



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
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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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AND THEIR ACTIVATION

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PATRICK HERBERT CLEVELAND

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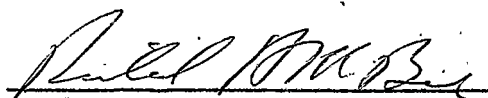
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ABSTRACT

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[®] 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.

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

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

	Simplex	Varicella/zoster	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	ILT
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 pseudol latent, (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 reoccurrence 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 venereal 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 L₂ 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 inflammation of the dorsal nerve roots and ganglia, resulting in pain and pruritus accompanied by vesicular lesions on the areas of skin corresponding 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 ganglia, 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. Chronic and pseudolatent infections

Briefly, the evidence for chronic infections relates to the observations 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 et al. (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 et al. have suggested 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 presented by Ashe and Rizzo (1964) in their studies on herpes simplex in rabbits, where they inoculated the virus onto the duct of the submaxillary 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 ¹²
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 reoccurrence 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 somewhat different light than that mentioned earlier. That is, viruses that were inoculated intracerebrally lie latent as 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 persistent 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 preceding 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 interferon. 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 reinfection by either virus. The equilibrium between the growth of the viruses and the resistance was unstable, so that when the culture was "cured" of its 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 infecting 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, artificial fever therapy, menstruation, vaccine administration, emotional stress, trauma, severe sunburn, ultraviolet light, allergic states, 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), pyrogen (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, occurred 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 pseudol latent 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 diethylaminoethyl (DEAE) - Sephadex column chromatography. 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 hypersensitivity 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 lesions observed in herpetic infections may have an autoimmune 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 (Goodman and Gilman, 1955). This break down of the blood brain barrier would expose hitherto 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 environment 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 $3.3 \text{ Log}_{10} \text{ PFU/ML}$ 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 $3.9 \text{ Log}_{10} \text{ PFU/ML}$ 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 $6.0 \text{ Log}_{10} \text{ PFU/ML}$ 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[®] 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[®] (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[®] 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 intramuscularly, 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 intraperitoneally .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.

Methods for the isolation of virus from tissues of rabbits - 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°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 procedure was repeated. The tissue cultures were initiated in 199 medium, plus 10% lamb serum, .20% sodium bicarbonate 100 units of penicillin, streptomycin, and 100 units of mycostatin, and maintained with the above medium with 5% lamb serum and .22% sodium bicarbonate.

Method for the isolation of virus from mice and hamsters - 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 $1\frac{1}{2}$ 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 formaldehyde/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		totals
	intramuscularly	into the skin	intramuscularly	into the skin	
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°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 of 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 homologous 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 hypersensitivity 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 scarring 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).

The histological examination of 6 paralyzed rabbits (4 MS and 2 L2) was done after the inoculation of adrenalin. The adrenalin inoculations had no effect upon the histological findings as the morphology of the damage done to these rabbits was identical with the nature of the damage seen in rabbits prior to adrenalin treatment.

B. Mice

1. Paralysis of mice with the L2 virus

The subtype I herpes simplex strain L2 was inoculated intramuscularly into the left back leg (0.05 ml per animal) of 37 adult mice and 132 mice 28 days old. None of the 37 adult mice became paralyzed but 59 of the 132 young mice developed a paralysis of the left back leg 6 to 10 days post inoculation. In 52 mice, the paralysis progressed to other limbs and terminated fatally. Two injections of adrenalin (0.1 ml per animal) were given subcutaneously 47 days post inoculation to 3 permanently paralyzed mice and 40 mice receiving the virus previously but not becoming paralyzed. None of the permanently paralyzed animals displayed increased paralysis nor did the non-paralyzed mice become paralyzed.

Four permanently paralyzed mice and 11 non-paralyzed mice were given hydrocortisone acetate treatment 83 days post inoculation. After 44 days of the treatment, neither were clinical symptoms manifested nor was virus isolated from these mice.

