Herpes Simplex Virus in Rabbit Central Nervous System

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TABLE OF CONTENTS

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

Chapter 1. Comparison of Neurovirulence of Herpes Simplex Subtypes 1 and 2 in Baby Rabbits ............... 13
  Introduction ................................................................. 13
  Results ................................................................. 15
  Discussion ............................................................... 16

Chapter 2. Subtype 2 Herpes Simplex Virus in the Rabbit Spinal Cords .................................................. 18
  Introduction ................................................................. 18
  Results ................................................................. 19
  Discussion ............................................................... 22

Materials and Methods ...................................................... 24
  Primary Rabbit Kidney Tissue Culture ......................... 24
  Virus Inoculation ......................................................... 25
  Histology ............................................................. 26
  Hematoxylin and Eosin Staining ........................................ 27
  Media ................................................................. 28

Summary ................................................................. 30

References ................................................................. 39
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Neurotropic Property in Baby Rabbit of Subtype 1</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Strain of &quot;Herpes Simplex&quot; Inoculated I.M. Into</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Left Back Leg.</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Neurotropic Property in Baby Rabbit of Subtype 2</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Strain of &quot;Herpes Simplex&quot; Inoculated I.M. Into</td>
<td></td>
</tr>
<tr>
<td></td>
<td>a Left Back Leg.</td>
<td></td>
</tr>
</tbody>
</table>

LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>The Inflammation in the Left Dorsal Ganglia of</td>
</tr>
<tr>
<td></td>
<td>Rabbit's Spinal Cord.</td>
</tr>
<tr>
<td>II</td>
<td>Reisolation of Subtype 2 MS Strain of &quot;Herpes</td>
</tr>
<tr>
<td></td>
<td>Simplex&quot; from the Infected Rabbits after</td>
</tr>
<tr>
<td></td>
<td>Epinephrine Inoculation.</td>
</tr>
<tr>
<td>III</td>
<td>The Correlation Between the Length of Time Before</td>
</tr>
<tr>
<td></td>
<td>and After Epinephrine Inoculation.</td>
</tr>
<tr>
<td>IV</td>
<td>Numbers of Rabbits Showed the Positive Virus</td>
</tr>
<tr>
<td></td>
<td>Isolation at Different Period of Time After</td>
</tr>
<tr>
<td></td>
<td>Specimens Inoculated into the Primary Rabbit</td>
</tr>
<tr>
<td></td>
<td>Kidney Tissue Culture.</td>
</tr>
<tr>
<td>V</td>
<td>Numbers of Rabbits Showed the Positive Virus</td>
</tr>
<tr>
<td></td>
<td>Isolation at Different Dilution of Inocula in</td>
</tr>
<tr>
<td></td>
<td>the Primary Rabbit Kidney Tissue Culture.</td>
</tr>
<tr>
<td>VI</td>
<td>The Correlation Between the Length of Time After</td>
</tr>
<tr>
<td></td>
<td>Tissue Culture Inoculated and Inocula.</td>
</tr>
</tbody>
</table>
The herpesvirus group contains viruses from many different natural hosts. There are representatives from man, monkey, horse, cow, pig, cat, dog, guinea pig and chicken. The human herpes group is composed of Herpes simplex virus; Varicella/zoster virus (chickenpox/shingles); Cytomegalovirus (salivary gland virus) and "Burkitt's lymphoma" virus. The virus particle of herpes simplex has been studied in detail by electron microscopy using potassium phosphotungstate stain (Brenner et al., 1959; Wildy and Watson, 1962). The virus is composed of three main parts: core, capsid and envelope (Wildy et al., 1960). The core of the virus particles was shown to be double stranded deoxyribonucleic acid (Russell, 1962; Ben-Porat and Kaplan, 1962). The base composition was determined by the ultraviolet absorbing bands relative to a marker DNA in cesium chloride density gradient. The G + C percentage is 68; molecular weight 68 x 10^6; density 1.727 and the sedimentation coefficient is 44S (Russell and Crawford, 1964). The capsid of the particle is made of protein (Crick and Watson, 1956), and is constructed with 162 subunits (capsomeres) which are arranged in an orderly manner showing a 5:3:2 axial symmetry. The capsid is formed into icosahedral shape. The capsomeres themselves appear as hollow elongated prisms with mean dimensions of 95 x 125 Å and
holes running down the middle about 40 Å in diameter. According to the countour of the virus particle, capsomeres are grouped into 150 hexagonal and 12 pentagonal units. In normal conditions they are not closely packed. They have a mean spacing distance about 25-30 Å (Wildy et al., 1960). The outer surrounding part of the particle is an envelope. The overall diameter of the enveloped virion is about 1800 Å. This envelope probably assists in the attachment of the virus to the host cell, thus increasing infectivity (Homes and Watson, 1963; Smith, 1964). The envelope was found to be composed mainly or entirely of host cell membrane. It has no antigenic properties in common with the capsid of the virus (Watson and Wildy, 1963). The herpesvirus group is composed of viruses with structural properties similar to those of herpes simplex virus.

