National Library of Canada
Bibliothèque nationale du Canada

Canadian Theses Division
Division des thèses canadiennes

Ottawa, Canada
K1A 0N4

0-315-24858-0

PERMISSION TO MICROFILM — AUTORISATION DE MICROFILMER

• Please print or type — Écrire en lettres moulées ou dactylographier

Full Name of Author — Nom complet de l'auteur

MICHAEL JOHN GILL

Date of Birth — Date de naissance

7 MARCH 1951

Country of Birth — Lieu de naissance

ENGLAND

Permanent Address — Résidence fixe

APT 613
5204 DALTON DRIVE NW
CALGARY ALBERTA T3A 3H1

Title of Thesis — Titre de la thèse

STUDIES TO DEVELOP A RADIOISOTOPE SCAN FOR HERPES ENCEPHALITIS

University — Université

ALBERTA

Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

Master of Science

Year this degree conferred — Année d'obtention de ce grade

1984

Name of Supervisor — Nom du directeur de thèse

DR D.L.J. TYRRELL

Date

28 May 1984

Signature

M J Gill

THE UNIVERSITY OF ALBERTA

STUDIES TO DEVELOP A RADIOISOTOPE SCAN FOR HERPES ENCEPHALITIS

by

MICHAEL JOHN GILL

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

in

DEPARTMENT OF MEDICINE

EDMONTON, ALBERTA

FALL, 1984

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: MICHAEL JOHN GILL

TITLE OF THESIS: STUDIES TO DEVELOP A RADIOISOTOPE SCAN FOR HERPES ENCEPHALITIS

DEGREE FOR WHICH THESIS WAS PRESENTED: MASTER OF SCIENCE

YEAR THIS DEGREE GRANTED: 1984

(Signed) M. J. Gill

PERMANENT ADDRESS:

Apt 613
5204 Dalton Drive N.W.
Calgary, Alberta

DATED: May 1984

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Studies to Develop A Radioisotope Scan for Herpes Encephalitis submitted by Michael John Gill in partial fulfilment of the requirements for the degree of Master of Science.

David L J Tyrrell

Supervisor

DATE May 11, 1984

Abstract

Although relatively rare, biopsy proven herpes encephalitis is the commonest cause of fatal encephalitis in N. America. Untreated it has a mortality rate of 70-80%. In 1977 an antiviral agent adenine arabinoside (ara-A) was shown to reduce this mortality rate to 30%. The greatest effect of the drug was demonstrated when it was given early in the course of the infection. Unfortunately brain biopsy is the only accepted way of achieving a definitive diagnosis of herpes encephalitis. The invasive nature of the diagnostic test has naturally tempered enthusiasm for early diagnosis. Therefore, there is a considerable need for a non invasive diagnostic test for herpes encephalitis.

In 1977, a new antiviral agent, a nucleoside analogue, Acyclovir was first described which is selectively phosphorylated by a virally coded enzyme (thymidine kinase). This phosphorylation step selectively traps acyclovir within herpes infected cells. Since then several other nucleoside analogues such as Bromovinyldeoxyuridine have been described with similar characteristics. This research has been based on the hypothesis that the selective trapping of nucleoside analogues within herpes infected cells can be used to diagnose herpes encephalitis by using a radiolabelled nucleoside analogue and a brain scanning technique.

Initial studies involved establishing an animal model of herpes encephalitis suitable for study. In the literature a variety of animal models of herpes encephalitis have been described but information concerning the reproducibility of the induction of encephalitis, the clinical signs of disease, and the localization of infection is often

not reported. Seven variables were adjusted in this work to find models suitable for radioisotope scanning and pharmacokinetic studies. Finally a rabbit model of herpes encephalitis, following intracerebral injection of HSV-I, was established which appeared suitable for radioisotope scanning. A mouse model following intracerebral injection of HSV-I, was established for tissue distribution studies.

Four nucleoside analogues suitable for radiolabelling were synthesized by the Department of Chemistry (Dr. M.J. Robbins) and the Faculty of Pharmacy (J. Samuel, Dr. E. Knaus, Dr. L. Wiebe) at the University of Alberta. These agents were tested for antiviral activity in tissue culture and Iodovinyldeoxyuridine was selected as the most promising agent due to its high antiviral activity (ID_{50} = 0.007 μg/mL) and the ease of synthesis and radiolabelling. Subsequent experiments quantifying the uptake of radiolabelled Iodovinyldeoxyuridine in herpes infected cells in vitro have shown that the trapping is both selective and rapid, and also occurs at a concentration of virus likely to be present in the infected human brain.

Preliminary studies in vivo quantifying the uptake of this radiolabelled compound in rabbits have confirmed that the radiolabel is selectively concentrated in herpes infected brains but not in uninfected brains. However the magnitude of this trapping is not great and is masked by a persistently high blood level. This prolonged high blood level of radioactivity was unexpected and currently efforts are being undertaken to identify the cause. Should the uptake of radiolabelled IVDU allow detection by scanning of rabbits with herpes encephalitis, a clinical trial would appear indicated.

ACKNOWLEDGEMENTS

Although many people have been of assistance and help to me during this research, several deserve particular recognition. Firstly I must acknowledge the encouragement, company and understanding that my wife Carole has provided during this busy period. I would like to thank Dorothy, Jyy and Ian for their tolerance and assistance in the laboratory. I would also like to thank John Samuel and Drs. Wiebe, Knaus and Singh for their collaborative help. Their enthusiasm has enabled this research to flourish as a true collaborative project.

Finally I must thank my supervisor, Dr. Lorne Tyrrell. His breadth of knowledge and limitless energy are an example to all. He also possesses the remarkable ability to bring both perspective and humour to stressful situations.

This research was funded by the Alberta Heritage Foundation for Medical Research. I would like to thank them for their generous support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATE

List of Abbreviations

ACG	9-(2-hydroxyethoxymethyl)guanine, acyclovir
Ara-A	9-β-D-Arabinofuranosyladenine, adenine arabinoside
Ara-ADP	9-β-D-Arabinofuranosyladenine-5'-diphosphate
Ara-AMP	9-β-D-Arabinofuranosyladenine-5'-monophosphate
Ara-ATP	9-β-D-Arabinofuranosyladenine-5'-triphosphate
Br-ACG	8-Bromo-9-(2-hydroxyethoxymethyl)guanine
BVDU	E-5-(2-bromovinyl)-2'-deoxyuridine, Bromovinyldeoxyuridine
CPE	Cytopathic effect
CSF	Cerebrospinal fluid
CT	Computerized tomogram
DNA	Deoxyribonucleic Acid
EBV	Epstein-Barr virus
EEG	Electroencephalogram
FIAC	2'Fluoro-5-iodo-arabinosylcytosine
HCMV	Human cytomegalovirus
HSE	Herpes simplex encephalitis
HSV	Herpes simplex viruses
HSV-I	Herpes simplex Type I
HSV-II	Herpes simplex Type II
I-ACG	8-Iodo-9-(2-hydroxyethoxymethyl)guanine
i.c.	intracerebral
ID_{50}	50% Inhibitory Dose

i.p.	intraperitoneal
i.v.	intravenous
IVDU	E-5-(2-iodovinyl)-2'-deoxyuridine, Iodovinyldeoxyuridine
[^{131}I]-IVDU	[^{131}I]-E-5-(2-iodovinyl)-2'-deoxyuridine
NIAID	National Institute of Allergy and Infectious Diseases
pfu	plaque forming units
$TCID_{50}$	Tissue culture infective $dose_{50}$
TK	thymidine kinase
VZ	varicella-zoster virus

CHAPTER I GENERAL INTRODUCTION

A. HERPES VIRUSES

The herpetoviridae consists of a family of large, enveloped, DNA-containing viruses (1). Although many members of the family are pathogenic, only five members are significant human pathogens. They are herpes simplex virus types I and II (HSV-I, HSV-II), varicella-zoster virus (VZ), Epstein-Barr virus (EBV) and human cytomegalovirus (HCMV). All members of the family have a nucleic acid core 30-45 nm in diameter which possesses the double stranded DNA. The core is surrounded by an icosahedral capsid. An envelope studded with glycoprotein projections surrounds the capsid giving the complete virus particle a diameter of 120-250 nm (1). The individual viruses can be distinguished by the composition of the genome as well as by the antigenicity of the capsid and envelope proteins. Using restriction endonucleases, strains of each type of HSV can now be readily identified (2).

Herpes viruses can cause a variety of different illnesses in man but fortunately most infections are self limiting and rarely fatal. For many years HSV has been known to cause mucocutaneous infections but it was only in 1961 that it was first appreciated that there are two types of HSV, with both shared and distinct surface antigens (3).

HSV-I can cause gingivostomatitis, keratoconjunctivitis, whitlow, cold sore, genital infections and occasionally encephalitis. HSV-II is primarily seen in genital infections and much less frequently in oro-labial and whitlow infections (4). However with changing sexual habits these distinctions are becoming blurred and the site of isolation of the virus no longer automatically predicts the type of HSV (5). Both

HSV-I and HSV-II possess the remarkable ability to become latent after a primary infection and then to become reactivated at a later date and cause illness. About 30-50% of adults in higher socio-economic groups have HSV antibodies whereas in lower socio-economic groups 80-100% have antibodies to HSV (6).

Primary infection with HSV-I is usually in the form of a self limiting gingivostomatitis. The virus is believed to spread in a retrograde fashion back to the trigeminal ganglion where it becomes latent in neuronal cells (7). A variety of stimulants including sunlight, heat, local trauma, menstruation, infection, and stress have all been associated with reactivation of HSV-I. The exact mode of action of these triggering agents remains uncertain although transient changes in cell mediated immunity and shifts in intracellular prostaglandin levels have been noted (8,9,10). HSV-I usually recurs in the form of a cold sore and analysis of the viral DNA in recurrent episodes from individual patients has shown that the virus is genetically identical, confirming the concept of reactivation (11). Latent HSV-I has also been isolated from both superior cervical and vagus ganglions and these may be the sites of origin for recurrences causing herpes oesophagitis (12). Herpes simplex encephalitis may follow a primary infection but usually results from reactivation of a latent virus (13).

HSV-II has similar properties of latency. After a primary genital infection the virus becomes latent in the sacral ganglia to become reactivated intermittently and cause clinical illness. VZ, another herpes virus, causes chicken pox as the primary infection before becoming latent in the dorsal root ganglia. Reactivation of VZ usually

produces infection in the distribution of one dermatome and is called shingles (4). HCMV and EBV also possess the ability to become latent in the host and can recur to cause illness. However, unlike HSV and VZ they are not neurotropic and are believed to become latent in the host's leukocytes.

B. HERPES ENCEPHALITIS

1. Epidemiology

Many viruses are known to cause encephalitis in humans. In North America, togaviruses and bunyaviruses account for many of the clinically apparent cases. As they are arthropod-borne they have characteristic epidemiological features occurring during distinct seasons each year and only in certain areas of the continent. Enterovirus infections can also cause encephalitis and their pattern is also seasonal. Fortunately most cases of viral encephalitis follow a benign course and deaths are relatively infrequent; however, some togavirus infections can be severe, but are rare in North America.

Although it had been strongly suspected from early animal studies that herpes viruses could cause human encephalitis, it was not until 1941 that HSV was isolated and definitively identified from the brain of a child dying from herpes encephalitis (14). Since then herpes encephalitis has become accepted as the most common cause of fatal encephalitis in North America and England (15). However, despite considerable recent advances in both the diagnosis and treatment of herpes encephalitis, there still exists considerable controversy concerning both clinical spectrum of the disease and also the optimal approach to achieving a diagnosis (16,17).

The National Institute of Allergy and Infectious Diseases (NIAID)

Collaborative Antiviral Study Group has reviewed some epidemiological features of patients with biopsy-proven herpes encephalitis (18). In their series herpes simplex encephalitis occurred in all age groups, was equally common in both sexes, showed no obvious predisposition to affect any particular racial group and had no particular seasonal distribution. Untreated biopsy proven herpes encephalitis carried a mortality rate of 70% (19).

