman
et al., 1957; Carstairs and Edmond, 1963).

Infection of the central nervous system takes the form of inflam-
mation of the dorsal nerve roots and ganglia, resulting in pain and
pruritus accompanied by vesicular lesions on the areas of skin correspond-
ing to the ganglion or ganglia that are affected, (Head and Campbell,
1900). This syndrome is referred to as zoster or shingles.

Until recently it was felt that chickenpox and shingles were
caused by two different agents; but it is now thought that zoster is
the manifestation of latent varicella virus (Weller and Witton, 1958)
and thus parallels the cold sores that are associated with latent herpes
simplex infections. The nature of the varicella/zoster latency is not
understood although zoster can be precipitated by the inoculation of
certain drugs and arsenicals or by the growth of tumors. Due to the
common association of these viruses with the dorsal nerves, roots,
and ganglis, the question arises as to whether this is the only tissue
in which virus is able to establish latency?

3. Human Cytomegalovirus (salivary gland virus)

Human cytomegalovirus appears to cause a generally subclinical
infection in that 50 - 80% of the adult population possesses antibody
without obvious symptoms. The urine of mothers of children with
cytomegalic inclusion disease have often been found to contain virus

for several months (Medearis, 1964; Weller and Hanshaw, 1962). These

observations suggest that the infection is of long duration.

4. Equine herpesvirus Type I

Equine herpesvirus type I produces a rhinopneumonitis accompanied

by fever in young horses, but in older animals the infection is usually

subclinical. When a pregnant mare is infected, the virus may invade

the fetus and cause abortion (Doll et al., 1957). The period between

the respiratory infection of the pregnant animal and abortion ranges

from 1 to 4 months, though experimental intravenous inoculations

cause abortion in only 4 days (Doll and Bryans, 1962). There is

extensive viral invasion of the fetus with intranuclear inclusions

and areas of necrosis particularly noticeable in the liver.

The virus multiplies readily in suckling hamsters with marked

involvement of the liver and spleen. The infection is generally fatal

(Doll et al., 1953).

The involvement of the liver and spleen is reminiscent of human

cytomegalovirus infections. The ability of equine herpes type I to

establish a long term infection is uncertain.

5. Infectious bovine rhinotracheitis virus

Infectious bovine rhinotracheitis has many clinical properties

in common with other herpesviruses (see Table II). It is not clear if

the virus can cause a long term infection though Snowden (1964) was

able to recover it from the vagina of one cow on two occasions separated

by 11 months.

6. Mouse cytomegalovirus

Mouse cytomegalovirus inoculated intraperitoneally into young mice will produce death with lesions in the connective tissue, liver, spleen and adrenal glands. Inoculation of adult mice does not produce symptoms. An infection of long duration in young mice was apparently obtained by Brodsky and Rowe (1958). Eight days after inoculation, virus was isolated from mouth swabs and salivary glands of infected mice. Inclusion bodies were also seen in the salivary glands. After about 4 months no inclusion bodies were seen in the salivary glands, though virus could be isolated from these glands and from mouth swabs for up to a year after infection.

B. Theories of virus-host relationships

Little is known about the actual virus-host relationship during long term infections by the herpesviruses. There are 2 situations presently described in the literature which define certain virus-host relationships as chronic infections (Kaufman, 1967) and chronic latent infections (Paine, 1964; Plummer, 1967). In addition to these, the writer feels that a third situation termed pseudolatency may develop in infected animals. In order to discuss these relationships, a clear understanding of the terms involved is necessary.

Chronic infections are those of long duration, in which the virus causes the host to express clinical symptoms. In these cases the virus

is reproducing at a sufficient rate to allow virus isolation. Latent

infections are those in which virus is present but not reproducing,

and can not be isolated. Chronic latency is the persistence of virus

in the latent form for an extended period of time. Virus in this form

may manifest itself in response to a stimulus. Pseudolatent infections

are those in which the virus is reproducing at such a slow rate that

the host would not exhibit symptoms and virus can not be detected.