Herpes simplex virus has been studied in great detail by serology. It was demonstrated that the simplex virus was neutralized by the monkey B-virus antiserum, but not vice versa (Burnet et al., 1939). No cross-neutralizations were detected (Plummer, 1964) between herpes simplex, equine herpes virus Types 1 and 2, infectious rhinotracheitis virus and pseudorabies virus. Complement fixing antibody to the simplex virus was noted in the sera of people who developed chickenpox, giving evidence of a strong antigenic relationship between the two viruses (Ross et al., 1965). Classification of herpes simplex
into two serotypes (subtypes 1 and 2) seems justified on the basis cross-neutralization studies (Schneweis, 1962; Plummer, 1964). There are many other criteria besides serological characteristics for the distinction between subtypes 1 and 2 of simplex virus. Differing sites of primary virus recovery from the patients is an outstanding distinguishing feature. Serological subtype 2 is isolated from the genitalia but subtype 1, on the contrary, is isolated from the lips and mouths of the patients (Dowdle et al., 1967). Herpes simplex viruses, subtype 1 and subtype 2, can also be differentiated by their behavior in rabbit tissue cultures. The cytopathic effect produced by subtype 2 virus is characterized by cells which become swollen and rounded, taking on a smooth globular appearance. Subtype 1 virus produces a cytopathic effect in which most of the cells do not completely separate from their neighboring cells and hence retain some of their original shape and only a few cells appear rounded. The titers of infective virus attained in the culture fluid of subtype 2 is much lower than those attained by subtype 1 virus (Plummer et al., 1968). The density and hence base composition of DNA from strains of subtype 1 and subtype 2 seem to be dissimilar. The G + C percentage calculated from the density of DNA has been shown to be about 68.3 in subtype 1, while that for subtype 2 is 70.4 (Goodheart, et al., 1968). Subtype 2 herpes simplex virus is slightly less
sensitive to the inhibiting action of heparin than is the sub-
type 1 strain. The result is shown by the rate of development of
cytopathic effect in rabbit tissue culture and the infection of the rabbit (Lehel and Hadhazy, 1966; Plummer et al., 1968).

Tissue cultures and experimental animals have been used in studying herpes simplex virus in both research and diagnostic work. Many types of tissue cultures from the different animal species have been used. The cytopathic change and plaque formation served as indicators of virus infectivity. Inclusion bodies have often been observed in the nuclei of infected cells (Morgan et al., 1959).

Herpes simplex virus infects only man as its natural host. Subtype 1 virus causes the common fever blister or cold sore of the lip. In some instances also are clearly neurotropic (Goodpasture, 1929). The most common site for the primary infection is the mucous membrane of mouths of young children (Dodd et al., 1938) resulting in an acute stomatitis with vesicular eruption (Scott et al., 1941). An outbreak of simplex stomatitis has been reported to occur among children, aged 1 month to 35 months (Hale et al., 1963). The common herpetic infection of the skin appears as a single cluster of vesicles which characterize either recurrent herpes or the ordinary fever blister. Although lesions of this type may occur in many skin sites, the most common location is the lip or adjacent portion
of the chin, cheek or nose. The genital area and the buttock region are also common locations for infection (Wheeler and Cabaniss, 1965) by subtype 2 virus. The herpetic infection on the fingers was found to be very common among the neurosurgical nurses; presumably subtype 1 virus is most commonly involved in this condition. The finger lesion can be readily mistaken for a staphylococcal infection since it became secondarily infected with *Staphylococcus pyogenes* (Stern et al., 1959; Hambrick et al., 1962). The human cornea is another target for simplex virus. In early stages of keratitis a vesicle appears and enlarges into a series of branching processes to form the typical dendrite (Ormsby, 1957). It has been reported that the herpes virus caused a fulminating visceral disease and hepatitis in infants; the virus was isolated from the liver of one out of eight such cases (Zuelzer and Stulberg, 1952). Clear eosinophilic intranuclear inclusion bodies were demonstrated in the liver, adrenal gland and in the lower third of the esophagus of the two patients aged 8 days and 18 months. Both of them died after diagnosis of herpes simplex infection (Tacker and Scofield, 1961). Simplex virus has been isolated from a child with gingivostomatitis, and from the cerebrospinal fluid of a patient with a bacterial meningitis (Brunell and Dodd, 1964).

As mentioned above, herpes simplex virus also can infect the central nervous system causing an acute necrotising
encephalitis. This was shown by the brain biopsy and by the examination of cerebrospinal fluid for atypical inflammatory cells and sequential antibody formation (Harland et al., 1967). The virus of herpes simplex has been isolated from the glycero-\textit{lated} brain material from two cases of fatal encephalitis. Intranuclear acidophilic inclusion bodies were demonstrated in both cases by the method of Phloxine-Methylene Blue stain (Whitman \textit{et al.}, 1964). From 5 cases of encephalitis, the virus was isolated from the cerebral portion of the brain (Rawls \textit{et al.}, 1966). Herpetic infection of the central nervous system may manifest itself clinically as aseptic meningitis or meningoencephalitis. In the adult, it may result more in meningoencephalitis than in childhood. Residual neurological and emotional sequelae may be common (Ross and Stevenson, 1961). This encephalitis is characterized by rapid onset, a high frequency of seizures regardless of the age of the patient and the severity of clinical disease during the acute stage (Olson \textit{et al.}, 1967).