Although HSV-I is by far the commonest type of herpes virus causing herpes encephalitis in adults, HSV-II and VZ can also cause encephalitis in selected cases. Encephalitis in adults resulting from HSV-II is particularly rare, accounting for 3% of all adult cases (20,47). In neonates HSV-II encephalitis is usually one feature of a disseminated infection, but may also present as a localized infection. Both carry a significant mortality and morbidity even with optimal antiviral therapy (21). VZ encephalitis is rare and may be found in immunocompromised patients with disseminated infection. These patients usually do very poorly despite therapy (22).

Although most cases of herpes encephalitis in adults are due to HSV-I and in neonates are due to HSV-II, the relative incidence of each type of virus needs to be monitored. This has become increasingly important with the development of the very new highly potent antiviral agents, which have pronounced act[illegible] primarily against HSV-I e.g. E-5-(2-bromovinyl)-2'-deoxyuridine (23).

2. Pathology

Since the original description of [illegible] first documented case of herpes encephalitis in 1941, there has been considerable interest in the pathology of this disease (14,90). Even [illegible] the early studies, the

severity of tissue necrosis caused by the infection, and the localized and asymmetrical topography of the lesions were readily appreciated (93).

At post mortem examination, the brains of victims of herpes encephalitis frequently have a characteristic macroscopic appearance with a severe hemorrhagic necrosis and cerebral softening accompanying a destruction of gross architecture (92,93). The topography of the infection is particularly interesting with a predilection for affecting the medial temporal and frontal regions of the brain (92,93). However in rare cases the parietal lobe, occipital lobe and the brain stem have all been implicated as being the site of initial localization (94,95,96).

Both light and electron microscopy have been used to examine brain tissue from victims of herpes encephalitis. Lymphocytic infiltration, perivascular cuffing and neuronal and glial cell loss are commonly found but all are non specific, as they can be encountered in other conditions (47). Eosinophilic intranuclear inclusions of Cowdry Type A are observed in approximately 70% of all cases of herpes encephalitis and are used as a significant marker for the diagnosis (90). However, the value of inclusion bodies as a diagnostic marker was challenged when it was proposed that they could represent non specific artifacts of little diagnostic significance (91). The validity of using these intranuclear inclusion bodies as a marker for viral encephalitis has been confirmed by electron microscopy. Inclusion bodies have been shown to consist in most cases, of a granular matrix embedding viral particles undergoing replication and maturation (98,92).

In the NIAID collaborative study on herpes encephalitis 71/84

patients with proven herpes simplex infection had pathologic changes on a brain biopsy consistent with herpes encephalitis. Those changes were perivascular cuffing, lymphocytic infiltration and/or neuronophagia as described previously. Intranuclear inclusion bodies were found in 47/84 patients. Of importance in twenty-five of the seventy patients where the biopsy was negative for herpes infection changes compatible with viral encephalitis were identified. In ten of these specimens inclusion bodies were noted, and in seven of these cases a non-herpes but still viral cause for the encephalitis was identified (47).

3. Pathogenesis

There appears to be at least three different settings for the development of human herpes encephalitis. In neonatal infection, it is believed that the infection is acquired during delivery through a birth canal infected with HSV-II. The virus may cause a local superficial infection of the infant's skin, mouth or eye, or it may cause a localized encephalitis without evidence of visceral involvement. In a small number of cases it may penetrate through the nasopharynx and cause a viremia with multiple organ involvement including the brain (24).

In adults it was originally believed that herpes encephalitis resulted from a primary infection. However by 1965, many patients suffering from acute herpes encephalitis had been identified who had serological evidence of previous herpetic infection, and the concept of a primary infection had to be reviewed.

Herpes virus DNA has been identified in trigeminal, sacral, superior cervical and vagus ganglia (12,7,26). Infectious virus can be reactivated by culturing the ganglia removed from cadavers (104). This evidence has led to the belief that herpes encephalitis usually results

from reactivation of a latent virus in the trigeminal ganglion.

On most occasions a reactivated herpes virus in the trigeminal ganglion tracks peripherally to produce a cold sore. On rare occasions the virus may spread along the tentorial nerves, which originate from the trigeminal ganglia, to cause encephalitis (27). This route of infection would explain the asymmetrical characteristic topography of the lesions in herpes encephalitis, since the tentorial nerves supply the fascia near the temporal and frontal lobes. Another plausible route of infection which has been proposed is that of direct spread along the olfactory bulbs particularly in primary infections (29).

It is also conceivable that the virus causing herpes encephalitis could in fact originate from the reactivation of a virus latent within the brain itself. HSV-I nucleic acid sequences have been readily detected by in situ hybridization in thin sections of brains from mice inoculated (six months previously) with HSV-I. These mice were clinically well and virus could not be recovered by conventional culture (105). Examination of the DNA extracted from 11 human brains has shown HSV-I DNA sequences in six samples. In some cases a complete viral genome was detected while in others only a part of the genome, or terminal fragments could be detected (106).

Unfortunately it is not clear what proportion of adult herpes encephalitis is a result of primary infection. The NIAID study has revealed the complexity of the probl [illegible]13). HSV-I isolated from seven patients with simultaneous orolabial herpes and biopsy proven encephalitis were examined by DNA restriction endonuclease fingerprinting. Presumably if the encephalitis resulted from a virus latent in the trigeminal ganglion the patterns obtained from both

isolates would be identical. In four of the seven patients the viruses isolated from the brain and the oral lesions were identical by DNA restriction enzyme analysis. Three of these patients had a long standing history of cold sores and readily detectable antibodies in the acute serum suggesting that a reactivation of a latent virus was the cause. In the other patient, the absence of a history of previous cold sores and undetectable antibodies in the acute serum suggested that this could have been a primary infection. In the three remaining patients the authors felt that the differences in the fingerprint patterns were minor and did not exclude these being the same viruses (13). In conclusion, adult herpes encephalitis may follow a primary infection but it is more likely to result from a reactivation of a latent virus.

One major question on the pathogenesis of herpes encephalitis is still unresolved. It is not clear why virus (HSV-I) which is ubiquitous in nature and causes a relatively minor self limiting infection in the majority of people can, in a few people cause a fulminant lethal encephalitis. Until recently it was believed that differences in pathogenicity of viral strains might be significant. In 1923, it had been noted in a rabbit model that one strain of herpes could cause encephalitis while a second strain was non-pathogenic (30). Laboratory strains of HSV-I have also shown variable neurovirulence (31,107). Recent studies using DNA restriction enzyme fingerprint analysis, have failed to reveal any common denominator in the brain biopsy isolates from patients with HSV-I encephalitis (32,33). It now seems unlikely that specific strains of HSV-I with increased tropism or pathogenicity for neural tissue will be identified from HSV encephalitis patients.

Other hypotheses have been proposed to explain the pathogenesis of herpes encephalitis and include i) an innate or acquired immunological deficiency facilitating encephalitis, ii) a chance event permitting viral access to the brain via a neurotropic route. Unfortunately no hypothesis has been generally accepted (13).

4. Clinical Features

The full clinical spectrum of herpes encephalitis is poorly understood and highly controversial. The controversy arises from the differing criteria used to achieve a diagnosis (34,16).

Using the most stringent criteria for diagnosis, by culturing HSV-I from a brain biopsy, the NIAID Collaborative Antiviral Study Group have reviewed the clinical features of over one hundred cases of herpes encephalitis (18). As previously mentioned no predisposition to herpes encephalitis was noted in either sex, in any age group or ethnic background or at any particular season. The commonest clinical findings both historically and at presentation are listed below.

Alteration of consciousness	- 97%
CSF pleiocytosis	- 97%
Fever	- 92%
Personality change	- 85%
Headache	- 81%
Dysphasia	- 76%
Seizures	- 67%
Autonomic dysfunction	- 60%

These figures have confirmed the clinical impression that herpes encephalitis most frequently presents as a febrile illness in a patient

with a history of headache, personality change and diminished level of consciousness, who may proceed to develop localizing signs and symptoms usually to one or both temporal lobes. The natural history of biopsy-proved HSE is depressing with a 70% 6 month mortality rate and with a high morbidity in the survivors (19).

A major controversy exists over the possibility that herpes may cause a mild diffuse encephalitis. Klapper, Bailey and Longson in the United Kingdom argue that HSE is common, but in most cases the mildness of the disease precludes a brain biopsy and the cause of the encephalitis goes unidentified (34). The diagnostic criterion for HSE in these studies was based on a radioimmune assay (RIA) for HSV antibodies and they believe that a certain ratio of cerebrospinal fluid (CSF) to serum antibodies is diagnostic of herpes encephalitis (36). Concern has been expressed that in the study which claims to validate this test, the controls were poorly selected and were inappropriate to the clinical setting where the test might be used. Secondly, it is argued that the number of patients in the brain biopsy proven group was too small to make this significant conclusion (16).

In summary, the clinical features of herpes encephalitis are non-specific and where a brain biopsy confirms the diagnosis, the outlook without treatment is particularly poor. The presence of a mild form of herpes encephalitis has yet to be confirmed.

5. Diagnosis

The diagnosis of herpes encephalitis cannot be made with certainty using clinical assessment and non-invasive tests. The only accepted and definite means of diagnosing herpes encephalitis pre-mortem is by demonstration of herpes simplex virus in tissue obtained by a brain

biopsy. The difficulty often arises in deciding which patients to biopsy. Non-invasive tests, although not definitive, are of some value in making the decision to proceed to biopsy and sometimes will assist in determining the site of biopsy. The NIAID collaborative study has provided valuable information by comparing the value of the non-invasive diagnostic tests to the results of brain biopsy (18).

a) Electroencephalogram (EEG)

The EEG in herpes encephalitis may show generalized abnormalities, but it is of particular help when it becomes localized with predominantly spiked and slow waves in the temporal region (37,38). In the NIAID series 81% of patients with herpes encephalitis had localization on EEG (18). In our series, 7 of 8 patients had localizatio n EEG (64). It is important to stress that localization on EEG is not specific since 59% of the HSV negative biopsied patients had focal signs on EEG. (18). The localization of the lesion on EEG often precedes changes detected on brain or CT scanning, and is therefore particularly useful in assisting with the choice of biopsy site. Some authors have suggested that slow wave complexes on EEG are associated with a poor prognosis (87,88). The number of patients evaluated in these studies was small. In view of the poor prognosis of all patients with HSV encephalitis, we do not feel there is presently sufficient evidence to correlate EEG findings with prognosis.

b) Brain Scan

It has been suggested that technetium brain scans may be of value in localizing the side involved in herpes encephalitis (40). In one study of 11 patients with presumed herpes simplex encephalitis (5 biopsy proven, 6 based on serology), [redacted] EEG scan was positive in 8 of 11, CT

scan negative in all patients and static radionucleotide brain scan was positive in all 10 patients in which it was performed. In our study of nine patients with herpes simplex encephalitis, brain scan was positive in the 4 patients in which it was performed (64). In NIAID study, technetium brain scans showed localizing features in 50% of cases, but was falsely positive in 14% of cases (18). The value of technetium brain scanning in herpes simplex encephalitis appears to be that it will localize the site of infection early, often before the computerized tomographic (CT) scan abnormalities are detectable.

c) Cerebral angiography

There are few studies of herpes simplex encephalitis in which cerebral angiography has been done routinely. In one study the role of cerebral angiography was reviewed in achieving a diagnosis of herpes encephalitis in 11 patients (10 biopsy proven) (39). The cerebral angiograms show characteristic changes of mass effect, localized areas of hypervascularity and venous blush. These findings are non-specific for herpes encephalitis and no studies have compared the value of cerebral angiograms with less invasive non-specific diagnostic techniques such as scans, EEGs or technetium brain scans.

d) Computerized Tomography

Computerized tomography has provided major advances in the early diagnosis of many neurological conditions. However the CT scan is neither sensitive nor specific enough to be used alone to achieve a diagnosis of herpes encephalitis (41). In one study all 11 patients studied with CT scan early in their disease had negative CT scans. In that study, Kim attributed the negative CT scanning results to the short interval between the onset of neurological symptoms and the first scan

(40). The commonest abnormality is a low absorption area in the temporal lobes with or without a mass effect (42). Unfortunately the CT scan is often normal in early cases and in the NIAID study showed a localization in only 60% of cases. False positive scans were obtained in 22% of cases (18). Unfortunately, the CT scan is most often negative in the early stages of herpes simplex encephalitis at the time when diagnosis is most crucial to the eventual outcome.

e) Antibody Studies

About 30% of patients with herpes simplex encephalitis are seronegative at the onset of disease implying that their encephalitis is the result of a primary herpetic infection. Seroconversion in these patients commonly ranges between 80-90%, depending upon the sensitivity of the test used (47). As many as 70% of patients who are seropositive at the onset of disease may show a four fold boost in antibody titres. There are some problems in the interpretation of these results, since 40% of the biopsy negative encephalitis patients who were seropositive at the time of biopsy also demonstrated significant increase in antibody titres. The serological tests used in the NIAID study were neutralization titres, passive hemagglutination or immunofluorescence for IgG antibodies (47). These tests are very sensitive and this may account for the relatively low specificity of the test. These results emphasize the difficulty in interpreting serum antibody studies in HSV encephalitis.