1. <u>Chronic and pseudolatent infections</u>

Briefly, the evidence for chronic infections relates to the obs-

ervations that viruses can be frequently isolated over a period of time

from the host, i.e. herpes simplex virus from rabbits and humans,

human cytomegalovirus from humans and mouse cytomegalovirus from mice.

Kaufman <u>et al</u>. (1967) have reported frequent (15%) but not

continuous viral isolations from the precorneal tear film of rabbits

infected corneally with herpes simplex, during a period 25 to 95 days

post inoculation. The episodes of viral shedding were only occasionally

accompanied by lesions detectable with the slit-lamp microscope. They

also observed a similar situation in precorneal tear film and saliva of

35 human volunteers. From these observations Kaufman <u>et al</u>. have sug-

gested that chronic virus multiplication in structures such as the

lacrimal and salivary glands, rather than latency, may cause recurrent

herpetic disease.

An analagous study on human cytomegalovirus infections of infants

and their mothers revealed a much higher frequency of virus isolation

than Kaufman obtained (Medearis, 1964). In addition to more frequent
virus isolations Medearis also was able to detect the virus in some of
the subjects 33 months after the initial isolation (see Table III).

Another interesting observation along these lines was that of
Schmidt and Rasmussen (1960). The experimental procedure entailed
intramuscular inoculation of rabbits with herpes simplex to build up a
protective immunity followed by a subsequent intracerebral inoculation.
Some weeks or months later some of the rabbits were given adrenalin.
Six of 10 rabbits receiving adrenalin died of herpes encephalitis.
Rabbits not receiving adrenalin did not exhibit any central nervous
system symptoms, but virus could be isolated from their brains. This
report suggests that in a chronic infection the severity of symptoms
can be increased by adrenalin inoculations.

Perhaps the best evidence for pseudolatent infections was pre-
sented by Ashe and Rizzo (1964) in their studies on herpes simplex in
rabbits, where they inoculated the virus onto the duct of the sub-
maxillary gland. Virus was frequently isolated from the saliva of
some animals up to 43 days after inoculation, but afterwards no virus
was isolated nor was there histological evidence of the virus. The
fluctuation of the titers of neutralizing antibody however, indicated
viral activity was continuing in some of the rabbits. The reliability
of neutralizing antibody as an indicator of viral activity was apparently
supported by their observation that, when ultraviolet inactivated herpes
simplex was injected into rabbits, the neutralizing antibody that arises

TABLE III. - Isolation of Cytomegalovirus from Throat Swabs and Urine Specimens (Adapted from Medearis, 1964)

	Throat Swabs			Urine Specimens		
	Number of positive infants or mothers/ number of subjects	Maximum duration of excretion	Percentage positive of total number of samples	Number of positive infants or mothers/ number of subjects	Maximum duration of excretion	Percentage positive of total number of samples
Infants with neonatal disease	6/7	17 months	40	6/6	33 months	87
Their mothers	6/7	10 months	17	5/7	10 months	53
**						

failed to persist for more than 4 to 5 weeks.

An interesting sidelight of Ashe and Rizzo's work was the isolation of herpes simplex from the saliva of 2 rabbits 747 and 996 days following intraperitoneal inoculation.

Perdrau (1938) reported that attempts to isolate virus from the brains of rabbits with chronic herpes encephalitis were unsuccessful if the specimens were fresh; but after a month of storage at 4°C in 50% glycerol solution, these same specimens were positive. Perdrau hypothesized that this was due to the inactivation of some immune component of the brain during the storage period.