Experimental animals are also susceptible. Paralysis was shown in rabbits in which the MS strain subtype 2 of herpes simplex virus was inoculated into the back leg. The paralysis in the rabbits appeared to be the spastic type (Plummer and Hackett, 1966). Hamsters inoculated by the vaginal route developed a posterior paralysis (Burnstein, 1965). The patho-
genesis of simplex virus encephalitis and myelitis was studied in suckling mice (Johnson, 1964). After intracerebral inoculation, the virus was found to multiply in meninges, ependyma and then to invade the underlying parenchyma infecting both neurons and glia. By extraneural inoculations, the virus supposedly gained access to the central nervous system by both hematogenous and neural pathways. The virus will traverse sensory, motor or sympathetic nerve pathways to the brain or the cord depending on the nerves supplying the peripherally infected area. The mode of transit is by the way of axis cylinder, rather than perineural spaces and is not by passive transportation (Goodpasture and Teague, 1923). There is no evidence that the simplex virus enters the central nervous system by the way of lymphatics or by direct extension through the tissue (Wildy, 1967). A mouse-passed strain of herpes instilled intranasally reaches the brain by both the trigeminal and olfactory pathways (Slavin and Berry, 1943).

Herpes simplex is one of the viruses which can persist in an occult form; and following alteration of the virus-host equilibrium by certain unknown factors, again become clinically active (Enders, 1964). Both experimental and circumstantial clinical data indicate that the herpes virus does exist in a latent stage within the human body (Goodpasture, 1929; Findlay and MacCallum, 1940). A latent herpetic encephalitis can be
demonstrated in rabbits by partially immunizing them prior to intracerebral injection of the virus (Perdrau, 1938).

Following a change in the physiological condition of the patient, the recurrent form of herpes simplex infection was often found after a period of latency of the virus. The altered factors are both external and internal environment including sunburn or irritation from the cold winds; fever; menstruation; nerve injury and emotion (Scott, 1957). Recurrent herpes simplex primarily involves the lip but may affect the conjunctiva and cornea. These ocular lesions often arise in association with herpetic vesicles of the skin in adjacent or remote areas during the course of a febrile or chronic debilitating disease (Brain, 1956). The clinical and experimental evidence indicate that a recurrent herpes may be caused by skin graft (Findlay and MacCallum, 1940); an operation (Carton and Kilbourne, 1952); a psychologic disturbance (Shearer and Finch, 1964). Recurrent herpetic infection can be produced also in rabbits by stimuli. Herpetic encephalitis can be precipitated by anaphylactic shock (Good and Campbell, 1945) and the virus can be recovered during a period of spontaneous exacerbation (Good and Campbell, 1948). Encephalomyelitis was precipitated after intramuscular inoculation of epinephrine in rabbit with latent herpes virus (Schmidt and Rasmussen, 1960). Epinephrine did reactivate the paralysis and in some cases stimulated
paralysis in infected rabbits (Plummer et al., 1967). Epinephrine could be responsible for initiating virus reactivation in the rabbit eye too (Laibson and Kibrick, 1966).

Recovery from primary herpetic infection has been shown in a guinea pig, to be accompanied by the development of immunity to this virus. The serum contained neutralizing antibodies against simplex virus. It has been found that the viricidal effect was high in hyperimmune serum (Bedson and Crawford, 1927). The neutralizing antibody is a 7S antibody (Yoshino and Tanigushi, 1965). About 75% of normal humans have the neutralizing antibody to the herpes virus (Andrewes and Carmichael, 1930). Forty-five percent of a group of fifty one persons with or without a history of herpes labialis, showed antibodies that fixed the complement in the presence of a herpetic antigen (Brain, 1932).

It was found that the complement fixing antibodies disappeared earlier, perhaps because the complement fixation was less sensitive than the neutralization technic in discerning the small level of antibodies. Human sera were diluted in the range used in the complement fixation test, and 95% showed neutralization (Holzel, 1953). The complement fixing antibody does appear by the fourth day after the primary infection in man and animals and may persist in high titer over 15 months (Gajdusek et al., 1952). Simplex virus has been shown to
produce passive hemagglutination in the presence of specific immune serum (Scott et al., 1957).

There is apparently no increase in the titer of antibodies during recurrent herpetic infection (Dudgeon, 1950). The presence of high levels of neutralizing antibodies in the blood of individuals did not prevent the appearance of the recurrent herpetic lesions (Garabedian and Syverton, 1955). Infected individuals seem to carry the virus throughout the whole life and their sera contain the antibodies to the virus.

The diagnosis of the herpes infection may be confirmed or established by the virus isolation or by the demonstration of an increase in antibody to the serum of the patient during the illness. The estimation of the serum antibody titer has been done by neutralization and/or complement fixation tests (Hayward, 1950). Observation of intranuclear inclusion bodies combined with the clinical picture is also convenient in diagnosis of the encephalitic manifestations of herpes simplex virus infection (Smith et al., 1941; Drachman and Adams, 1961; Olson et al., 1967).

The microneutralization technic, a modification of macroneutralization, has been shown to be useful for the diagnostic virology laboratory and also for the analysis of the antigens of herpes simplex strains (Pauls and Dowdle, 1967). The colorimetric neutralization test has been used for the diagnosis
of primary herpetic infection (Schmidt and Lennette, 1961). Simplex virus from the infected rabbit kidney cell tissue culture was found to contain a high titer of precipitating antigen which proved suitable for the diagnostic test for primary and recurrent infection of herpes simplex using patient serum (Tokumaru, 1965).