In an attempt to overcome these problems, CSF antibody levels have been examined both in isolation and also as a ratio to serum levels as possible diagnostic tests (46,47). Normally there is a barrier to the diffusion of antibody from blood to the CSF so that a much lower level

of antibody, usually less than 200-300 fold, is found is the CSF (58). However, if there is an HSV infection within the brain, specific antibody production in the CNS should dramatically increase levels. A ratio of serum to CSF of <20 has been proposed as being useful in diagnosing acute herpes encephalitis (46). The NIAID study group have reviewed their data and have shown that using simultaneous assays of serum and CSF antibodies, a lowered ratio of serum to CSF antibodies has a 50% sensitivity and 81% specificity for HSV encephalitis during the first ten days of infection. Unfortunately, the low sensitivity during the early phase of infection and the occasional false positive limits its value as a diagnostic tool for early HSV encephalitis.

f) Cerebrospinal Fluid Studies

Routine studies are not very helpful in the diagnosis of herpes encephalitis. There are no characteristic differences in either protein or glucose levels in the CSF that distinguish the HSV positive from the HSV negative groups. In general, there is a mild elevation of CSF protein and the CSF WBCs are frequently elevated to 50-500 cells/mm^3 and the CSF:blood glucose ratio was greater than 50% in 95% of the cases (18).

Several investigators have attempted to utilize the presence of viral antigens or proteins in the CSF to achieve a diagnosis. HSV-I specifies an enzyme thymidine kinase, which can readily be distinguished from cellular enzymes (51). In a rabbit model with early herpes encephalitis, virally specified TK can be detected in the CSF. In a limited study of human HSV encephalitis, viral TK has been detected in CSF (52,53). However these results have yet to be reproduced or confirmed in other studies.

A similar promising approach is to detect HSV antigens in the CSF of patients with HSV encephalitis using either radioimmune assay or enzyme link immunoassay (54,55). However these tests have still to be evaluated in a clinical trial.

Although Dyan and Stokes (56) claimed that immunofluorescent staining of CSF lymphocytes could be of value in the rapid diagnosis of HSV encephalitis, these results have been challenged (57). There has been no further studies suggesting that this technique would be useful in early and specific diagnosis of HSV encephalitis.

g) Brain Biopsy

Positive identification achieved by culturing herpes simplex virus in brain biopsy tissue is the only definitive diagnostic test for herpes encephalitis. The biopsy detects in 98% of cases (50).

Traditional tissue culture methods for isolation of herpes simplex virus may take 2-3 days and may even be slower particularly if taken from an inappropriate site (44,99). This delay has resulted in the development of a number of techniques to give a more rapid diagnosis while the culture result is awaited. Histological examination of the brain biospy in herpes encephalitis classically shows perivascular cuffing, lymphocytic infiltration and neuronophagia. These features were found in 71 of 84 patients in whom herpes simplex virus was subsequently cultured. In these patients only 47/84 patients had intranuclear inclusion bodies Cowdry Type A. In biopsy negative patients 25/70 also had evidence of acute encephalitis and ten of these patients had intranuclear inclusion bodies. These findings demonstrate the poor specificity of histology (47).

Electron microscopy has been used to examine brain biopsy

specimens. Traditional examination using thin section blocks takes 2-3 days and has a sensitivity of 45% with a specificty of 98% (47). However, more rapid attempts at diagnosis are being made by examining directly a freeze thawed preparation of homogenized tissue.

Touch preparations of the biopsy can be used for direct or indirect immunofluorescence to detect herpes virus antigen. In the NIAID study this test is positive in 70-80% of cases with a specificity of 91% (45,47). This test offers a diagnosis within hours of the time of biopsy. Selection of the most appropriate test for any given hospital depends upon the availability of equipment and technical expertise.

One study has quantified the virus recovered from 18 brain biopsy specimens. Eleven patients had $>10^4$ Tissue Culture Infective Dose $_{50}$ ($TCID_{50}$) per g brain, while 7 patients had $<10^4$ $TCID_{50}$ per g brain (47). Those patients with a large quantity of virus at the time of biopsy had a poorer outcome (47).

The principle argument against performing a brain biopsy when herpes encephalitis is suspected is that it is an invasive procedure (48). The risks associated with brain biopsy have been quantified in two series covering 90 consecutive brain biopsies (49,97). The major risks encountered are those of anaesthesia, intracranial bleeding, infection and post operative seizures (49,97). This risk of serious morbidity from a brain biopsy is in the neighbourbood of 0.5 to 2% (17).

One unexpected bonus from performing brain biopsy, is that in over one quarter of the herpes negative group, a previously unsuspected diagnosis which had not been achieved by conventional investigations, was made. In NIAID study 20 of the 76 biopsy negative patients had a new diagnosis made as a result of the biopsy (50). It cannot be

emphasized too strongly that these patients then received appropriate therapy based on the new diagnosis and 13 of the 20 patients made a complete recovery (50).

The importance of performing a brain biopsy to achieve the diagnosis of herpes encephalitis remains both an emotional and a controversial subject. Several factors including the risk of the procedure, the criteria used to select patients, the benefits of achieving a diagnosis and the risks of blind drug therapy all have to be considered in making this decision.

There is an argument by some clinicians that blind therapy with ara-A in patients with the clinical picture and non-specific tests supporting the diagnosis of HSV encephalitis should be an accepted practice (106). This argument will gain more support if and when less toxic antiviral agents become available for the treatment of HSV encephalitis. However, the principle of doing a brain biopsy to diagnose HSV encephalitis should not be based on the relative toxicity of antiviral agents, but on the principle that, wherever possible, therapy should be directed at an identifiable etiological agent. The clinical and non-invasive diagnostic tools currently available will identify a group of patients in which HSV is the etiological agent in only about 60% of cases (50). The benefit of the biopsy has been clearly demonstrated in the NIAID study where the biopsy confirmed HSV encephalitis in 98% of HSV encephalitis patients, and also diagnosed other treatable diseases in 20 of the 76 HSV biopsy-negative patients (50). Many of these patients received appropriate therapy with favourable results. Such patients could have suffered irreversible neurological damage or death if they had been given blind antiviral

therapy.

6. Therapy

For many years antiviral therapy for herpes encephalitis was not attempted since the only available antiviral agents caused excessive host toxicity when used systemically. In 1970 idoxuridine, a nucleoside analogue used topically for herpes keratitis, appeared to be beneficial if given systemically in treating herpes simplex encephalitis (109). However, this was an uncontrolled study and the criteria for the diagnosis of herpes simplex encephalitis were not sufficiently rigid. Despite this, for several years idoxuridine was the accepted standard therapy for herpes encephalitis. (108). In 1975, idoxuridine was shown to be both ineffective and toxic in a well designed placebo-controlled study with firm diagnostic criteria for herpes simplex encephalitis (59). Two similar nucleoside analogues have also been evaluated in the treatment of herpes encephalitis. Cytosine arabinoside has proved to be ineffective and highly toxic (34). However, adenine arabinoside (ara-A) has been shown to have a considerable therapeutic effect and currently is the only drug licensed for the treatment of herpes simplex encephalitis (19,50). In the initial trial of ara-A, 28 patients with biopsy proven herpes simplex encephalitis were randomised to receive either placebo (10 patients) or ara-A (18 patients). Of the ten patients treated with placebo, seven died within one month and a further two within six months (35). Only five of the eighteen patients treated with ara-A died. The placebo arm of this study was discontinued on ethical grounds (19). The study was continued with ara-A being given to all adult patients with biopsy proven herpes simplex encephalitis (50). In the 75 patients with biopsy proven herpes simplex encephalitis

treated with ara-A, cumulative mortality after one year was 39%. Two major prognostic indicators were noted. Patients under the age of thirty, who were only lethargic at the time of biopsy responded well and often returned to normal life. However, older patients, comatose at the time of biopsy faired badly and had an 80% mortality rate (50). These figures indicating an improved overall mortality with therapy, although encouraging, have not always been reproduced in other centres (60). A similar study has demonstrated the efficacy of ara-A in neonatal herpes simplex virus infection (21).

When the diagnosis of herpes simplex encephalitis has been confirmed by a brain biopsy, standard therapy now consists of ten days of treatment with intravenous ara-A at a dosage of 15 mg/kg/day. Several recent case reports have noted "relapses" or "recurrences" after antiviral therapy (99,100,101). In one case, a patient responded to ten days of ara-A therapy following a clinical diagnosis of herpes simplex encephalitis, but eight weeks later developed an encephalitic illness and HSV-I was recovered from a brain biopsy (99). In the one study where viral titers were made of the brain biopsy and subsequently of autopsy material, ara-A therapy resulted in a substantial reduction in viral titers in seven of eight patients, while in all patients receiving placebo viral titers increased (48). Although several hypotheses could readily explain these cases of relapsed encephalitis, a continuous review of both the duration and dosage of any regimen is prudent.

Unfortunately ara-A is a toxic agent. Its side effects include nausea, vomiting, diarrhea, bone marrow suppression, weakness, tremor and confusion (50,61). Furthermore, concern has been raised that the fluid load required to administer a therapeutic dose of ara-A may

be causing significant morbidity (62). For this reason considerable research has been undertaken into alternate antiviral therapy.

Adenosine arabinoside-5'-monophosphate (ara-AMP) is metabolised to the same active component as ara-A, but it has the advantage of a much greater solubility. In animal studies it appears to be effective in treating herpes simplex encephalitis (63). However, in the NIAID study, ara-AMP has recently been withdrawn because of "no demonstrable benefit" (120). In one recent local trial of ara-AMP treatment in biopsy proven HSV encephalitis, eight of nine patients were alive after one month and five of nine after one year and no marked toxicity was noted (64).

Several other new agents have recently been developed which are highly selective in inhibiting HSV replication and have little host toxicity. The efficacy of 9-(2-hydroxyethoxymethyl) guanine (acyclovir, ACG) and of E-5-(2-Bromovinyl)-2'-deoxyuridine (BVDU) in treating HSV encephalitis in animals has been demonstrated (65,66). Currently ACG is being evaluated by the NIAID in a controlled trial in humans.

Many patients with herpes encephalitis will require ventilatory assistance during decreased levels of consciousness. Cerebral oedema can adversely affect the outcome. Surgical decompression and insertion of pressure monitors may be necessary (34). The use of high doses of steroids have been advocated for the control of cerebral oedema, however the value of steroids in treating cerebral oedema associated with encephalitis remains controversial (110).

7. Animal Models

For over sixty years animals have been used as models for herpes infections. Initially animal models were used to investigate the pathogenesis of herpes infections but more recently they have been used

extensively for the testing of antiviral agents. The use of animal models has been of invaluable benefit in improving the understanding of herpes infections.