2. Latent and chronic latent infections

The evidence for the latent infection theory is based primarily upon negative results. If virus is latent in a provirus state it could not be isolated as the virus would be non-infectious (Lwoff et al., 1959; Paine, 1964). The possibility of the viral DNA attaching itself to the host DNA does not appear unreasonable as a similar situation exists with certain bacteriophages, (Adams, 1959). The guanine/cytosine (G/C) content of the nucleic acid has been measured for several of the herpesviruses (Russell and Crawford, 1964; Crawford and Lee, 1964). The G/C content is high (about 70 moles per 100 moles nucleic acid) for herpes simplex, pseudorabies, and infectious bovine rhinotracheitis, but for the two horse viruses and for human cytomegalovirus it is significantly lower (about 56 moles per 100 moles nucleic acid). A possible explanation for this difference would be the incorporation of large

portions of host cell DNA (which has a low G/C content) into their

genomes. This would be definitely suggestive of human cytomegalovirus

DNA - host DNA attachment, thus supporting the provirus theory of

latency (Plummer, 1967).

The evidence in favor of completely latent infections is

unsatisfactory, in that neutralizing antibody titers were not observed

nor were sufficient numbers of viral isolations attempted (Ormsby

and Montgomery, 1948; Warren et al.,1940; Keddie et al., 1941; Abraham,

1934; Blum, 1926; Van Rooyen, et al., 1941; Dunbar, 1938; Schmidt

and Rasmussen, 1960; and Good and Campbell, 1948). The activation of

zoster by tumors, arsenical and drugs as mentioned earlier, is however

indicative of the activation of a latent infection. The common reoccur-

rence of cold sores due to non-specific stimuli is also indicative of the

activation of a latent infection.

The work of Schmidt and Rasmussen (1960) may also be interpreted

in a some what different light then that mentioned earlier. That is,

viruses that were inoculated intracerebrally lie latent as an infectious

particles in the cytoplasm of brain cells. The subsequent injection

of adrenalin then somehow stimulates the viruses into active reproduction.

This alternate mechanism of latency would explain why virus could be

isolated from rabbits not receiving adrenalin.

The isolation of infectious bovine rhinotracheitis virus from the

vagina of a cow on two occasions separated by 11 months, and the 1 to

4 month delay from respiratory infection to abortion with equine herpes

type I in pregnant horses, represent two, of many cases where the study
was not extensive enough to determine the approximate nature of the
long term virus-host relationship.

3. Long term infection in tissue culture

To what degree the observations on persistant infections "in vitro"
can be applied to "in vivo" infections is uncertain, but there are
suggestive analogies which support and clarify some of the speculations
made in the preceeding sections. In tissue culture, the standard
technique used to produce a chronic infection is to add neutralizing
antibody to the medium (Wheeler, 1960). The effect of changing tissue
metabolism on the establishment and reactivation of a latent infection
was presented by Pelmont and Morgan (1959). These authors showed that,
when HeLa cells were placed in a nutritionally deficient medium,
infection with herpesvirus did not result in the expected cytopathic
effect, nor could virus be isolated by passage to fresh cells (latent
infection). If, however, the missing nutrients were restored to the
infected culture, after several passages in the deficient medium, the
virus became detectable and caused the characteristic cytopathic effect.

Another mechanism contributing to the development of cellular
resistance to destruction by herpes simplex is the production of inter-
feron. In 1962 Barski and Cornefert reported that when a low cancer line
of mouse cells (N_2) was infected with polyoma virus, a latent infection
was regularly produced and that these cells resisted further infection
both by polyoma and herpesviruses. This they attributed to the

production of a virus-inhibiting substance similar to interferon.
Glasgow and Habel (1963) described a continuous line of mouse embryo
cells, chronically infected with polyoma virus (carrier culture 23-P),
which partially resisted challenge by herpes simplex. After infection
with herpes simplex at low multiplicity, an incomplete cytopathic effect
resulted and cells "grew out", leading to a double carrier culture that
elaborated both polyoma and herpes simplex and was resistant to reinfec-
tion by either virus. The equilibrium between the growth of the
viruses and the resistance was unstable, so that when the culture was
"cured" of it's polyoma infection, it was immediately destroyed by
the herpes simplex. It was suggested that polyoma and herpesviruses were
weak producers of endogenous interferons so that resistance was only
achieved by the additive effect of both viruses. A herpesvirus carrier
culture could be produced in polyoma-free susceptible cells by adding
sufficient exogenous interferons. In rabbits, if ultraviolet-irradiated
influenza (Lee) virus was applied to one eye within 24 hours after infect-
ing both corneas with herpes simplex, and reapplied 4 times daily for
4 days, the lesion of the (Lee) virus treated eye was much less marked
than in the untreated eye. This was presumably the result of the activity
of interferon produced by the cells infected with the attenuated
influenza virus (Tommilia and Penttinen, 1962).