Rabbits, immunized with herpes simplex virus by corneal route, developed complement-requiring neutralizing antibodies at an early stage. It usually reaches a peak titer before the complement-nonrequiring neutralizing titer appears (Yoshino and Tanigushi, 1964). The examination of patients' sera and normal sera showed that humans also develop complement-requiring neutralizing antibodies but only primary herpetic patients develop this type of antibody. Two sera out of fifty three whose diagnosis was not possible with usual methods, demonstrated this type of antibody (Yoshino and Tanigushi, 1966).

The treatment of herpes simplex infection is still unresolved. Some chemicals were used experimentally but there were no significant therapeutic results. The idoxuridine and 5-iodo-2'deoxyuridine offer some encouragement experimentally. Idoxuridine is an analogue of thymidine. Experiments indicate that the analogue interfere with DNA synthesis only in actively dividing cells (Buckely and MacCallum, 1967; Evan et al., 1967). In routine treatment of keratitis, 5-iodo-2'deoxyuridine has
proved its effectiveness, yet attempted treatment of herpes simplex encephalitis cases with a systemic 5-iodo-2'deoxyuridine provided little benefit. Hence it is not recommended for routine therapeutic use (Breeden et al., 1966).

The research presented in this thesis has been planned to investigate the herpes simplex virus along two different lines. In Chapter 1 the study has been done on the comparison of neurovirulence of herpes simplex, subtypes 1 and 2, in baby rabbits. In Chapter 2, the study of subtype 2 herpes simplex virus in the rabbit spinal cords has been performed.
Comparison of Neurovirulence of Herpes Simplex Subtypes 1 and 2 in Baby Rabbits

It has been mentioned previously that herpes simplex virus is antigenically differentiated into subtype 1 and subtype 2. Each subtype has its own characteristics including differences in the pock lesions on the embryonic egg chorioallantoic membrane and in neurotropism in experimental animals. Nahmias et al. (1968), showed the different size of pock lesions on the embryonated egg chorioallantoic membrane produced by subtypes 1 and 2 herpes simplex virus. Subtype 1 simplex virus produces small size of pock lesions with a diameter of less than 0.5 mm while subtype 2 virus produces comparatively large pocks with a diameter greater than 0.5 mm. By histological study, small pock lesions produced by subtype 1 herpes simplex virus showed predominance of hyperplastic basal cells in the ectoderm. The nuclei of these cells are rounded or oval with slightly irregular nuclear membranes. No defined intranuclear inclusion bodies were demonstrated. On the contrary, subtype 2 herpes simplex virus pock lesions contained hyperplastic fibroblasts in the ectoderm and numerous giant syncytial cells were observed. Nuclei of these cells are characteristically molded against
each other and have prominent thickened, regular nuclear membranes. Distinct inclusion bodies are demonstrated in nuclei. The ability of herpes simplex virus to invade the central nervous system following peripheral inoculation of mice or rabbits, resulting in encephalitis, has long been known. Plummer and Hackett (1966), demonstrated that 19 to 30 percent of mice developed paralysis of the appropriate limb when they were inoculated into the posterior femoral muscle of the back leg or muscle of the upper part of the front leg with either MS strain of subtype 2 herpes simplex virus or L2 strain of subtype 1 herpes simplex virus. All those receiving MS virus proceeded to a fatal encephalomyelitis; although 38 percent of those paralyzed by the L2 strain virus survived with permanent paralysis of the limb had no ensuing encephalomyelitis. MS as well as L2 viruses were also inoculated intradermally into the shaved skin on the femoral part of the left back leg of rabbits. About 70 percent of the rabbits receiving MS virus developed paralysis of the inoculated limbs; none of the rabbits receiving L2 virus was paralyzed. Figeuro and Rawls (1969) found that subtype 2 herpes simplex virus showed more neurovirulence in intracranial inoculated three-week-old Swiss mice than subtype 1 herpes simplex virus when the LD$_{50}$ was expressed in the terms of TCD$_{50}$: LD$_{50}$ or PFU: LD$_{50}$ ratios. But on the basis of the number of virus particles inoculated, the LD$_{50}$'s of the two subtypes of virus were not significantly different.
Plummer *et al.* (1968), showed that the ability of subtypes 1 and 2 herpes simplex virus to invade the central nervous system of one-month-old Swiss-Webster mice. They inoculated 197, Watson and L2 strains of subtype 1 as well as Wiggins, Dawson and MS strains of subtype 2 herpes simplex viruses into the femoral muscle of the left back leg of mice. They injected $10^3$ PFU log$_{10}$ per 0.2 ml of virus and found that 37 to 60 percent of subtype 2, MS strain, inoculated mice developed paralysis.