Doerr and Vochting in 1920, described that inoculation of herpes virus onto a rabbit's cornea led to the rabbit developing encephalitis (67). They believed that the initial infection caused a viremia which seeded in the brain. This view was corrected in 1923 when Goodpasture and Teague documented transmission of herpes virus from a peripheral focus to the central nervous system along sensory, motor and even sympathetic nerves (30). By injecting herpes into peripheral sites and subsequently examining central nerves for intranuclear inclusion bodies characteristic of infection, transmission of virus from the masseter muscle to the trigeminal nerve and from the vitreous humour to the optic tract were documented. Injections into other sites confirmed rapid transmission along almost any nerve.

Furthermore they developed a model of herpes encephalitis by inoculating herpes onto the scarified cornea of a rabbit. They found characteristic pathological changes along with sensory branch of the trigeminal nerve spreading into the pons and caudal medulla. The lesions in later stages of infection involved the ventral surface of the telencephalon including Ammon's horn. The inclusion bodies were usually found in tissue on the side of the infected eye although contralateral lesions were demonstrated (30).

Animal models of herpes encephalitis were not used to a great extent until the 1960s when the identification of herpes as a significant cause of human encephalitis kindled interest in both the pathogenesis of herpes encephalitis and in possible forms of therapy.

In 1970 some of the original studies were repeated using electron microscopy to confirm that herpes could pass from the cornea to the trigeminal ganglion and subsequently produce a localized encephalitis (68). Since then several different models of herpes encephalitis have been described.

a) Intraocular injection

Direct injection of herpes into the eyes has been described as one route of inducing encephalitis in 18 day old albino rabbits (69,70). Seven days after the injection of virus into the eye most animals became ataxic with head jerking movements. 10% of these animals proceed to die from convulsions but in the remaining 90% the signs completely resolve after 1-2 days. The virus spreads down the optic nerve by cell to cell infection of the neuroglia at a rate of 1-1.5 mm per day. By day 16 pathological lesions are found in the contralateral side of the chiasma in the lateral geniculate body and in both optic nerves.

This route of infection has been used in one study to correlate fluorescein staining for HSV and uptake of a ^{14}C labelled antiviral drug (71). Studies quantifying the severity of viral infection in this model have not been performed to date.

b) Intranasal Inoculation

Two different research groups have used intranasal inoculation of HSV into weanling mice as a model of herpes encephalitis (72,73). Both groups point out that entry through the nasopharynx is an important portal of entry in man. The model was primarily used for testing the activity of a number of new antiviral agents, when the infecting dose of virus would kill 99% of untreated mice. Of interest one group noted that older mice were resistant to the lethal effects of intranasal

inoculation of HSV (73).

c) Intracerebral Inoculation

Direct intracerebral inoculation of HSV has been used to precipitate encephalitis in mice, rats and guinea pigs (74,75). These models have been used primarily to test the efficacy of new antiviral agents. In one study 500 g guinea pigs inoculated with HSV intracerebrally reproducibly died between days 5 to 7. By immunofluorescent staining, a high concentration of viral antigen in the anterior cortex, and moderate concentration of antigen in both hemispheres was demonstrated (74).

d) Intraperitoneal Inoculation

Intraperitoneal inoculation of HSV in weanling mice has been used to test antiviral agents (77). The encephalitis that develops is only one of the many sites of infection throughout the body.

e) Corneal Inoculation

Inducing encephalitis by corneal inoculation has two obvious advantages. Firstly, the introduction of the virus to the brain is in a non-invasive fashion, and it avoids a variety of artifacts and side effects caused by direct inoculation. Secondly, as the route of infection centres on the trigeminal ganglion, it probably simulates human infection more closely (68).

Barringer using rabbits age 3-6 weeks has found that most animals after having HSV inoculated onto the scarified cornea developed a twisting of the head towards the inoculated side after 7-9 days. By day 10-12 a few animals had repeated twitching of the side of the face, retraction of the head and clonic movements of the forelimb. In these animals viruses could be identified in the inferior frontal portions of

both cerebral hemispheres (68).

A mouse model of herpes encephalitis following corneal inoculation has been fully described (78). About 50% of weanling mice infected by corneal inoculation developed encephalitis. The first signs were apparent at 6-10 days and death occurred at 9-13 days. In this study, various areas of the brain from the animals were studied each day by histological sections for pathological lesions, by electron microscopy and by immunofluorescent staining for viral antigen and by plaque-assay for viral titers. Of particular interest was the finding that viral titers in the trigeminal ganglion were very high reaching 10^7 pfu/g of pooled tissue (78).

C. NEW ANTIVIRAL CHEMOTHERAPEUTIC AGENTS

Initial attempts at antiviral chemotherapy used agents developed by the oncologists for inhibiting DNA replication. Understandably these agents lacked specificity and induced considerable host toxicity (59). However the demonstration in 1963 that herpes viruses infecting mouse fibroblasts induced a unique thymidine kinase (TK) opened a new opportunity for chemotherapy (79). The demonstration that this virally specified enzyme had biochemical differences distinct from the host enzyme and it could phosphorylate nucleoside analogues, while host cell enzymes failed to recognize them as substrates, has made specific antiviral chemotherapy a real possibility (80,51).

Acyclovir (9-(2-hydroxyethoxymethyl)guanine, ACG) is the first of a new class of antiviral agents that use viral TK to enhance their action (81). ACG is only phosphorylated by virally specified TK to its monophosphate derivative ACG-MP (82). Cellular enzymes further phosphorylate ACG-MP to its di and triphosphate derivatives (83). The

ACG-TP preferentially inhibits virally specified DNA polymerases (84). The phosphorylation of ACG causes it to be selectively trapped within HSV infected cells. In cells infected with either HSV TK-ve viruses or other viruses that do not specify TK, ACG is not phosphorylated and the ACG trapping within the cells is not significant.

The specificity of uptake of nucleoside analogues into virally infected cells is of major importance in reducing the toxicity of these drugs. Many new antiviral agents, which are similar to ACG in that they rely on viral TK for activation and uptake, are currently under development. For example, BVDU holds considerable promise in that only very small concentrations, in the order of 0.007 μg/mL, are required to significantly inhibit viral replication (23).

The studies conducted so far in both animal and clinical infections suggest that these agents are very potent, have only minor toxicity to the host and live up to their considerable potential which was first recognized in in vitro studies.

D) THE PROBLEM AND THE APPROACH

Achieving a definitive diagnosis is the cornerstone of good medical therapy. It removes concern about "missed" diagnoses, allows both treatment and response to be properly evaluated and avoids subjecting a patient to the potential side effects of drugs that offer no benefit. In herpes encephalitis, the diagnosis can only be made at the expense of undergoing a brain biopsy, a procedure which carries a small but real risk to the patient. Furthermore only one half of the patients judged by other non specific tests to have features compatible with herpes encephalitis actually have this infection. Therefore, there is a great need for a sensitive and specific, non-invasive test to diagnose herpes

encephalitis. The aim of this research has been to use the selective trapping of antiviral agents within HSV infected cells as a tool to develop a brain scan for herpes encephalitis.

As the new generation of antiviral agents are recognized only by herpesvirus specified TK, they are effectively trapped within HSV infected cells. If an appropriate gamma emitting label could be substituted into or attached to these antivirals and it became subsequently concentrated within HSV infected cells it could be used for diagnostic imaging. Preliminary studies using ^{14}C labelled antiviral agents have recently been reported in HSV infections (71). However, adequate quantitation of the viral infection with the radiolabel uptake were not reported. Furthermore, substitution of a gamma-emitting radioisotope into 2'fluoro-5-iodo-arabinosylcytosine (FIAC), the agent used in these studies, may be both technically difficult and could change the configuration of the drug so radically that the preliminary study to demonstrate the selective trapping would have to be repeated.

The approach to the problem has been initially to investigate and evaluate a variety of animal models of herpes encephalitis for their ability to simulate the human infection. The ideal animal model would be one that developed focal HSV encephalitis localized around the temporal lobe that could be induced in 100% of animals with little artifact. An animal with a large brain would offer obvious advantages in requiring less resolution in the scanning procedure.

Next, a variety of cold antiviral agents were examined in tissue culture for their ability to inhibit viral replication. The radiolabelled agents were used in uninfected and HSV infected tissue culture to provide data on the uptake of label into infected cells, on

the optimal timing and dosage of drug administration and on the predicted sensitivity and specificit the test.

These studies have been followed by tissue distribution studies of the labelled antiviral compound in an infected and uninfected animal model so that optimal conditions for a scan could be established. The ultimate aim of this research would be to compare in a clinical trial the sensitivity and specificity of the selective trapping of the radiolabelled nucleoside in a brain scan to that of the brain biopsy for the diagnosis of herpes encephalitis.

CHAPTER II MATERIALS AND METHODS

A. CHEMICALS AND REAGENTS

Agarose and L-glutamine were purchased from Sigma Chemical Company, St. Louis, Missouri. Disodium ethylenediamine-tetraacetate (EDTA) and sodium bicarbonate powder were purchased from Fisher Scientific Company, Fair Lawn, New Jersey. Penicillin G was bought from Glaxo Pharmaceuticals, Toronto, Ontario, and streptomycin was bought from Allen and Hanburys, Toronto, Ontario. The Basal Medium Eagles (BME), Minimum Essential Medium (MEM), Foetal calf serum and bovine calf serum were all purchased from Flow Laboratories, Virginia. All bottles of serum were heat inactivated at 56°C for 30 minutes and maintained at 4°C until use.

Sodium phenobarbital was purchased from Abbott Laboratories, Montreal, Quebec. Lidocaine Hydrochloride 2% was purchased from Astra Laboratories, Montreal, Quebec. Trypsin (1:250) was bought from Difco Laboratories, Detroit, Michigan and neutral red was bought from J. Baker Chemical Company, Phillipsburg, New Jersey. All tissue culture plates, tubes and flasks were purchased from Beckton-Dickinson, Oxnard, California.

8-Iodo-9-(2-hydroxyethoxymethyl)guanine (I-ACG) and 8 - Bromo-9-(2-hydroxyethoxymethyl)guanine (Br-ACG) were provided generously by Dr. M.J. Robbins, University of Alberta. [^{131}I]-Iodovinyl-deoxyuridine and [^{125}I]-Iodovinyldeoxyuridine were synthesized and provided by J. Samuel, under the supervision of Dr. L. Wiebe and Dr. E.E. Knaus, Unversity of Alberta (114).

B. ANIMALS AND VIRUSES

1. Animals

Guinea pigs (Hartley strain), mice (Balb/cCr strain), rats (Lewis strain) and rabbits (Dutch strain) were all obtained through the University of Alberta Small Animal Breeding Program. The date of birth was known for each animal and all animals were kept under standard conditions.

2. Viruses

Herpes simplex Type I viruses, KOS strain, and B2006 strain which is TK deficient (121), were obtained from Dr. W.C. Leung, University of Alberta. Herpes simplex Type I (JLJ strain) was obtained from the culture of the brain biopsy of a local patient with herpes encephalitis. Typing had been previously performed by immunofluorescence.

C. CELL PREPARATION AND MAINTENANCE

1. Continuous cell lines

Vero cell lines were maintained in Eagle's minimal essential medium (MEM) supplemented with 100 IU./mL of penicillin G, 100 µg/mL of streptomycin, 2 g/L $NaHCO_3$, 2 mM of glutamine and 4% calf serum. The monolayers were disrupted by washing twice with ATV solution (Trypsin 1:250 at 0.25%, 0.5 mM dextrose, 100 mM KCl, 100 mM naCl, 50 mM $NaHCO_3$ and 1 mM EDTA) and divided in the ratio of 1 to 3. All cultures were maintained at 37°C in either tissue culture plates in a humidified 5% CO_2 incubator or screw capped culture flasks.