C. Methods of activating long term infections

There have been numerous reports in the past concerning factors which activate herpes infections. A list of factors which activate long term infections include: febrile diseases, artifical fever therapy, menstruation, vaccine administration, emotional stress, trauma, severe sunburn, ultraviolet light, allergic stages, chemical irritants, secondary dentition, coitus, trichlorethylene anesthesia, digestive disturbances, adrenalin and anaphylactic shock (Ormsby and Montgomery, 1948; Warren et al., 1940; Keddie et al., 1941; Abraham, 1934; Blum, 1926; Van Rooyen, et al., 1941; Dunbar, 1938; Schmidt and Rasmussen, 1960; and Good and Campbell, 1948). Only a few of these have been adequately documented.

Schmidt and Rasmussen (1960) used rabbits with chronically infected brains to test several chemicals, to determine whether they could activate the infection in rabbits. The chemicals tested included suprarenin (a synthetic adrenalin), pyromen (a bacterial pyrogen), hydrocortisone acetate (an immunosuppressive drug), glutathione (a reducing agent), and sterile saline.

The chemicals were injected over a relatively short period of time (1 to 6 days depending upon the chemical). The activation of the virus, manifested as symptoms of the central nervous system prior to death and the isolation of relatively high virus titers from the brain, occured in 6 to 10 rabbits receiving adrenalin 24 to 160 days after intracerebral

inoculation.

Good and Campbell (1948) were also able to activate a latent

herpetic encephalitis in guinea pigs by anaphylatic shock.

The immunosuppressive drugs, hydrocortisone acetate and

methotrexate when inoculated over an extended period into mice infected

with mouse cytomegalovirus, promoted more extensive tissue damage and

increased the titer of recoverable virus (Medearis, 1964; Henson, 1967).

The mode of cortisone action to enhance viral infections is relatively

unknown, because of the diversity of effects it produces within the

body. It has been shown that cortisone has an inhibitory effect on the

production of interferon in embryonated eggs, in a continuous line

of rat embryo cells, and in chick embryo cells in tissue culture,

(Kilbourne et al., 1961; Demaeyer and DeMaeyer, 1963; and Reinicke, 1965).

The suppression of interferon production may simply be a manifestation of

cortisone's ability to stimulate or inhibit RNA and protein synthesis

"in vivo", depending on the organ considered. The liver is an example

of a tissue in which RNA and protein synthesis are stimulated, while in

the thymus and lymph nodes, they are inhibited (Tremolieres et al., 1954;

Feigelson, et al., 1962; Feigelson and Feigelson, 1963; Pena, et al., 1964;

Kidson, 1965).

It would seem quite likely in view of the action of cortisone against

the immune mechanism that its use in a chronic or pseudolatent infection,

would upset the balance between the virus' invasiveness and the host's

immune mechanism, thus giving the virus an advantage (Goodman and Gilman,

1955; Medearis, 1964; Henson, 1967). In truly latent infections the use

of cortisone would presumably have no effect.

An alternative theory to viral activation is an autoimmune response.