**RESULTS**

Inoculation of Rabbits with Subtypes 1 and 2 Strain Viruses. *

The results are summarized in Tables I and II (Pages 31 and 32). In Table I, the neurotropic properties of subtype 1 strains of virus are shown. Only 1 of 46 inoculated rabbits, or 2.2 percent, showed paralysis of the inoculated limb; although histological study revealed 5 out of 46 rabbits, or 10.8 percent, to have inflammation of left dorsal ganglia and horns of the spinal cords. Two of the 010 and two of L2 rabbits showed changes which were not the typical limb paralyses of subtype 2.

*Inoculum size and route of inoculation are shown on page 25.*
rabbits, but which can be described as ataxia and marked behavioral changes, such as gnashing of the teeth. In contrast, the neurotropic property of subtype 2 strain virus is shown in Table II (page 32). Twenty-four of 38 inoculated rabbits, or 63.2 percent, demonstrate the spastic type of paralyses of the inoculated leg. By histological study, 28 of the 38 inoculated rabbits were observed to have inflammation in the left dorsal ganglia and horns of the spinal cord, shown in Fig. I (page 33). The number of paralyzed rabbits seems to correspond to the number of rabbits with inflammed left dorsal ganglia and horns. All rabbits with inflammation of the left dorsal ganglia were free from the inflammation of the right dorsal ganglia.

DISCUSSION

73.6 percent of rabbits inoculated with strains of subtype 2 viruses showed the inflammation of left dorsal ganglia and horns of spinal cords whereas only 10.8 percent of rabbits inoculated with strains of subtype 1 viruses showed. This clearly showed that strains of subtype 2 viruses are more active in producing damage to these sites than strains of subtype 1 viruses. The inflammed ganglia produced by some strains of subtype 2 viruses showed a large area of inflammatory cells ranging from 7 to 10 microns in diameter, shown in Figure I (page 33).
No patch of inflammation of such size have been observed in ganglia of rabbits inoculated with strains of subtype 1 viruses, despite the inocula of subtype 1 strains ranging from 4.5 to 5.2 PFU log_{10}, while strains of subtype 2 herpes simplex viruses ranged from 2.9 to 3.8 PFU log_{10} per 0.2 ml. Thus, of strains of subtype 1 virus, the inocula were about 100-fold higher than subtype 2 virus inocula.

We do not of course know whether the subtype 2 strains are more neurotropic in man, although it is true that the MS strain was isolated from the central nervous system of a case of multiple sclerosis (Gudnadottir et al., 1964).
Subtype 2 Herpes Simplex Virus in the Rabbit Spinal Cord

Herpes simplex virus persists in human tissues and periodically produces skin lesions in some individuals. The form in which it persists during the clinically quiescent periods is not known; the virus may be slowly multiplying or may be in a non-replicative state. There is similar uncertainty about varicella virus which is believed to persist in the dorsal ganglia, with occasional activation as zoster. How these viruses are clinically activated is not understood. There is no evidence one way or the other as to whether herpesviruses of animals persist in their host.

A number of experimental studies have been done of herpes simplex virus in rabbits. Chronic infections of the brains of rabbits by herpes simplex virus subtype 1 have been experimentally produced. Encephalitis developed spontaneously in a proportion of such rabbits, although anaphylactic shock (Good and Campbell, 1948), or inoculation of adrenalin (Schmidt and Rasmussen, 1960), seemed to increase the likelihood of this. Other attempts to study the activating ability of adrenalin have employed chronic infections of the rabbit eye by herpes simplex virus (Laibson and Kibrick, 1966, 1967); very few spontaneous
releases of virus were observed during these experiments but intramuscular inoculation of adrenalin considerably increased the incidence of virus release. Kaufman et al. (1967), reported very frequent spontaneous releases of herpes simplex virus from the eyes of experimentally infected rabbits, such as would make exceedingly difficult the assessment of the activating potential of any stimulus.

This study was done on (a) detectability of the virus, and (b) the ability of adrenalin to activate virus in the adult rabbits.

RESULTS

Forty-three 2-month-old rabbits were inoculated with MS strain of subtype 2 herpes simplex virus, 5.0 log_{10} PFU into the femoral muscle of the left back leg. All the animals developed some degree of paralysis; 5 of them were killed because of severity of the paralysis. After 6 to 11 months, 24 of the rabbits were dosed with epinephrine in aqueous form. Three died as a result of epinephrine. Five of the remaining 21 rabbits showed a considerable increase in their paralysis 3 to 10 days later. They were killed immediately; there was inflammation of the lumbar dorsal ganglia on the left hand side. The
cells of the lumbar ganglia and spinal cord not actually used for the histology were separated by the conventional trypsinization technic using 0.25 percent concentration trypsin, and then inoculated into tubes of primary rabbit kidney tissue culture, 12 tubes per specimen. The cultures were maintained for 8 weeks and the medium partially changed at intervals; an effort was made to retain the cells from the ganglia and spinal cords in tubes which was not too difficult because they tended to adhere to the established cell sheet. By this method MS strain of subtype 2 herpes simplex virus was isolated from 4 of the 5 rabbits. The results are shown with the filled rectangular marks in Fig. II (page 34).

The remaining 16 rabbits, which received the epinephrine but did not show the severe increase in paralysis, were killed between 10 and 25 days after the epinephrine injection. All but one showed inflammation of dorsal ganglia; virus was isolated from 5 of them. Results are shown with the opened rectangular mark in Fig. II (page 34).