2. Primary rabbit kidney cells

Three day old rabbits were sacrificed by cervical dislocation. The kidneys were removed, washed several times in sterile phosphate buffered saline (PBS) and cut into small pieces. The pieces were incubated for

40 minutes at 37°C in 10 mL BME containing 0.25% Trypsin 1:250, 100 IU./mL penicillin G, 100 µg/mL streptomycin and 2 g/L $NaHCO_3$. The supernatants were collected from three sequential incubations and added to 10 mL of Foetal calf serum. After a cell count, tissue culture flasks were seeded at a concentration of 5×10^6 cells per 25 cm^2 flask. Cells were maintained in 10 mL of BME supplemented with 10% foetal calf serum, 100 µg/mL streptomycin, 100 IU./mL penicillin G, 2 g/L $NaHCO_3$ and 2 mM glutamine. Cells were split three times, 24-48 h apart, usually at a ratio of 1:2 and were used after 3 cell passages.

D. VIRUS PREPARATION

1. Stock Virus

Monolayers of Vero cells were infected at a multiplicity of infection of 0.1 plaque forming units (pfu) per cell. At approximately 20 hours, when the cells showed marked cytopathic effect (CPE), the virus was harvested by subjecting the cells to three cycles of freezing and thawing, followed by centrifugation at 1000g for 10 minutes. The supernatant which contained the virus was stored in aliquots of 5 mL at -70°C.

2. Brain samples

The brains were removed from the animals aseptically and were bisected and each half divided into three geographical areas. Samples were weighed, diluted to 10% w/v with MEM, supplemented with 2 mM glutamine, 2 g/L $NaHCO_2$, 100 IU./mL penicillin G, 2% calf serum and 100 µg/mL streptomycin and sonicated at 200 watts for 3 minutes in a Braunsonic sonicator. Following centrifugation at 1000g for 10 minutes serial dilutions of the supernatant were made. All samples were stored at -70°C until plaque assays were performed.

E. PLAQUE ASSAYS

1. Virus Titrations

Vero cells were grown to confluency in 60 mm tissue culture dishes. The media was removed and 0.1 mL of the virus was spread over the cells' surface. After a 1 h incubation period in a humidified 5% CO_2 incubator at 37°C, an overlay of 4 mL of preheated 1.2% agarose and MEM (supplemented with 100 IU./mL penicillin G, 100 µg/mL streptomycin, 2 g/L $NaHCO_3$, 2 mM glutamine and 2% calf serum) was made. After three days, a 2 mL solution of preheated 1.2% agarose, MEM and 1% neutral red was added on top of the overlay.

Plaques were counted macroscopically 6 h after staining and CPE was confirmed by microscopic examination.

2. Antiviral activity assays

Primary rabbit kidney cells were passed and grown to confluency as previously described. After infection with 30-50 pfu of HSV-I in 0.1 mL, the cell monolayer was incubated for one hour at 37°C in a humidified 5% CO_2 atmosphere. Various dilutions of each drug were prepared in MEM (supplemented as previously described) mixed with an equal volume of 1.2% agarose in double strength supplemented MEM solution and were added to monolayers to give a final volume of 4 mL.

F. ANIMAL INFECTION TECHNIQUE

Mice and rats were anesthetized in an ether chamber. Guinea pigs and rabbits were sedated by intraperitoneal injection of phenobarbital (0.08 mg per g body wt.). Following removal of fur with an electrical shaver, the scalp was cleaned with povidone iodine solution 5%, anesthetized with 2% lidocaine and a small burr hole was made with a 16 G needle midway between the eye and the ear and 3 mm to the LEFT of the

midline. A 26 G needle was inserted to a depth of 4 mm through the burr hole and 0.1 mL of MEM containing the virus was injected into the left temporal lobe.

G. QUANTITATION OF CELLULAR UPTAKE OF [^{131}I]-IVDU BY RABBIT KIDNEY CELLS

The rabbit kidney cells were grown to confluency in 25 cm^2 flasks (approximately 5 x 10^5 cells/flask). The medium was removed and the cell monolayer was infected with a known infecting dose of either HSV-I (TK +ve), HSV-I (TK -ve) or mock infection. After 1 hour, 2 mL of medium was added to the flask which was incubated at 37°C. A known activity and concentration [^{131}I]-IVDU in 0.2 mL was added at 7 h after infection. The dose and activity of the [^{131}I]-IVDU was deliberately chosen to be well below the range where its antiviral activity may inhibit viral replication, but was sufficient to provide adequate counts. Unless otherwise stated, the flasks were incubated for a further 4 h. Early CPE was confirmed microscopically prior to harvesting. The supernatant was decanted and the cells washed twice with 1 mL of phosphate buffered saline. The washings were added to the supernatants for quantitative counting. Radio immune precipitation assay (RIPA) buffer (1 mL) was added to each flask to dissolve the cells (85). The flask was left for five minutes and the fluid removed. After two further washes with RIPA buffer (1 mL/wash), quantitative counting was performed on the solubilized cells. All samples were counted within six hours of collection in a Beckman 8000 gamma counter.

CHAPTER III EVALUATION OF ANIMAL MODELS

Many variables are involved in establishing an animal model of herpes encephalitis. Seven variables were examined either alone, or in a small number of the possible combinations in the effort to establish an animal model suitable for pharmacokinetic studies and radioisotope scanning.

The variables were:

i) Animal species eg. rat, mouse, guinea pig, rabbit

ii) Age of animal eg. weanling, adult

iii) Route of infection eg. corneal inoculation, intracerebral injection

iv) Titer of infecting dose eg. 10^6 pfu, 10^4 pfu.

v) Strain of infecting virus eg. HSV-I (JLJ), HSV-I (B2006), HSV-I (KOS)

vi) Volume used to deliver the infection eg. 0.01 mL, 0.1 mL

vii) Time at which the animal brains were examined eg. 2 days, 5 days

The ideal model would be an inexpensive animal in which encephalitis could be induced atraumatically with reproducibility. Preferably the encephalitis would be localized to one area of the brain in a similar distribution to that found in human herpes encephalitis and the animal should exhibit clinical signs suggestive of encephalitis. An identical animal model with the infection caused by a TK -ve deficient mutant of HSV-I would also be desirable as it would provide an excellent control to test the specificity of the scanning approach which depends upon viral thymidine kinase (TK) activity.

A. ANIMAL SPECIES

Several pilot experiments were initially performed using a small

number of animals. Four different species of animals were examined as potential models. One strain of each animal species always provided by the same supplier was used during this research. This was a deliberate policy to avoid any of the variations in resistance to HSV-I infections that have been demonstrated between even closely related strains of the same animal species (111). The following basic criteria were used to assess the suitability of the animal for further study:

1. The ease and safety of inducing anesthesia.
2. The ease of inducing infection by a given route.
3. The ability of the animal to exhibit recognizable clinical signs of encephalitis.
4. The ease with which the brain could be removed, its areas recognized, and detailed dissection performed.

1. Rat

Six adult Lewis strain rats (wt. approx. 300 g) were anesthetized by ether. Considerable difficulty was encountered in achieving a suitable level of anesthesia for a duration adequate to give an intracerebral injection of 10^7 pfu HSV-I (KOS). Two rats failed to revive after anesthesia. Two rats were alive but had not exhibited any clinical signs of encephalitis after three months. Two rats died at 96 and 120 h after injection, neither having shown signs of infection prior to death. Virus could be recovered from both brains at a titer of 10^5 pfu/g. However the brain was relatively small for detailed dissection and subsequent culture of the various geographical areas. In view of the size of the brain, the variable response and difficulty with anesthesia, the rat was not used for further evaluation.

2. Guinea Pig

Seven young adult Hartley strain guinea pigs (wt. approx. 550 g) were anesthetized by intraperitoneal injection of barbiturates as previously described. All animals were infected by intracerebral injection of HSV-I (KOS) 10^7 pfu in 0.1 mL. Four animals failed to develop any illness and survived a minimum of three months. The three remaining animals developed encephalitis which manifested itself as disinterest in eating, drinking, decreased preening activity, lethargy and ruffled fur. These animals died at 48, 120 and 156 h after infection. Virus was recovered in the brain of each animal at an average titer of 10^5 pfu/g tissue. Each brain was divided into six areas. In all animals the titer from the left cerebrum, which was the site of viral inoculation had a titer ten fold higher than in other areas of the brain. The guinea pig brain could be removed easily and as it weighed approximately 5 g in the adult animal, it was of sufficient size to facilitate easy dissection. However, the guinea pig was too small for the brain scanning apparatus available.

3. Rabbits

Eight freshly-weaned Dutch rabbits (wt. approx. 900 g) were readily anesthetized with intraperitoneal injection of barbiturates. All animals were infected by intracerebral inoculation of 10^7 pfu of HSV-I (JLJ) using the technique described in Chapter II. Seven of the eight animals developed overt encephalitis and/or neurological defects which resulted in death within 3-7 days after infection. The neurological manifestations included laboured breathing, grinding/gnashing of teeth which progressed to episodic extension on the front legs with a simultaneous extension of the neck backwards with a twisting to the side

of the initial infection (left). Each episode lasted about 60 seconds and was followed by a period of relative normal behaviour. Within several hours the episodes became almost continuous and the two animals which were closely observed with these signs died within twelve hours. One animal showed a unilateral hind limb paralysis one day prior to developing the obvious signs of encephalitis. The brain (wt. approx. 7 g) could be removed and dissected easily. HSV-I was detected in the brains of all the animals which died. The titer of virus was approximately 10^5 pfu/g of brain tissue, and was ten fold to one hundred fold greater on the left side of the brain per g tissue.

4. Mice

Young Balb/cCr mice (age 25 days) were readily anesthetized with ether. A burr hole was easily made through the skull using a 25G needle and various titers of HSV-I (JLJ) in 0.05 mL were injected through the same needle into the left side of the brain. Only two of fifty animals failed to recover from anesthesia. All of the animals injected with HSV-I (JLJ) 100 pfu or greater died from encephalitis within one week. The survival curve of these animals is shown in Figure 1. Only 40% of the mice showed clinical signs of encephalitis prior to death, which usually occurred between days 3 and 7. The signs manifested in these animals were disinterest in eating, drinking, diminished activity, hyperexcitability to stimuli, ruffled fur and huddling. The brain was small (wt. approx. 0.3 g) but easy to remove. However, due to its size detailed dissection was not feasible.

Conclusion

From the results of these preliminary experiments it was decided that two animals appeared suitable for further studies. The rabbit

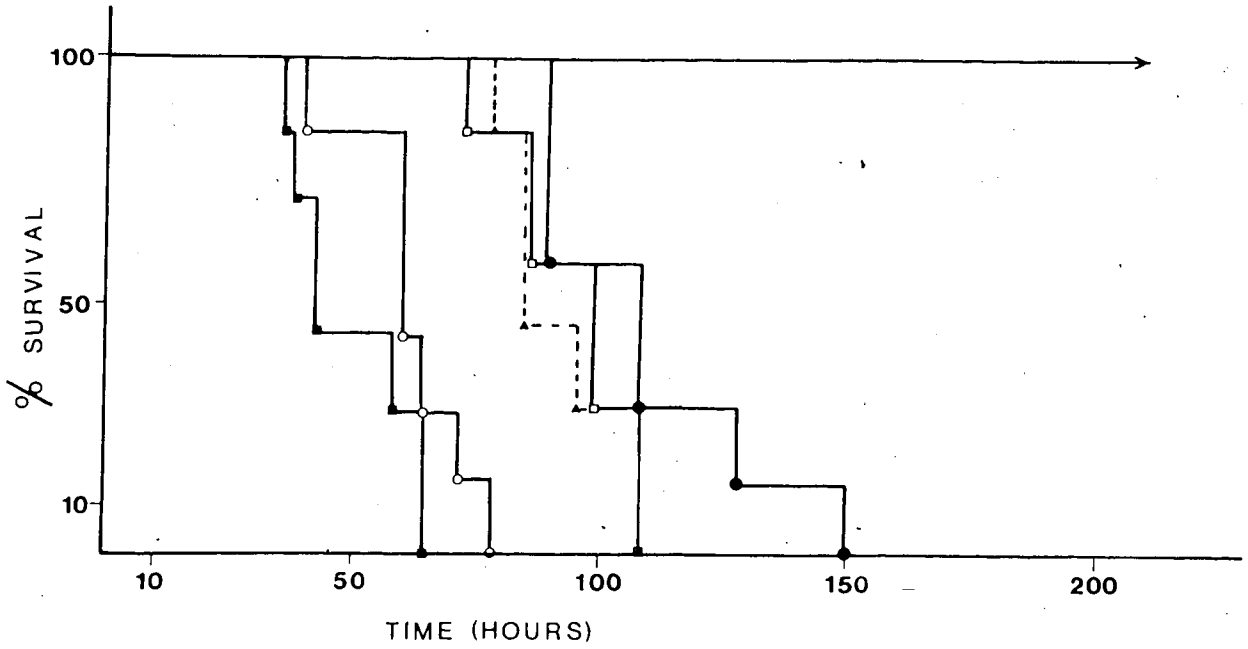

Figure 1

Survival curves of Balb/cCr mice age 3 1/2 weeks infected with various titers of HSV-I (JLJ) given by intracerebral inoculation in 0.05 mL. Seven animals were infected in each group.