This theory is supported by Nagler's report in 1944 of the development

of a tuberculin-like skin test in recovered patients who were inoculated

intradermally with heat killed herpes simplex. This hypersensitivity has

also been confirmed by Brown (1953) in guinea pigs. Since then, Tokumaru

(1963) has separated the heat killed virus into 3 fractions by diethyla-

minoethyl (DEAE) - Sephadex column chromatrography. One fraction,

eluted in 0.11 m NaCl, had a high sensitizing ability but a low

complement fixing ability; another, eluted in 0.27 m NaCl, had a high

complement fixing activity and high sensitizing ability. The viral

antigen, eluted in 0.35 m NaCl, had little sensitizing ability but a

high complement fixing activity. It may be inferred from this report

that the antigenic fraction responsible for the delayed type hyper-

sensitivity is not the virus specific antigen but may be a group specific

antigen or possibly a new viral induced host cell antigen. Roane and

Roizman (1964) have demonstrated that herpes simplex infected cells do

indeed have a new host antigen induced by the virus, present on their

cytoplasmic membranes.

The histological picture of delayed-type hypersensitivity reactions

showed a dense collection of cells, consisting in the main of perivascular

masses of mononuclear cells (i.e, macrophages and lymphocytes) (Humphrey

and White, 1964). It is interesting that this description fits Head and

Campbell's (1900) observations on zoster. It should also be pointed
out that delayed-type hypersensitivity reactions do not exhibit a
readily demonstrable relation to circulation antibody (i.e. it is
usually associated with a cell fixed antibody).

With evidence for the presence of a new cell antigen induced by
herpes simplex and the ability of heat killed herpes simplex virus to
cause a delayed-type hypersensitivity reaction in mind, it seems likely
that the lessions observed in herpetic infections may have an auto-
immune etiology.

One of the theories for adrenalin activation fits nicely into
the autoimmune theory. It has long been established that small amounts
of adrenalin serve to contract blood vessels. When large doses are
applied, the contraction is more dramatic and damages the vessel walls
causing a later dilation resulting in hemorrhage, thus allowing white
blood cells and antibody access to central nervous system tissues (Good-
man and Gilman, 1955). This break down of the blood brain barrier would
expose hither to unexposed antigens to the immune mechanism, and thus
precipitate an autoimmune disorder.

Another theory of adrenalin activation would be that of a direct
chemical stimulation of latent viruses. Yet another theory was
proposed by Schmidt and Rasmussen (1960). This theory suggests that
the vasoconstrictive activity of adrenalin creates a reducing environ-
ment by impeding the flow of oxygen to the tissues. Perdreau (1931)
had shown earlier that herpes simplex which was inactivated in an

oxidizing environment could be reactivated in a reducing environment.

D. Purpose

The purpose of the work done in this thesis was to determine the nature of long term herpes virus-host relationships and the subsequent activation of clinical symptoms with adrenalin and immunosuppressive drugs. The problem was approached by inoculating adrenalin and immunosuppressive drugs into rabbits and mice that had been previously inoculated extraneurally with one of several herpesviruses.

METHODS AND MATERIALS

Viruses - The MS strain of herpes simplex subtype 2 was isolated
by Dr. M. Gudnadottir of the University of Iceland from the central
nervous system of a patient suffering from multiple sclerosis. Since
that time, the virus has been passed fourteen times in rabbit kidney
tissue cultures, and at present, yields a titer of 3.3 Log_{10} PFU/ML in
rabbit kidney tissue culture. The L2 strain of herpes simplex subtype
1 and the US strain of herpes simplex subtype 2 were isolated in Russia
(Shubladze, Maevakaya, Ananov and Volkeva, 1960). The number of passages
performed in tissue culture is unknown. For these studies the virus
stocks of L2 and US were grown in rabbit kidney tissue culture and had
titers of 6.0 and 3.9 Log_{10} PFU/ML respectively. The equine herpes-
virus type 1 was isolated by Doll, Bryans, McCullum and Crowe (1947).
Neither the number of passages performed in tissue culture nor the
passages performed in live hamsters are known. The virus stock was
grown in rabbit kidney tissue culture and had a titer of 6.0 Log_{10} PFU/
ML in rabbit kidney tissue culture.