The 14 rabbits which did not receive epinephrine were killed between 6 and 11 months after MS strain of subtype 2 of herpes simplex virus was inoculated. All but one showed inflammation of the dorsal ganglia; virus was isolated from seven of them.
There was no correlation between the length of time before and after epinephrine inoculation (Fig. III, page 35). The length of time between the inoculation of the ganglia and spinal cells into the tissue culture of primary rabbit kidney cells, and the appearance of herpes simplex CPE was surprisingly long. From Fig. IV (page 36), the first and the last tubes with CPE appearance were shown. Six of 9 rabbits showed the first appearance of CPE about 7 or 10 days after inoculation. Two of 9 rabbits showed the first CPE appearance 38 and 42 days after inoculation. Five of 9 rabbits showed the last appearance of CPE around 36 to 38 days postinoculation. Only 1 of 9 rabbits who showed the last CPE appearance 3 days after the first appearance.

After the trypsinization of the ganglion and spinal cord of rabbits, specimens were diluted into 1:2, 1:4, 1:8 and 1:16 dilutions. Three tubes of primary rabbit kidney cell tissue cultures were inoculated with each dilution. Fig. V (page 37) showed the numbers of tubes with CPE in each dilution of rabbits. All rabbits showed positive virus isolation in 1:2 diluted culture. Only 6 of 9 rabbits showed positive virus isolation in 1:4 and 1:16 dilution, and 3 of 9 rabbits showed positive result of virus isolation in 1:8 dilution.

There was no correlation between dilutions of cultures and length of time after virus was inoculated into the primary rabbit kidney tissue culture (Fig. VI, page 38).
According to the results, rabbits that received epinephrine inoculation showed positive virus isolation from 9 of 21 rabbits or 42.9 percent while the rabbits without epinephrine inoculation showed positive virus isolation from 7 of 14 rabbits or 50.0 percent. Whether epinephrine activated the MS virus or not, cannot be proved at the present time. In spite of this, epinephrine did seem to markedly increase paralysis in some of the rabbits whose spinal cords were chronically infected with herpes simplex subtype 2, which is of course in harmony with the precipitation by epinephrine of encephalitis in rabbits chronically infected with simplex virus (Schmidt and Rasmussen, 1960). Thirty-three out of 35 rabbits showed the inflammation of the lumbar dorsal ganglia. These results were corresponded to Plummer and Hackett (1966), who inoculated rabbits with MS strain of subtype 2 herpes simplex virus and the inflammation was found in the dorsal ganglia.

The length of time between inoculation of the ganglion and cord cells into the tissue culture of primary rabbit kidney cells and the first appearance of CPE, was surprisingly long especially for the herpes simplex virus which has an eclipse period of under 10 hours. This suggests that extracellular virus was not present in the inoculum, unless an inhibitor
capable of suppressing virus activity for a lengthy period of time was also present. The only alternative explanation is that the virus is in a cell-associated, non-replicative form perhaps in association with inflammatory white cells, which are certainly present in the ganglia infected with subtype 2 herpes simplex virus even many months after primary inoculation of the virus into the primary rabbit kidney tissue culture.
MATERIALS AND METHODS

Primary Rabbit Kidney Tissue Culture. Virus isolations had been done on the primary rabbit kidney tissue culture. Four-week-old healthy rabbits were supplied from Abrams Small Stock Breeders Co., Chicago, Illinois. Their kidneys were removed and decapsulated under sterile technic. A kidney was minced before trypsinization. The trypsinization was done in a sterile 250 ml trypsinized flask, from Bellco Biological Glassware, in which a sterile magnetic bar was used for spinning the kidney tissue. The trypsin, the product of Delco, 2.5 percent concentration in physiological saline, 25 ml, was used for separating cells from the 2 minced rabbit kidneys. This procedure was performed by spinning the minced kidney tissue at room temperature for 30 minutes. The trypsinized cell suspension was transferred from the flask into the sterile centrifuged flask and centrifuged at 1500 rpm for 10 minutes. Cells were layered on the bottom of the flask while the trypsin fluid was on the top above the cells. The fluid was poured off and the excess amount of trypsin was washed with 10 percent initiated 199 media by resuspending the cells. The cell suspension was centrifuged at the same speed and period of time. After pouring the media off, cells were resuspended in fresh 37 C warm 10 percent lamb serum initiator media in the sterile cornwall
pipette (Becton, Dickinson and Company), with magnetic stirring bar. Two rabbit kidneys were usually suspended in 400 ml of 10 percent lamb serum initiator media.

During the spinning of the magnetic bar, cell suspension was distributed into sterile Kimax tubes, size 15 cm long and 1.5 cm in diameter. The tubes were plugged with sterile rubber stoppers. All tubes were placed in a stationary metal rack and incubated at 37 C incubator until cells formed a sheet which normally took about 5 days. Five percent lamb serum maintenance 199 media was used to refeed the cells in maintaining them for virus isolation.

VIRUS INOCULATION

Chapter 1 Comparison of Neurovirulence of Herpes Simplex Subtypes 1 and 2 in Baby Rabbits.