■ = 10^6_5 pfu
○ = 10^5_4 pfu
▲ = 10^4_3 pfu
□ = 10^3_2 pfu
● = 10^2 pfu

offered the advantage of i) ease of handling ii) ease of anesthesia iii) showing pronounced clinical signs characteristic of encephalitis and iv) having a brain of a size suitable for detailed dissection. The mouse model offered the advantages of i) ease of handling and anesthesia and ii) its low cost allowed its use in considerable numbers making it particularly suitable for dose-response curves and tissue distribution studies. However the weanling mouse (age 25 days) was too small for easy blood sampling from its tail vein, so in subsequent experiments, older mice (49 days) were used.

B. ROUTE OF INFECTION

The five routes used in inducing herpes encephalitis have been described earlier. Intraperitoneal injection causes a disseminated infection which would be inappropriate for this work, since the comparison uptake of nucleoside analogues in HSV infected brain tissue to other uninfected tissues would be of value in evaluating a model for adult herpes encephalitis. The intranasal route of infection appeared inappropriate not only because the infection is often systemic, but also because encephalitis can o- be induced by this route in neonatal animals, restricting the size of animals that can be used (73). Intraocular inoculation produces an infection that is relatively localized, but is usually self-limiting and the animal seldom shows features compatible with encephalitis. Therefore the two models evaluated were corneal inoculation and intracerebral injection. It was decided to evaluate these routes in both the rabbit and guinea pig as both animals appeared suitable at that time.

1. Corneal Inoculation

Four young adult male guinea pigs (wt. approx. 450 g) and two

weanling male rabbits (wt. approx. 850 g) were anesthetized by intraperitoneal injection of barbiturates. After 30 minutes the left cornea of each animal was scarified with a 21G needle and a 0.1 mL of a stock virus solution containing 10^8 pfu of HSV-I (KOS) was dropped onto and massaged into the cornea. The animals were observed daily, initially for herpetic keratitis and subsequently for the development of any neurologic signs.

The features exhibited were:

Day 1 - eye open: scarifications on clear cornea

Day 2-4 - eye open: small vesicles visible adjacent to scarifications: increasing purulent exudate

Day 5-10 - eye closed: caked exudate: cornea opacifying

Day 10-12 - eye open: cornea opaque

Day 20-60 - eye open: corneal opacity slowly resolving

None of the animals infected by this route developed neurological signs during the period of observation (60 days). One guinea pig was electively sacrificed after seven days to see if virus could be isolated from the brain. A plaque assay was performed and no virus was detected.

2. Intracerebral Injection

Four young adult male guinea pigs and two weanling male rabbits were anesthetized by an intraperitoneal injection of barbiturates. A small burr hole was made in the skull and 0.05 mL containing 10^7 pfu of HSV-I (KOS) was injected into the left cerebral cortex. The animals were observed daily for encephalitis. Two guinea pigs and both of the rabbits died from encephalitis between 4 and 5 days. The brains from all of the animals were removed and plaque counts for virus performed on 6 areas of the brain. The results are depicted in Table 1.

Table 1. Titer of Virus in Anatomical Areas of Rabbit and Guinea Pig Brain

Area of Brain	Rabbit		Guinea Pig	
	1	2	1	2
Left Anterior	*$4x10^5$	$5x10^5$	$7x10^5$	$7x10^4$
Left Middle	$1x10^6$	$3x10^6$	$1x10^6$	$2x10^6$
Left Posterior	$4x10^4$	$1x10^3$	$1x10^5$	$1x10^5$
Right Anterior	$5x10^5$	$1x10^4$	$6x10^4$	$4x10^5$
Right Middle	$6x10^5$	$1x10^6$	$9x10^5$	$2x10^6$
Right Posterior	$2x10^4$	$1x10^4$	$8x10^4$	$1x10^5$
Time of death	100 h	120 h	96 h	94 h

* Plaque forming units per gram of brain tissue

Conclusion

From these preliminary experiments, it appeared that the corneal route of introducing infection with HSV-I (KOS) did not produce clinically overt infection in either guinea pigs or rabbits. The intracerebral inoculation route appeared substantially more promising and merited further evaluation.

Infection by the route of corneal inoculation has been well described in the literature (30,68). Strain virulence appears to be of major importance. The KOS strain used in these early experiments has recently been shown to be less pathogenic than previously assumed (107, 117). Subsequent experiments using HSV-I (JLJ strain) inoculation onto

a scarified cornea was successful in producing disease. However, by the time this was realized, we had adopted the intracerebral route of inoculation of HSV-I (JLJ) for the subsequent studies.

C. ANIMAL AGE/TITER OF VIRUS

Following these initial studies it appeared that the most promising animal models for this work would be the rabbit and the mouse infected by the intracerebral route. The effects of the age of the animal and the titer of infecting virus were next evaluated, as both might affect the reproducibility of the model.

1. Rabbit Model

Eight weanling and eight adult rabbits were infected with a known titer of HSV-I (JLJ) by intracerebral inoculation. Four rabbits in each group received 10^7 pfu and four received 10^4 pfu of virus. The survival of the animals is shown in Table 2.

Six areas from each brain of all fifteen rabbits were assayed for virus by plaque counting. In all animals an average of 5×10^4 to 5×10^6 pfu/g brain were detected. No difference was detected between the viral counts in the old or weanling animals, or between the animals infected with different doses of virus. However it was noticed the titers were usually significantly higher in the left side of the brain (particularly, the left middle zone). This will be shown in more detail in Section E of this chapter. A five fold variation in viral titers on plaque counting was not unusual, on both duplicate or repeat assays.

2. Mouse Model

Fifty 25 day old and fifty old Balb/cCr mice were anesthetized and injected with mL of a known titer of HSV-I (JLJ) virus intracerebrally. Ten animals from each group were inoculated at

Table 2. Effect of Age of Animal and Titer of Virus on Herpes Encephalitis Model in Rabbits

a. Adult rabbits survival

	Rabbit Number			
	1	2	3	4
10^7 pfu	115 h	126 h	140 h	survived
10^4 pfu	143 h	148 h	148 h	188 h

b. Weanling rabbits survival

	1	2	3	4
10^7 pfu	74 h	85 h	120 h	102 h
10^4 pfu	120 h	120 h	120 h	120 h

each titer. The survival curves for the weanling mice are shown in Figure 1 and for the 6 week old mice in Figure 2. The brains of all the mice in Figures 1 and 2 were removed at death and the virus quantified by plaque assay. In all animals, virus could be readily isolated at titers varying from 5×10^4 pfu per g to 1×10^6 pfu per g of brain. As can be seen from comparing curves, the larger the infecting dose of virus the shorter the survival of the animal. Similarly it can be appreciated from the survival curves that the younger animals developed encephalitis earlier than older animals inoculated with an identical titer of virus.

A further unexpected but interesting finding was found in examining the plaque counts from the brain at the time of death when a relatively

narrow range of viral titers was detected. This range was constant regardless of species of animal (rabbit or mouse), duration of infection (time of death), age of animal, or of titer of initial viral inoculation. These results suggest that there may be a critical average titer of virus (around 1 x 10^6 pfu/g of brain) above which the brain cannot function normally and maintain life.

D. STRAIN OF VIRUS

The pathogenicity of a virus can vary dramatically both with the species of the host and the tissue that is infected. In view of this, the pathogenicity of three strains of HSV-I were evaluated in the mouse model. HSV-I (JLJ) a wild strain which has only undergone three passages in vitro, HSV-I (B2006) a thymidine kinase deficient (TK -ve) strain and HSV-I (KOS) a well described strain that has undergone many passages in vitro were evaluated. It was particularly important to establish a TK -ve animal model as this would provide an excellent control for the scanning approach which relies on viral TK.

The pathogenicity of the three viruses was examined by establishing survival curves for all three strains in the mouse model. Figures 2,3 and 4 show the survival curves for mice infected with HSV-I strains JLJ B2006 (TK -ve) and KOS respectively. All animals were Balb/cCr mice age 6 weeks infected by a known titer of the appropriate strain given by intracerebral injection in 0.05 mL. From these curves it can be seen that the wild strain HSV-I (JLJ) was more pathogenic at high titers in that it caused death more rapidly than the other two strains, and at lower titer, it was more pathogenic causing a higher percentage of animals to die. By comparing Figures 1 and 5 to 2 and 3 it can be seen that this effect is not age dependent.

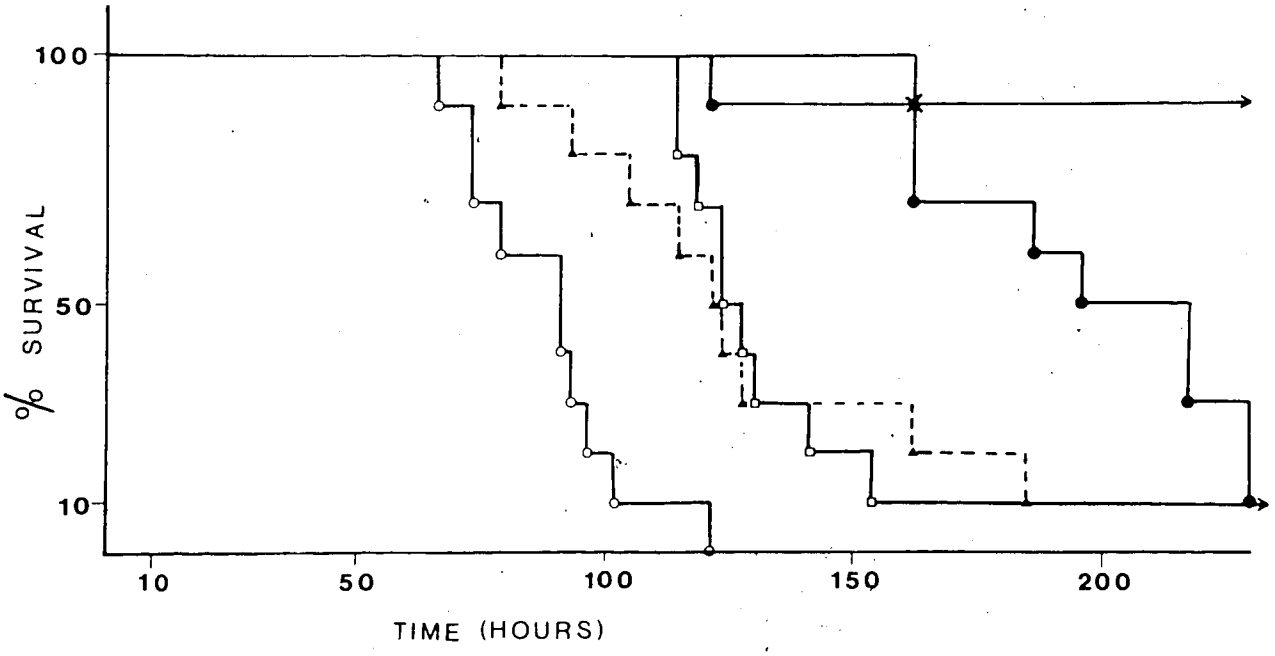

Figure 2

Survival curves of Balb/cCr mice age 6 weeks infected with various titers of HSV-I (JLJ) given by intracerebral inoculation in 0.05 mL. Ten animals were infected in each group.