Animals - The rabbits used in this work were obtained from rabbitries
surrounding Bozeman and possess varied pedigrees. The adult rabbits
weighed from 2.5 to 4.5 kilograms. Rabbits of either sex were used.

The mice were all of the Swiss Manor strain and were originally
obtained from the Manor farms Statsburg, N.Y. in 1964. The hamsters were
classified as Golden Syrian hamsters.

Inoculation of virus, adrenalin and predef 2X[R] into rabbits - The
inoculation of virus into the muscle or skin is described in the results.

Adrenalin (1/1000 solution, Parke, Davis and Co.) was administered

subcutaneously, 2.5 or 3.0 mg per animal, given as 5 inoculations of

0.5 ml, or as 3 inoculations of 1.0 ml, each injection was separated

by 3 hours. The areas of the body used for these inoculations were

the nuchal region or the legs, though never the left back leg which

was the site of virus inoculation. Predef 2x$^{\text{®}}$ (2 mg per cc, Upjohn)

was inoculated intraperitoneally, 2 ml per animal and given every day

for the first week, then every other day for 3 months. The drinking

water of rabbits receiving predef 2x$^{\text{®}}$ contained either 300 units/ml

of penicillin, streptomycin or 0.2 mg/ml of terramycin (Pfizer) to

prevent bacterial infections.

Inoculation of virus, adrenalin, and hydro-cortisone acetate into

mice and hamsters - The inoculation of the various viruses intra-

muscularly, intraperitoneally, intracerebrally and subcutaneously is

described in the results. Adrenalin (1/1000 solution, Parke, Davis and

Co.) was administered subcutaneously in doses ranging from 1 mg to 3 mg

per animal, given as 2 to 3 inoculations each separated by 4 hours. All

injections were administered subcutaneously at the nuchal region. Hydro-

cortisone acetate (5 mg/ml, Wolins) was inoculated intraperioneally .02

ml/gram of body weight, and given every day for the first week and every

3rd day thereafter. The drinking water of these animals was supplemented

with either penicillin, streptomycin, or terramycin as previously

described for the rabbits.

<u>Methods for the isolation of virus from tissues of rabbits</u> - The

lower parts of the spines and the proximal parts of the spinal nerves

were aseptically removed from freshly chloroformed rabbits, and

approximately 20% suspensions were made in sterile physiological saline

using Tenbrock grinders. Volumes of 0.1 ml of undiluted, 10^{-1} and 10^{-2}

dilutions were inoculated into petri dish cultures of rabbit kidney

tissues. The medium was changed 24 hours later. The cultures were

observed for cytopathic effect for one week. The remaining undiluted

suspension was stored at $4^{o}C$ in a 50% glycerol solution for one month.

At this time it was inoculated again on to cultures of rabbit kidney

tissue and embryonic mouse tissue culture, and the above preceedure was

repeated. The tissue cultures were initiated in 199 medium, plus 10%

lamb serum, .20% sodium bicarbonate 100 units of penicellin, streptomycin,

and 100 units of mycostatin, and maintained with the above medium with

5% lamb serum and .22% sodium bicarbonate.

<u>Method for the isolation of virus from mice and hamsters</u> - Brain,

liver, lung, and salivary gland specimens were aseptically removed from

either freshly chloroformed animals or animals that had just died. The

tissues were minced finely in 1 ml of sterile saline with surgical scissors.

Urine and blood specimens were also obtained and diluted 1:10 in sterile

physiological saline. A volume of 0.2 ml of the resultant suspensions

were inoculated into petri dish cultures of rabbit kidney tissue. The

medium was changed 24 hours later. The cultures were observed for a

cytopathic effect for 9 days.

Serum neutralization tests - Doubling dilutions of rabbit serum
were incubated for 1½ hrs at 37°C with 50 PFU/0.2 ml of virus in each
dilution. The mixture was then inoculated into petri dish cultures of
rabbit kidney cells and a methocel/199 medium overlay was applied
to facilitate plaquing.