Ten strains of subtype 1 and 8 strains of subtype 2 herpes simplex virus which had been done 2 or 5 passages in primary rabbit tissue culture cells before, were grown in the same type of tissue culture for fresh culture inoculum. A half ml of fresh culture inoculum of each strain was inoculated into the femoral muscle of left back leg of two-week-old baby rabbits by 3 ml plastic (Tomac) disposable syringe with needle gauge No. 20 was attached at the tip. Scratches were also made on the skin around inoculated area. Rabbits then were observed every
alternate day for 17 days. Spinal cords at the lumbar region were removed for histology study of inflammation by Hematoxylin and Eosin staining method.

Chapter 2 Subtype 2 Herpes Simplex in the Rabbit Spinal Cord.

Forty-three 2-month-old rabbits were supplied from Abrams Small Stock Breeders, and were inoculated with MS strain of subtype 2 herpes simplex virus. This strain had been isolated from a brain of a patient with multiple sclerosis by Gudnadottir et al. (1964). The fresh culture inoculum was done by making a passage on primary rabbit kidney cells about 48 hr before inoculation was performed. All rabbits were kept in good care, and were observed weekly. After 6 to 11 months, a number of rabbits were inoculated with 1:1000 diluted aqueous synthetic epinephrine (adrenalin chloride, Park Davis). Injections of epinephrine were given on three occasions one day, and two the next day; on each occasion 0.5 ml was given subcutaneously in the shaven scapular region, and 0.2 ml intramuscularly in the front legs. Increased paralytic rabbits were killed immediately and their spinal cords were removed under sterile conditions for virus isolation and histology study of inflammation.

HISTOLOGY

Spinal cords at lumbar regions of rabbits were sectioned with a cryostat (Model CTD-International-Harris-Cryostat). A
block of spinal cord with left and right dorsal ganglia was fixed in a drop of water on a metal holder and then placed in the cryostat at -20 C for 2-3 hr before sections were cut. Sixteen micron thick sections were cut by a cold sharp blade. Three sections were placed directly on a glass slide 25 x 75 mm, from Scientific Products, and air dried, done for Hematoxylin and Eosin staining method.

**HEMATOXYLIN AND EOSIN STAINING**

After the sections on the glass slide were air dried, they were fixed in a fixative solution which was composed of 1 part of 40 percent concentration of formalin in 9 parts of absolute ethanol, for 1 minute. Then they were rinsed briefly with 70 percent, 50 percent ethanols and water respectively. In Harris' hematoxylin, sections were immersed for 5 minutes; rinsed with tap water and then immersed in 1 percent lithium carbonate until sections turned blue. Several changes of water were done for rinsing the sections before immersing them into 1 percent alcoholic eosin. Sections were then left in the alcoholic eosin for 3 minutes and passed through the dehydrated processes. Starting with 2 changes of 95 percent ethanol; 5 dips for each change, and 5 dips of 2 changed absolute ethanol. Sections were, next step, dipped into the equal parts of ethanol and
xylene for 5 times before being passed to the 2 changed pure xylene. Finally, sections were mounted under the cover glass by permount.

MEDIA

There are two different kinds of media, initiator or growth media and maintenance media. The initiator media is composed of Medium 199-10X which is an Earle's base from Grand Island Biological Co.; Glutamine C.P. from Pfanstiehl Laboratory, Inc.; Sodium bicarbonate powder from J. T. Baker; Penicillin G in Potassium buffer from Eli Lilly; Streptomycin sulfate from Eli Lilly; Fungizone from E. R. Squibb; and Lamb serum from Grand Island Biological Co.

The composition of initiate media in 100 ml.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium 199-10X</td>
<td>14 ml</td>
</tr>
<tr>
<td>Glutamine</td>
<td>29 mg</td>
</tr>
<tr>
<td>Penicillin and Streptomycin</td>
<td>5 mg</td>
</tr>
<tr>
<td>Fungizone</td>
<td>0.05 mg</td>
</tr>
<tr>
<td>7.5% Sodium bicarb</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>Lamb serum</td>
<td>10 ml</td>
</tr>
<tr>
<td>Sterile Deaminized distilled water</td>
<td>85 ml</td>
</tr>
</tbody>
</table>
The maintenance media are composed of the same quantity and chemical substance as initiate media except 2.5 ml of 7.5 sodium bicarbonate instead of 1.5 ml in 100 ml working media and 5 ml of lamb serum instead of 10 ml.

Three percent of Fetal calf serum maintenance media is used in virus isolation prior to the inoculation of each specimen. This type of media is composed of the same quantity and chemicals as regular maintenance media except using 3 ml of Fetal calf serum per 100 ml of media.
SUMMARY

Seventy-four percent of rabbits inoculated with 8 strains of subtype 2 Herpes simplex virus showed the inflammation of the left dorsal ganglia and horns of spinal cords whereas only 11 percent of rabbits inoculated with 10 strains of subtype 1 herpes simplex virus showed the inflammation of left dorsal ganglia and horns. This indicated that the strains of subtype 2 herpes simplex virus are more active in producing damage to the dorsal ganglia than strains of subtype 1 herpes simplex virus.