○ = 10^5 pfu
▲ = 10^4 pfu
□ = 10^3 pfu
● = 10^2 pfu
✕ = 10 pfu

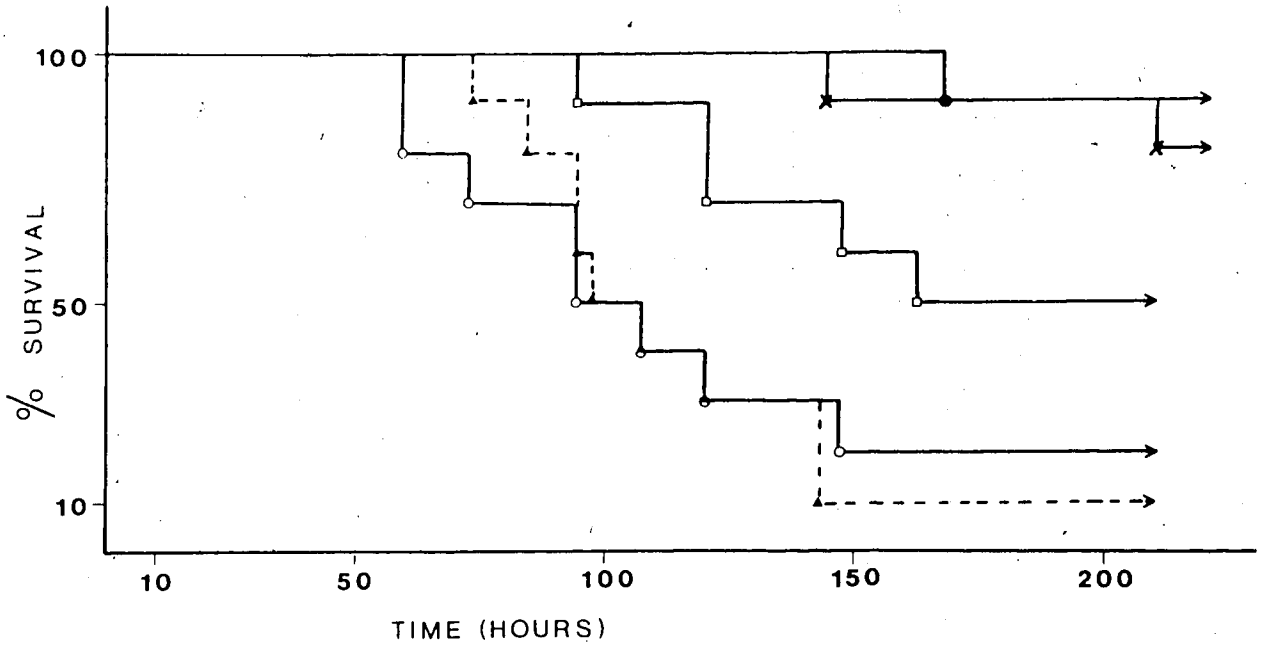

Figure 3

Survival curve of Balb/cCr mice age 6 weeks infected with various titers of HSV-I (B2006, TK -ve) given by intracerebral inoculation of 0.05 mL. Ten animals were infected in each group.

○ = 10^5 pfu
▲ = 10^4 pfu
□ = 10^3 pfu
● = 10^2 pfu
✕ = 10 pfu

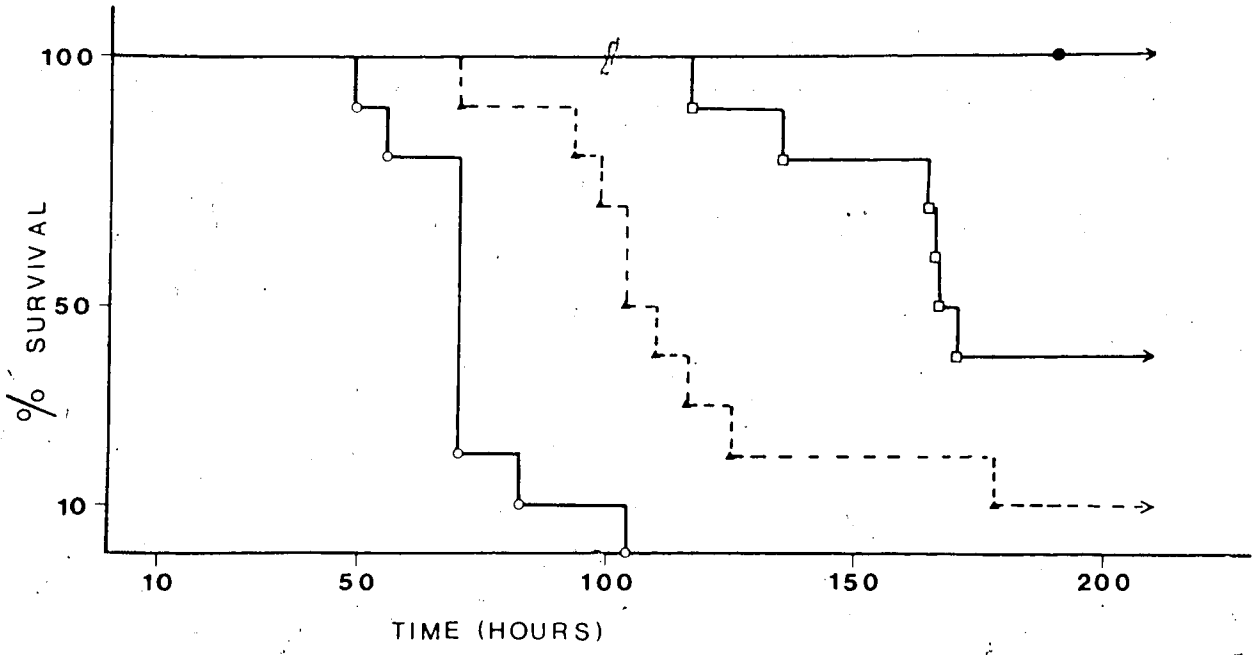

Figure 4

Survival curves of Balb/cCr mice age 6 weeks infected with various titers of HSV-I (KOS) given by intracerebral inoculation of 0.05 mL. Ten animals were infected in each group.

○ = 10^5 pfu
▲ = 10^4 pfu
□ = 10^3 pfu
● = 10^2 pfu

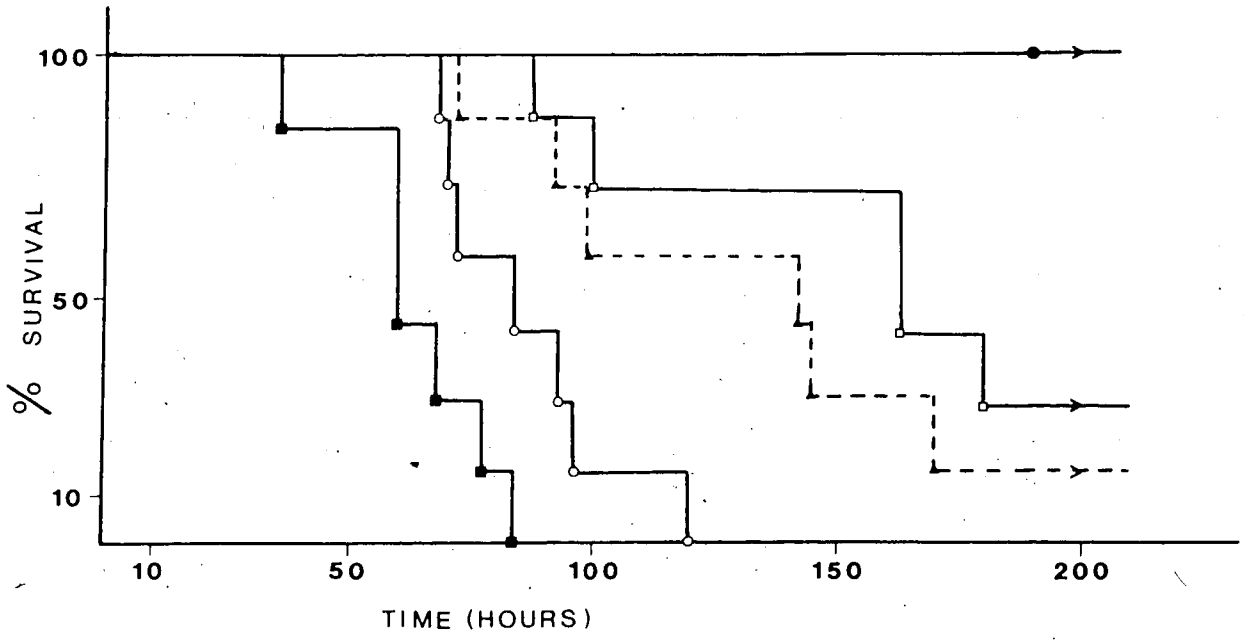

Figure 5.

Survival curves of Balb/cCr mice age 3 1/2 weeks infected with various titers of HSV-I (B2006, TK -ve) given by intracerebral inoculation of 0.05 mL. Seven animals were infected in each group.

■ = 10^6 pfu
○ = 10^5 pfu
▲ = 10^4 pfu
□ = 10^3 pfu
● = 10^2 pfu

Conclusion

Using a high titer of HSV-I (B2006) an animal model of herpes encephalitis caused by a TK -ve virus was established and this appears suitable for further use as a control model in the attempt to develop radioisotope scan for herpes encephalitis. The studies with different strains of virus clearly demonstrated the varying pathogenicity of these strains. The wild strain HSV-I (JLJ), at a high titer, caused death from encephalitis more rapidly than similar infections with the other two strains. At lower titers the HSV-I (JLJ) caused death in more animals than the other two strains. The diminished pathogenicity of the TK -ve strain has previously been described, but the low pathogenicity of KOS strain has only recently become appreciated (112,107).

E. VOLUME OF INFECTING DOSE/TIME COURSE

From the previous results it appeared that encephalitis was readily induced in a weanling rabbit infected by an intracerebral injection of 0.1 mL containing 10^7 pfu HSV-I (JLJ). In this model the titers of virus recovered from the area initially infected (the middle zone of the left cerebral hemisphere) were ten fold greater than in other areas of the brain. As it would be advantageous for scanning purposes to have one localized area which has much more viral replication than other areas of the brain, two factors were examined in an attempt to further increase the localization of infection.

1. Volume adjustment

In order to evaluate the importance of the volume of the intracerebral injection in influencing the localization of infection, four weanling rabbits were infected by intracerebral injection with 10^7 pfu of HSV-I (JLJ). In two rabbits the inoculum was given in 0.1 mL and

in two it was given in 0.01 mL. All four rabbits died between 92 and 108 hours after infection and the average viral titers of the animals are depicted in Table 3.

Table 3. Effect of Volume of Infecting Virus on Localization of Infection

	Rabbits (0.1 mL)		Rabbits (0.01 mL)	
	1	2	1	2
Left Anterior	*$1x10^5$	$3x10^5$	$3x10^5$	$2x10^5$
Left Middle	$4x10^5$	$7x10^5$	$1x10^6$	$8x10^5$
Left Posterior	$8x10^4$	$1x10^5$	$1x10^4$	$5x10^4$
Right Anterior	$1x10^5$	$4x10^4$	$2x10^4$	$1x10^4$
Right Middle	$4x10^4$	$7x10^4$	$1x10^4$	$1x10^5$
Right Posterior	$5x10^4$	$3x10^4$	$4x10^4$	$3x10^3$

*Plaque forming units per gram of brain tissue.

Conclusion

From these results it appeared unlikely that minimizing the volume of the infecting dose would significantly improve the localization of infection.