II. Results of equine herpesvirus type I in mice and hamsters

A. Attempts to establish paralysis in mice with equine herpesvirus type I

Equine herpesvirus type I was inoculated intramuscularly into the left back leg (0.05 ml per animal) of 46 mice that were 4 weeks old. The mice had been pretreated with hydrocortisone acetate 1 week prior to the virus inoculation and the treatment was continued for an additional week. Fourteen of these mice died 1 to 8 days post inoculation. The deaths of the experimental mice were presumably due to the side effect of the cortisone since six of 30 control mice on cortisone treatment also died. None of the mice became paralyzed nor could virus be isolated from their spinal cords or brains.

Intracerebral inoculations of equine herpesvirus type I (0.03 ml per animal) into 36 mice 4 weeks old produced paralysis of both back legs and hyperexcitability in only one mouse 4 days after viral inoculation. The paralysis progressed in this animal and death ensued one day later. Virus was isolated from the brain of this mouse but not from the brains of 9 other mice in the same group. An identical experiment was performed on 29 other mice that were pretreated with hydrocortisone acetate one week prior to virus inoculation. Twenty four of the 29 mice died three to 10 days post inoculation. Before death most of the mice exhibited such signs of central nervous system involvement as paralysis of the limbs or hyperexcitability. Virus was isolated

from the brains of 4 mice from a group of 16 that were cultured. Results of virus isolation attempts from 10 of the remaining 12 mice were masked by bacterial contamination of the tissue culture. The bacterial contamination may have prevented more positive virus isolation, or possibly the bacteria were responsible for the symptoms of central nervous system involvement.

Adrenalin (.03 ml per animal) was twice inoculated intramuscularly into the right back leg of 21 mice which had been inoculated intracerebrally with the virus 30 days previously. None of the mice displayed symptoms of central nervous system involvement.

The results of intracerebral inoculations of 4 day old mice with .01 ml of 10^{-0} , 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} dilutions of equine herpesvirus type I in sterile saline are summarized on Table V. The minimum number of days post inoculation until death varied from 6 to 8 days depending upon the dilution. The period of time required for all the mice given a particular dilution to die increased with the increasing dilution as illustrated in Figure 1. To establish the cause of death, viral isolations were performed on animals that had died. The lethal dose₅₀ for intracerebral inoculations of 4 day old mice as calculated by Karber equation was $10^{4.83}$ LD₅₀/1 ml which converts to 1 LD₅₀ equals 17 PFU.

TABLE V. - Mortality among 4 day old mice inoculated intracerebrally with varying dilutions of equine herpesvirus type I.

Virus dilution	Mortality	Positive virus isolation from the brain
10^0	13/13	3/3
10^{-1}	11/11	2/2
10^{-2}	9/9	3/3
10^{-3}	3/9	3/3
10^{-4}	0/10	0/3
10^{-5}	0/11	ND*
Sterile Saline	0/12	0/3

(Results expressed as vulgar fractions; numerator = positive viral isolation, denominator = number of animals examined)

*ND - not done.