Histopathology - Rabbits were perfused with approximately 300 ml of
a 10% formaldehyde 1% acetic acid fixative. Sections of the spinal cord
were then removed and allowed to fix 48 hrs in the formaldehyde/acetic
acid solution. Brain, liver, salivary glands and kidney specimens from
mice and hamsters were also fixed for at least 48 hours in the form-
aldehyde/acetic acid solution. The fixed specimens were dehydrated in
graduated solutions of alcohol and xylene, mounted in paraffin and
sectioned to 6μ on a Spencer Model 820 microtome. The sections were
stained with hematoxylin/eosin or thionine.

RESULTS

The results of this work are divided into two parts. The first part contains the results of studies on long term infections with herpes simplex strains and the activation of these infections. The second part consists of attempts to establish long term infections with equine herpesvirus type I, and a description of the pathogenesis of this virus in mice and hamsters.

I. Results of herpes simplex strains in rabbits and mice

A. Rabbits

1. Paralysis of rabbits with different strains of herpes simplex

The subtype 2 herpes simplex strains MS and US were inoculated intramuscularly into the femoral region of the left back leg (0.5 ml per animal) of 6 and 10 adult rabbits respectively. Paralysis of that leg developed in 5 of the 6 MS inoculated rabbits and 7 of the 10 US inoculated rabbits. The paralysis became evident 8 to 15 days after inoculation. The paralysis seemed to be of the spastic type and varied in severity from slight to almost total. In the more severely paralyzed animals there was considerable loss of sensation in the paralyzed limb.

The MS virus was introduced into the shaven skin of the lower femoral region of the left back leg of 18 rabbits by scratching a drop of virus suspension into the skin. Ten developed paralysis between 13 and 33 days (two of these failed to show the paralysis until 30 and 33

days).

Ten rabbits (8 weeks of age) were inoculated intramuscularly with
0.5 ml of the L2 virus. Nine of the 10 developed spastic type
paralysis (with one proceeding to encephalitis and death) between 7
and 17 days. Five of the 10 rabbits inoculated into the skin also
developed a paralysis. The severity of the paralysis varied over the
same range as with the MS virus.

MS virus was isolated from the lower spinal cord and nerve roots
of 5 additional animals that had been paralyzed 2 to 4 days as a result
of intramuscular inoculation of MS virus.

About 62% of the paralyzed rabbits regained some of the lost
function of the leg during the weeks subsequent to the paralysis.

2. Response of rabbits to inoculation with adrenalin

Adrenalin was inoculated (into both paralyzed and non-paralyzed
rabbits) in 5 injections of 0.5 ml, 8 weeks to 18 weeks following the
inoculation of either MS virus or L2 virus by the intramuscular or the
skin routes (Table IV). The number of animals shown in the first two
columns does not necessarily correspond to the numbers indicated in the
early part of the "results" as some were killed for histological
examination). A notable observation was that the paralysis in all
but 1 of 24 animals paralyzed by either MS or L2 became worse. Those
rabbits that had regained some of the lost movement of the leg developed
a more severe paralysis than they originally had. Eleven of 12 animals
that had not previously shown clinical symptoms developed paralysis of

TABLE IV. - The paralysis of rabbits due to the inoculation of herpes simplex viruses (MS and L2) and the subsequent increase in paralysis due to the inoculation of adrenalin.

	MS virus inoculated		L2 virus inoculated		
	intramuscularly	into the skin	intramuscularly	into the skin	totals
Number of animals inoculated	5	14	9	8	36
Number developing primary paralysis-	4	8	8	4	24
Number of days between the inoculation of virus and the inoculation of adrenalin	128	86	56	56	326
Number of animals paralyzed after the inoculation of adrenalin	5	13	9	8	35

the left back leg after receiving adrenalin. The time taken for the increase in paralysis to become apparent after adrenalin inoculations was 8 to 14 days. None of the animals died or became encephalitic, though two of them seemed to show slight paralytic involvement of the right back leg.