Rabbit spinal cords could be chronically infected with MS strain of subtype 2 herpes simplex virus. The viruses could be re-isolated over subsequent months from about half the animals without prior stimulation; the interval between inoculation of trypsinized spinal tissue into tissue cultures and the development of cytopathic effect was often long, varying from 7 to 42 days.
## TABLE I

Neurotropic Property in Baby Rabbit of Subtype 1 Strain of "Herpes simplex"

Inoculated I.M. Into a Back Leg

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pass Level</th>
<th>Inoculum (PFU log$_{10}$)</th>
<th>Number Paralyzed</th>
<th>Percent</th>
<th>Number with Inflammation of dorsal ganglia and horn</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>6</td>
<td>4.7</td>
<td>0/4</td>
<td>0.0</td>
<td>0/4</td>
<td>0.0</td>
</tr>
<tr>
<td>03</td>
<td>6</td>
<td>4.8</td>
<td>0/5</td>
<td>0.0</td>
<td>0/5</td>
<td>0.0</td>
</tr>
<tr>
<td>05</td>
<td>6</td>
<td>4.7</td>
<td>0/4</td>
<td>0.0</td>
<td>0/4</td>
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</tr>
<tr>
<td>06</td>
<td>6</td>
<td>4.5</td>
<td>0/5</td>
<td>0.0</td>
<td>0/5</td>
<td>0.0</td>
</tr>
<tr>
<td>07</td>
<td>3</td>
<td>4.9</td>
<td>1/5</td>
<td>20.0</td>
<td>1/5</td>
<td>20.0</td>
</tr>
<tr>
<td>09</td>
<td>3</td>
<td>4.9</td>
<td>0/5</td>
<td>0.0</td>
<td>0/5</td>
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<tr>
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<td>3</td>
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<td>0/5</td>
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<td>1/5</td>
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</tr>
<tr>
<td>L2</td>
<td>? more than 15</td>
<td>5.2</td>
<td>0/4</td>
<td>0.0</td>
<td>2/4</td>
<td>50.0</td>
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<tr>
<td>03 var.</td>
<td>?</td>
<td>4.8</td>
<td>0/5</td>
<td>0.0</td>
<td>0/5</td>
<td>0.0</td>
</tr>
<tr>
<td>Roiz.MP</td>
<td></td>
<td>4.9</td>
<td>0/4</td>
<td>0.0</td>
<td>0/4</td>
<td>0.0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>1/46</td>
<td>2.2</td>
<td>5/46</td>
<td>10.8</td>
</tr>
<tr>
<td>Strain</td>
<td>Pass Level</td>
<td>Inoculum (PFU log_{10})</td>
<td>Number Paralyzed</td>
<td>Percent Paralyzed</td>
<td>Number with Inflammation of dorsal ganglia and horn</td>
<td>Percent</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
<td>-------------------------</td>
<td>------------------</td>
<td>-------------------</td>
<td>--------------------------------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>G1</td>
<td>3</td>
<td>2.9</td>
<td>1/5</td>
<td>20.0</td>
<td>2/5</td>
<td>40.0</td>
</tr>
<tr>
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<td>100.0</td>
<td>5/5</td>
<td>100.0</td>
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<tr>
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<td>6</td>
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<td>1/5</td>
<td>20.0</td>
<td>2/5</td>
<td>40.0</td>
</tr>
<tr>
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<td>5/5</td>
<td>100.0</td>
<td>5/5</td>
<td>100.0</td>
</tr>
<tr>
<td>G9</td>
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<td>3.4</td>
<td>3/4</td>
<td>75.0</td>
<td>4/4</td>
<td>100.0</td>
</tr>
<tr>
<td>G10</td>
<td>3</td>
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<td>4/5</td>
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</tr>
<tr>
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<td>2/5</td>
<td>40.0</td>
<td>2/5</td>
<td>40.0</td>
</tr>
<tr>
<td>G12</td>
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<td>3/4</td>
<td>75.0</td>
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<td>75.0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>24/38</td>
<td>63.2</td>
<td>28/38</td>
<td>73.6</td>
</tr>
</tbody>
</table>
Figure I. The Inflammation in the Left Dorsal Ganglion of Rabbit's Spinal Cord
Figure II. Reisolation of Subtype 2 MS Strain of "Herpes Simplex" from the Infected Rabbits after Epinephrine Inoculation.

Rabbit showed a considerable increase in paralysis after epinephrine inoculation.

Rabbit showed no considerable increase in paralysis after epinephrine inoculation.
Figure III. The Correlation Between the Length of Time Before and After Epinephrine Inoculation.

△ Number of rabbit showed the positive virus isolation.
Figure IV. Numbers of Rabbits Showed the Positive Virus Isolation at Different Period of Times After Specimens Inoculated into the Primary Rabbit Kidney Tissue Culture

Δ First tissue culture tube showed CPE.

× Last tissue culture tube showed CPE.
Figure V. Numbers of Rabbits Showed the Positive Virus Isolation at Different Dilutions of Inocula in the Primary Rabbit Kidney Tissue Culture

□ Rabbit tissue culture with CPE
**Figure VI.** The Correlation Between the Length of Time after Tissue Culture Inoculated and Inocula

![Diagram showing correlation between days after tissue culture inoculation and dilutions with CPE](image)

- △ Rabbit tissue culture with CPE.
REFERENCES


APPROVAL SHEET

The thesis submitted by Anan Phuangsa has been read and approved by members of the Department of Microbiology.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

11/10/70
Date

Signature of Advisor