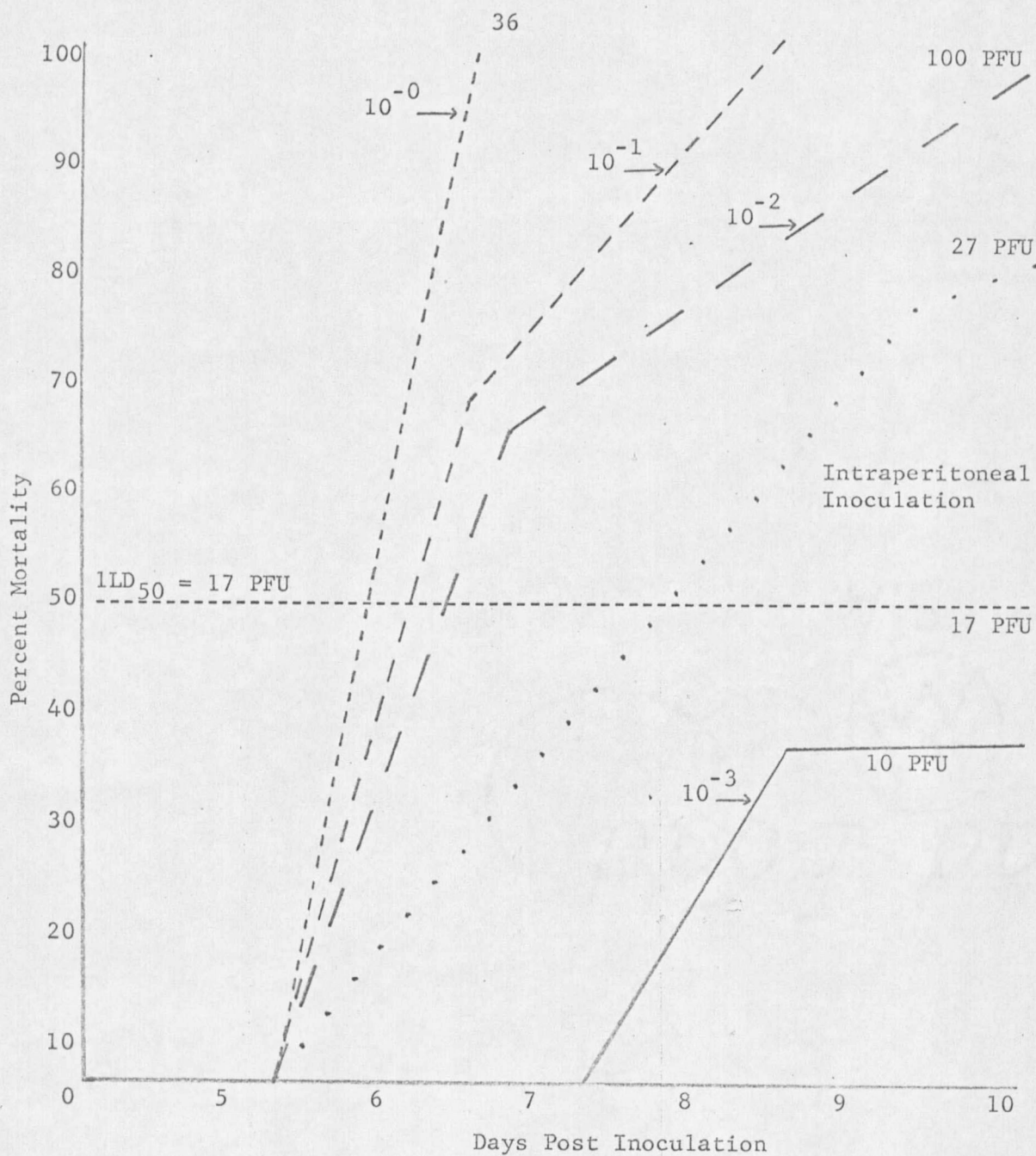


Figure 1. The figure depicts the increase in mortality in relation to time when new born mice are inoculated intracerebrally with varying dilutions of equine herpesvirus type I and when inoculated intraperitoneally with undiluted equine herpesvirus type I.

B. Pathogenesis of equine herpesvirus type I in mice

Equine herpesvirus type I was inoculated intraperitoneally (0.05 ml per animal) into 241 mice from 1 to 7 days old. Eighty percent of the mice died between the 5th and 14th day post inoculation (Figure 1). Attempts to isolate virus from brain, liver, lung, salivary glands, urine and blood specimens of 8 mice 3 to 5 days post inoculation were unsuccessful with the exception of the brain specimen of one mouse. On the other hand, positive virus isolations were obtained from brain tissues of 9 of 13 mice 6 to 13 days post injection, but none from the other specimens. The mice selected for viral isolations were chosen whenever possible from animals that appeared to be in a terminal stage of infection (i.e. symptoms of central nervous system involvement such as loss of equilibrium, drowsiness, hunching of the back, etc.).