Six MS and 5 L2 rabbits were sacrificed in an attempt to isolate virus from their lower cords and nerve roots but no infective virus was detected from either fresh specimens or from ones that had been stored at $4^{o}C$ in 50% glycerol. This was in marked contrast to the ease of isolation at the time of primary paralysis. However, at the time of the isolation attempts (i.e. shortly after the development paralysis stimulated by the adrenalin) 58% of the MS rabbits and 75% of the L2 rabbits had neutralizing antibody at a serum dilution of 1:8, whereas none of the 5 rabbits from which virus was isolated at the time of the primary paralysis had detectable neutralizing antibody at a serum dilution of 1:4.

The successful stimulation of paralysis by the first series of adrenalin injections raised the question as to what would happen to rabbits receiving additional injections of adrenalin. When 9 paralyzed rabbits were given adrenalin as three injections of 1 ml three weeks after the first adrenalin injections, none of the rabbits exhibited a significant increase in paralysis.

In an attempt to clarify the mode of action of the adrenalin, 11 adult rabbits were inoculated intramuscularly into the femoral

region of the left back leg with the MS virus (0.5 ml per animal). After

12 days none of the rabbits were paralyzed. At 13 days following

inoculation adrenalin was inoculated as 3 injections of 1 ml. Paralysis

developed in 4 of the 11 rabbits 2 to 7 days after adrenalin treatment.

In 2 of the 4, paralysis worsened and the rabbits died 9 and 15 days

following treatment with adrenalin. Attempts to isolate virus from

these 2 rabbits were unsuccessful. None of the rabbits possessed

neutralizing antibody at a serum dilution of 1:4, 13 days after viral

inoculation. At 32 days post inoculation the only rabbits that had

neutralizing antibody (1:8 and 1:16 serum dilutions) were the 2

surviving paralyzed rabbits.

3. Response of rabbits to hypersensitivity tests

A test to determine if these rabbits were hypersensitive to MS

virus or to nervous tissue was performed on 6 rabbits, 2 of which were

paralyzed. The rabbits were shaved 43 days post inoculation and .02 ml

of homologus virus and rabbit spinal cord suspensions were inoculated

intradermally into 2 sites on the backs of these rabbits. None of the

animals developed the characteristic signs of a delayed type hyper-

sensitivity reaction.

4. Control measures

Ten rabbits not receiving virus were given 3 injections of 1.0 ml

of adrenalin to determine if adrenalin by itself could induce paralysis.

None of the 10 animals became paralyzed.

Preliminary experiments to determine the most successful stimulating dose of adrenalin have indicated that the larger the dose the better the stimulation.

5. Response of rabbits to the inoculation of predef 2X®

Predef 2X®, an immunosuppressant, was used to treat 9 paralyzed rabbits (4 MS and 5 L2). The rabbits at the start of the treatment were 218 and 176 days post inoculation respectively, and had received two series of adrenalin injections. The rabbits were treated with the predef 2X® for 72 days. After 46 days of the treatment, 1 of the L2 rabbits showed an increased paralysis and died on the 47th day. Virus was isolated from the central nervous system of this rabbit. A control experiment to determine the effect of Predef 2X® on uninoculated rabbits was not performed due to shortage of rabbits and Predef 2X®.

6. Histological examination of paralyzed rabbits

Histological examination of 14 paralyzed rabbits (10 MS, 1 US and 3 L2) revealed, in each case, inflammation or scaring of some of the left dorsal ganglia of the lumbar region, with actual destruction of some of the nerve bodies. The infiltrated cells taking part in the reaction were primarily lymphocytes. In each of the MS animals and the US rabbit, the inflammation extended into the left posterior horn and in none of these was there damage to the anterior horn. Two of the animals paralyzed by L2 virus, however, showed inflammation of the left anterior horn but not to the dorsal horn (although, as indicated above, the sensory ganglia were involved).