The brain, liver, lung, salivary glands, urine and blood specimens in 10 of the 48 mice that survived to the 31st day post inoculation were tested for virus and their sera checked for neutralizing antibody to equine herpesvirus type I. The specimens of all 10 mice were negative in respect to virus and neutralizing antibody was not detected at the 1:4 dilution. Sixteen other survivors were put on extended hydrocortisone acetate treatment from the 72nd to the 104th day post inoculation. Nine of the 16 died during this period but virus could not be isolated from any of their specimens.

1. Attempts to isolate equine herpesvirus type I using the glycerol technique

The unsuccessful attempts to isolate virus from the visceral specimens of the 1 to 7 day old mice inoculated intraperitoneally prompted the use of Perdrau's (1938) glycerol isolation method.

Tissue specimens obtained from 8 mice 3 to 5 days post inoculation and 13 mice 6 to 13 days post inoculation as described earlier were placed into 199 medium plus 50% glycerol. After storage at 4°C for 1 month the specimens were minced and 0.2 ml of the mixture was placed upon embryonic mouse tissue cultures as well as the usual rabbit kidney tissue cultures. The results of these isolation attempts were identical to those obtained without glycerol.

2. Pathogenesis of equine herpesvirus type I in mice pretreated with cortisone

Forty five mice were treated with hydrocortisone acetate from the 1st to 6th day of age and then inoculated intraperitoneally with 0.05 ml of equine herpesvirus type I on the 7th day. All except one of these mice died between the 1st and the 10th day post inoculation due to either the virus or to the side effects of the cortisone. The mouse that survived, received its last cortisone injection 6 days post inoculation. Virus was isolated from 5 of 10 livers, 1 of 10 lungs and 1 of 10 urine specimens of mice 3 to 5 days post inoculation. Virus isolation attempts from brain, salivary glands and blood specimens from these mice were unsuccessful. Virus was recovered from 5 of 7 brain specimens, but in

none of the other specimens from the 6th to 8th day post inoculation group. The results obtained from precortisonized mice challenged with equine herpesvirus type I are summarized on Table VI.

C. Pathogenesis of equine herpesvirus type I in hamsters

Equine herpesvirus type I was inoculated intraperitoneally (0.05 ml per animal) into 14 hamsters 10 days old. Three days after inoculation virus isolations were attempted on 4 hamsters that appeared to be ill. The types of tissue and fluid specimens cultured were the same as those described for the mice. Virus was recovered from all the tissues and fluid specimens of 3 of the 4 hamsters except the urine specimens. Eighty seven percent of the hamsters inoculated died between the 2nd and 6th days post inoculation.

D. Mouth swab and urine isolations

The results of 18 mouth swabs and 57 urine isolation attempts taken 3 to 32 days post inoculation from non-cortisonized mice and hamsters inoculated intraperitoneally, intranasally, and subcutaneously in the neck were all negative. The urine specimen taken from the survivor of the 45 cortisone treated mice receiving virus when they were 7 days old was positive 22 days post inoculation. The only other positive urine isolation came from a 3 day post inoculated animal in the same group.

TABLE VI. - The recovery of infective virus from various tissues and fluids of mice pretreated with cortisone and subsequently challenged at 7 days of age with equine herpesvirus type I.

DPI*	Specimens					
	(Brain)	(Liver)	(Lung)	(Salivary)	(Urine)	(Blood)
3	0/5	4/5	0/5	0/5	1/5	0/5
4	0/5	0/3	0/3	0/3	0/3	0/3
5	0/2	1/2	1/2	0/2	0/2	0/2
6	3/5	0/5	0/5	0/5	0/5	0/5
8	2/2	0/2	0/2	0/2	0/2	0/2

(Results expressed as vulgar fractions; numerator = positive viral isolation, denominator = number of animals cultured.)

* Days post inoculation.

E. Control measures

To ascertain that the viruses being isolated were indeed equine herpesvirus type I, several of the isolates were mixed with rabbit anti-equine herpesvirus type I serum at a final serum dilution of 1:16. In all cases the viral isolates were neutralized by this procedure. To rule out natural infections of the mouse colony by equine herpesvirus type I, the tissue and fluid specimens of 12 control mice varying in age from 2 to 42 days old were cultured for virus on rabbit kidney and embryonic mouse tissue culture systems. All cultures were negative.

Intracerebral inoculations of 0.01 ml of sterile saline were performed on 4 day old mice to eliminate the possibility of traumatic death or sepsis contributing to the results, yielded entirely negative results.

F. Histological examinations of mice and hamsters

Histological examination of brain, liver, kidney and salivary gland specimens of all the mice and hamsters that had been used for virus isolations after receiving equine herpesvirus type I intraperitoneally are summarized on Table VII. The specimens were examined for the presence of (1) eosinophilic intranuclear inclusion bodies and (2) perivascular cuffing or infiltration of leucocytes. As seen in Table VII, only the liver of cortisone treated mice and non-treated hamsters

TABLE VII. - The results of histological examination of mice and hamsters receiving 0.05 ml of equine herpesvirus type I intraperitoneally.

Animal treatment	DPI*	Specimens							
		(Brain)		(Liver)		(Kidney)		(Salivary Glands)	
		PVC*		PVC		PVC		PVC	
		or		or		or		or	
		ICB*	INF*	ICB	INF	ICB	INF	ICB	INF
Mice inoculated from 1 to 7 days of age.	3-5	0/8	1/8	0/8	8/8	0/8	0/8	0/8	0/8
	6-13	0/12	7/12	0/12	5/12	0/12	0/12	0/12	0/12
Mice inoculated at 7 days of age (pretreated with cortisone).	3-5	0/8	4/8	6/8	8/8	0/8	0/8	0/8	0/8
	6-8	0/7	7/7	0/7	6/7	0/7	0/7	0/7	0/7
Hamsters inoculated at 10 days of age.	3	0/4	3/4	3/4	4/4	0/4	0/4	0/4	0/4

* DPI - days post inoculation
 ICB - inclusion body
 PVC - perivascular cuffing
 INF - infiltration of leucocytes

contained eosinophilic intranuclear inclusion bodies. The liver of the hamsters contained numerous inclusion bodies throughout, while those present in the cortisone treated mice were few in number and strictly confined to the area of leucocyte infiltration. The only tissue other than liver that contained any evidence of virus activity was the brain. In most of these specimens perivascular cuffing or leucocyte infiltration was evident. The number of inclusion bodies present in the liver of cortisone treated mice gradually diminished with an increase in days post inoculation until there were none present 6 days post inoculation.

DISCUSSION

The question of whether or not herpesviruses can reach the central nervous system from the skin and establish a long term infection there is important from a practical as well as from an academic point of view. The practical implications of the problem can be seen from a consideration of the frequent occurrence of herpes simplex infections in the human population. If long term herpes simplex infections of the central nervous system are established, and they are capable of being activated as suggested by Plummer (1967), then the use of agents capable of activating the infections should be contraindicated for patients with a history of herpes simplex infections.

There is little question that the MS, US, and L2 strains of herpes simplex are almost always able to reach the central nervous system after inoculation into the skin of rabbits. This observation is borne out by the adrenalin activation of paralysis in 11 of 12 rabbits that had received the virus but did not become paralyzed. The results of histological examination of the spinal cords and ganglia of these rabbits corresponds perfectly with those described in Head and Campbell's (1900) original clinical account of zoster ganglia, they include 1. extremely acute inflammation with the exudation of small round deeply staining cells, 2. destruction of ganglion cells and fibres, 3. inflammation of the sheath of the ganglion. The similarity between the activation of herpes simplex induced paralysis in these rabbits and the activation of a latent varicella virus to produce zoster is most striking.

The increased paralysis induced in one of 9 rabbits by prolonged predef 2X[®] treatment and subsequent virus multiplication suggest that long term infection established by herpes simplex virus is a chronic latent infection. This conclusion is supported by the fact that if the infection in these rabbits was chronic or pseudol latent the suppression of the immune mechanism by predef 2X[®] should have produced paralysis and death in a much larger percentage of the treated animals. Moreover, the time elapsed after predef 2X[®] treatment until paralysis should have been much shorter for the rabbit that did eventually develop paralysis. The serum antibody that was present within these animals was apparently not capable of eliminating the virus from the host, because the virus can spread from cell to cell without coming in contact with antibody (Baron, 1966). Subsequently the only immune mechanism that would be capable of controlling the infection is interferon, but interferon production is inhibited by the predef 2X[®] treatment.

The means by which adrenalin is able to increase the paralysis in paralyzed rabbits and stimulate paralysis in symptomless rabbits is obscure. Several theories may explain this phenomenon; (1) an autoimmune theory or the (2) chemical activation of a chronic latent infection.

Adrenalin acts as a vasoconstrictor when given as a small dose but when administered in larger doses the constriction is severe and damages the integrity of the vessel walls thus breaking down the blood-brain barrier (Goodman and Gilman, 1955). This characteristic of

adrenalin action supports the autoimmune theory of paralysis. The chemical similarity between adrenalin and sympathin, may also be construed to support one of the theories of chemical activation. Another theory of chemical activation was expressed by Schmidt and Rasmussen (1960). They felt that the activation via adrenalin was due to the reducing environment brought about by the vasoconstriction.

The 8 day delay between adrenalin inoculation and the first symptoms of paralysis may be interpreted as the time required for the immune system to marshal it's forces against the newly exposed antigens in the nervous tissue, or as the time required for the activated latent virus to destroy enough cells so as to cause symptoms.

The inability of the second administration of adrenalin to increase the paralysis is also obscure. When interpreted in the light of the autoimmune theory, it may be because virus has not ascended any farther up the spinal cord. If an autoimmune reaction did take place it would be at the site of the original reaction and consequently no new nerves would be affected, and no additional paralysis would develop. The interpretation of these results by the chemical activation theory is that just three weeks after the first adrenalin injection the activated latent virus is quickly controlled by a "primed" immune mechanism.

The absence of positive viral isolations from the CNS of paralyzed rabbits shortly after paralysis following adrenalin treatment may be interpreted as paralysis due to an autoimmune reaction therefore, infectious virus need not be present. An alternative is that the

paralysis is due to chemical activation of virus but the virus can not be detected because it is masked by interferon, immune lymphocytes or circulating antibody. The negative results of the hypersensitivity tests on paralyzed and non-paralyzed rabbits, though not contradictory, are not in agreement with the autoimmune theory. Perhaps the observed paralysis is due to a combination of autoimmune reaction and latent virus activation, as this theory is also consistent with the results of the adrenalin tests.

The failure of adrenalin and hydrocortisone acetate to precipitate an increased paralysis in paralyzed mice is not in line with the results obtained with these agents in rabbits. The mechanism(s) responsible for adrenalin activation of paralysis in the rabbits is apparently absent in mice. Possibly herpes simplex does not cause chronic latent infections in mice, or the cortisone treatment was not continued long enough to detect a chronic latent infection. The absence of adrenalin induced paralysis in mice and positive virus isolation from paralyzed mice treated with cortisone indicates that if there is a long term infection, it is not chronic or pseudol latent, therefore it must be a chronic latent infection. Although this reasoning is a proof by negative results it is nevertheless, a valid argument.

The attempts to establish a long term infection in mice with equine herpesvirus type I and then activate the infection with adrenalin or hydrocortisone acetate met with the same fate as herpes simplex virus in mice. These results do not suggest whether or not

equine herpesvirus type I is capable of establishing a long term infection, but they do again imply that the mouse is not the ideal host for a long term herpesvirus infections.

The isolation of equine herpesvirus type I 22 days post inoculation from the urine of the cortisonized mouse that survived both the cortisone treatment and virus inoculation suggests that a long term infection is possible with equine herpesvirus type I but it is not common. The isolation of other herpesviruses, both human and mouse cytomegaloviruses chronically from the urine has been demonstrated by Medearis (1964 a & b).

An interesting observation that elucidates in part the method of virus spread to the central nervous system (i.e., via nerve tracts or via the hematogenesis route) is that the time interval from the challenge with virus to the onset of the death phase is the same for intraperitoneal and intracerebral inoculations (10^0 , 10^{-1} , 10^{-2} dilutions) of equine herpesvirus type I. This observation along with the failure to isolate virus from the visceral tissues of intraperitoneally inoculated mice suggests that upon inoculation, the equine herpesvirus type I is directly carried to the central nervous system via the vascular system. If the virus inoculated intraperitoneally traveled by nerve tract it would presumably take longer to reach the central nervous system and consequently longer to kill the mouse. The curves in Figure 1 are in complete agreement with the vascular transport hypothesis (Johnson, 1964). The actual method of transporting the virus particle may be as

Johnson (1964) has suggested for herpes simplex in the mouse. This is the carrying of the virus to the central nervous system by infected macrophages which lodge in the brain. The intraperitoneal inoculation curve in Figure 1 may be further interpreted as depicting the amount of virus being transported to the brain from the peritoneum by comparing it with the mortality curves of the different dilutions of intracerebral inoculated equine herpesvirus type I. By this method the number of infectious particles carried to the brain from the peritoneum is approximately 27 PFU.

A fairly clear picture of the pathogenesis of equine herpesvirus type I in mice was obtained as a result of this study. The inability to isolate virus or detect inclusion bodies in visceral specimens of intraperitoneally inoculated newborn mice suggests that the virus is unable to infect these tissues. The frequent viral isolation from the brain specimens of intraperitoneally inoculated mice, demonstrates that the virus is virulent, and strikingly neurotrophic. The results of intracerebral inoculation of 4 week old mice and 4 day old mice revealed that the older mice were more than 300 times more resistant to the virus than the younger group. This finding is consistent with the reports of age resistance for the rest of the herpesviruses (Plummer, 1967). This age dependent resistance may reflect the maturing of an immune mechanism, possibly the development of efficient interferon production. The enhanced virulence of the virus in intraperitoneally inoculated mice that were pretreated with cortisone may be the result of

interferon suppression by the cortisone (Medearis, 1964; Henson, 1967).

In conclusion it must be emphasized that the experiments failed to prove that chronic and pseudolatent infections are not established by strains of herpes simplex; but they indicate strongly that chronic latent infections are established by the virus in rabbits but not in mice. The mode of adrenalin activation of paralysis remains unknown. However, these studies of adrenalin activation suggest strongly an autoimmune etiology. The evidence that supported this theory was the inability to isolate virus, the similarity of the histological picture with that reported for experimental allergic encephalomyelitis (Humphrey and White 1964), and the nature of adrenalin action on the Blood brain barrier (Goodman and Gilman 1955).

The mode of predef 2X[®] activation of paralysis may be the inhibition of interferon synthesis or activity.

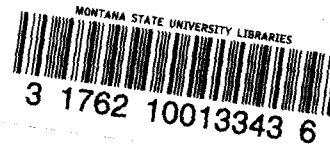
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