2. Time Frame

A second possible factor which could influence the localization of the infection could be time of examination after infection. It appeared possible that virus might spread out centrifugally from the initial site of infection, so if the animal was examined early in the course of its infection before major dissemination of the infection had occurred,

viral titers might reveal more localized infection.

Six weanling rabbits were infected with 10^6 pfu of HSV-I (JLJ) in 0.05 ml in the left cerebral hemisphere. Two animals were sacrificed at 24, 48 and 72 h. Following sacrifice the brains were removed, dissected into distinct anatomical areas, and plaque counts were performed. The plaque counts are depicted in Table 4.

Table 4. Time Course Study on [illegible]tion of Infection

Area of brain	Rabbit sacrificed at					
	24 h	24 h	48 h	48 h	72 h	72 h
Left Anterior	*$1x10^6$	$2x10^6$	$2x10^4$	$2x10^4$	$1x10^3$	$5x10^3$
Left Middle	$5x10^6$	$2x10^6$	$1x10^4$	$2x10^4$	$1x10^4$	$2x10^5$
Left Posterior	$1x10^5$	$2x10^5$	$2x10^4$	$1x10^3$	$1x10^2$	$1x10^2$
Right Anterior	$4x10^5$	$5x10^4$	$2x10^3$	$2x10^3$	$1x10^3$	$1x10^2$
Right Middle	$1x10^6$	$1x10^3$	$2x10^3$	$4x10^3$	$1x10^3$	$3x10^3$
Right Posterior	$1x10^6$	$8x10^4$	$1x10^3$	$2x10^3$	$1x10^2$	$4x10^2$

*Plaque forming units per gram of brain tissue.

These figures are of interest for several reasons. Firstly, all previous animals had appeared clinically well for the first 72 h after infection, despite a considerable number of viruses being present. Secondly, there does not appear to be a pronounced certrifugal spread of virus following an initial localized infection.

F. MACROSCOPIC APPEARANCE OF RABBIT BRAIN

To complete the evaluation of the weanling rabbit model of herpes

encephalitis two rabbits were examined as controls. Each rabbit was injected with 10^7 pfu of HSV-I (JLJ) that had previously been inactivated by exposure to ultraviolet light (113). These rabbits remained completely well and when they were electively sacrificed 120 h after infection, HSV could not be cultured from the brain of either animal.

The appearance of the uninfected rabbit brain was unremarkable, apart from a small scar on the site of injection. In contrast the brains of the infected animals showed several macroscopic changes (see Plate 1). At the site of injection in the left cerebral hemisphere a small area of induration was visible centred around the site of needle puncture. Most of the left hemisphere was hyperemic with a distinct loss of sheen. When dissected the left hemisphere was distinctly soft suggestive of cerebral necrosis.

G. SUMMARY

From these animal studies, it appeared that two different animal models would be needed for the *in vivo* studies in the development of a brain scan for herpes encephalitis. The main model would be that of the weanling Dutch rabbit. By the intracerebral inoculation of 10^7 pfu of HSV-I (JLJ), a clinically obvious illness could be induced in most animals by 4 days. In these animals, viruses could be recovered at a titer of between 10^5 to 10^6 pfu per g of brain tissue. The titers in the area around the injection site were usually ten fold higher than other areas of the brain. In the rabbit, both pathological and clinical pictures remarkable similar to that of the human condition were

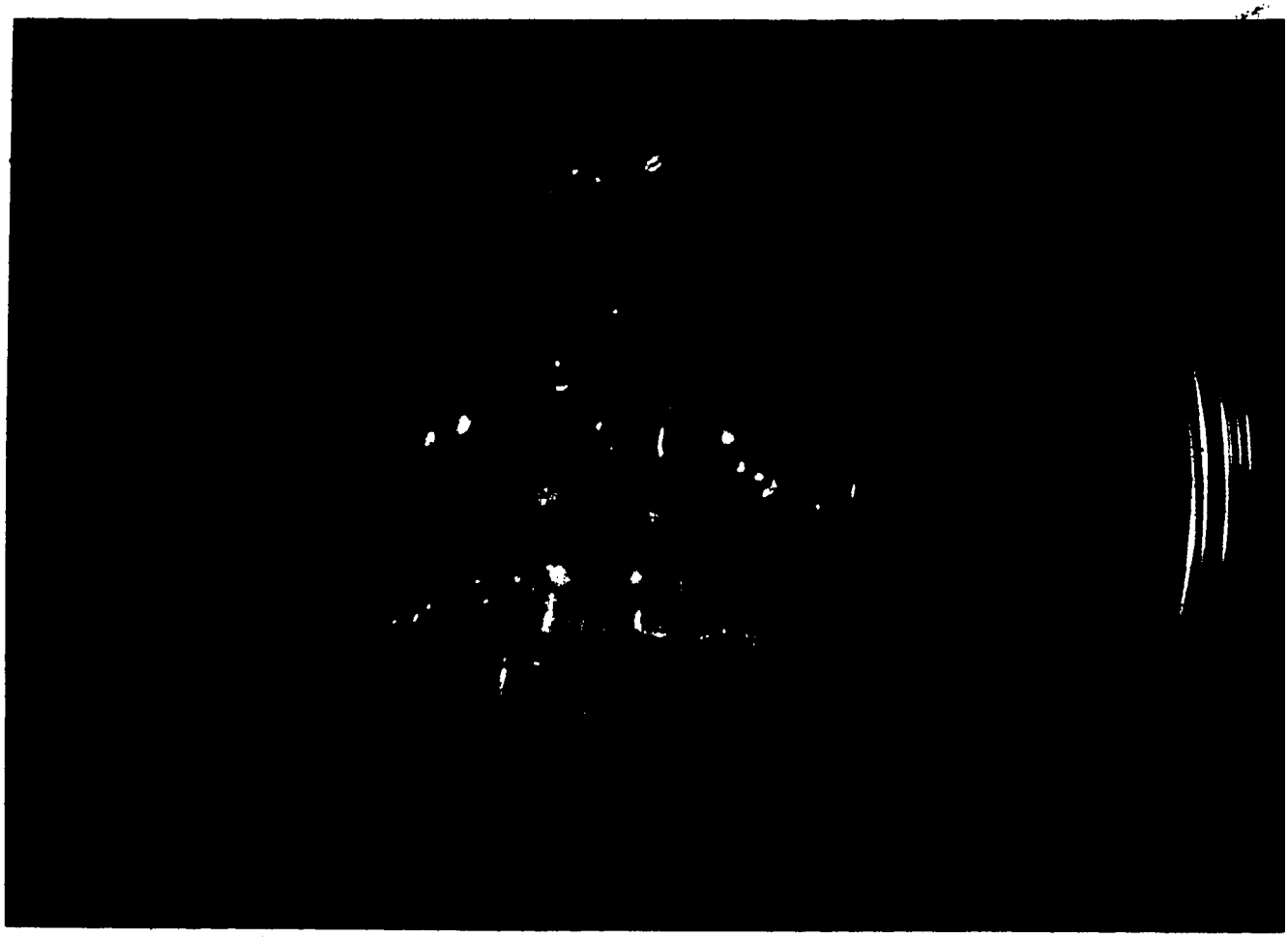

Plate 1.

Macroscopic appearance of the ventral surface of a weanling rabbit brain infected 110 h previously by an injection of HSV-I into left cerebral hemisphere. The left hemisphere (to the right of the picture) shows marked hyperemia, loss of sheen and a mild deformation of the contours of the brain around the site of injection.

produced. Therefore the rabbit appeared to be an ideal model in which to

i) measure blood levels after isotope injection since repeated blood sampling is easy.

ii) measure differential isotope uptake of various areas of a HSV-I infected brain.

iii) use for gamma imaging of the brain.

iv) perform preliminary tissue distribution studies.

The mouse model offered some alternate advantages in several situations which included:

i) Definitive tissue distribution. Many animals are required for each time point and the use of the inexpensive mouse model is more appropriate for this study.

ii) Comparative studies evaluating the specificity of radiolabelled nucleoside analogue uptake in TK +ve and TK -ve HSV infections. The established mouse models of both HSV-I, (TK +ve) and HSV-I (TK-ve) encephalitis appeared suitable for this purpose.

CHAPTER IV CELL CULTURE STUDIES WITH NUCLEOSIDE ANALOGUES

As outlined in Chapter I the aim of this research project is to utilize the phosphorylation and selective trapping of nucleoside analogues within HSV-I infected cells as a means of diagnosing herpes encephalitis. In HSV-I infected brain cells, a radiolabelled nucleoside analogue should be phosphorylated by the viral TK and selectively trapped allowing the easy detection of regions of HSV infection by a brain scan. In other causes of encephalitis, no selective trapping should occur and the brain scan should be negative.

Initial tissue studies consisted of two distinct p[illegible]ses

A. Selection of an appropriate radiolabelled nucleoside analogue.

B. quantitation of the uptake of the radiolabelled nucleoside analogue in HSV-I infected cells in vitro.

This approach allowed for an initial comparative trial of several antiviral agents and then a quantitation of in vitro uptake to be made of the nucleoside analogue selected as the most promising for the development of the selective brain scan.

A. NUCLEOSIDE ANALOGUE SELECTION

Several nucleoside analogues and radiolabels appeared likely candidates for this study. As the object of this work is to develop a brain scan for clinical use, the most promising radiolabels would appear to be Iodine and Bromine (116). Iodine is currently used as a scanning agent for thyroid disease. Two main groups of nucleoside analogues appeared suitable. Although ACG does not possess Iodine or Bromine within its structure, they have been substituted into the 8 position of

ACG (M.J. Robbins, personal communication). Therefore the 8-Iodo and 8-Bromo substitutions of ACG were both used in the preliminary studies. The second group of nucleoside analogues assessed were the halogenated vinyl deoxyuridines which contain an intrinsic Iodine or Bromine molecule.

Four nucleoside analogues were evaluated in a preliminary screen. The ideal analogue would have a high affinity for viral TK and would be concentrated to a great extent within the virally infected cell; where presumably it would have antiviral activity. The antiviral activity of each nucleoside analogue was assessed to confirm that it was selectively phosphorylated in the cell.

Antiviral activity can be detected in a variety of different ways. Unfortunately there is little standardization between the antiviral assays used. Antiviral activity is usually assessed by either dye uptake or by plaque reduction studies (88, 89). In view of familiarity with plaque counting, the plaque reduction technique was used in this work. This technique has been used by DeClercq et al to screen a wide range of antiviral agents against a variety of HSV viral strains (23).

1. ID_{50} Assay in Vero Cells

Vero cells at passage 192 were infected by a given infecting dose (approx. 100 pfu) of HSV-I (JLJ). One hour after infection the cell monolayer was covered by MEM/agarose (as described in Chapter II) which had been supplemented by a given concentration of antiviral agent. A broad range of concentrations of antiviral agents were used. After 3 days the plates were stained and the plaques counted. The number of plaques present in the absence of antiviral agent and at each concentration of antiviral agent were plotted on a graph and the

concentration required to reduce the plaque count by one half was calculated (ID_{50}).

The ID_{50} for ACG derived from this technique is well described in the literature as it lies between 0.07 and [illegible] µg/mL depending upon the strain of virus used in the assay. However in several assays the ID_{50} for ACG in this system appeared to be 1.0 to 10 µg/mL. Many factors have been shown to alter assays of antiviral activity (118,119,23).

Mycoplasmal contamination in a cell line which has undergone multiple passages in a laboratory appeared the most likely cause of this variability. Mycoplasma are known to contain enzymes which cleave nucleoside analogues between the sugar and base residues (DeClercq - personal communication). For this reason primary rabbit kidney cells were used for the subsequent antiviral activity assays.

2. ID_{50} Assay in rabbit kidney cells

Primary rabbit kidney cells after initial preparation as described in Chapter II were grown to confluency in 60 mm petri dishes. Each plate was infected with a given pfu of HSV-I (JLJ) and one hour after infection a known concentration of antiviral agent contained in the agarose/MEM overlay was added to each plate. Duplicate plates were made for each concentration of antiviral agent. After three days the plates were stained, the plaques counted and the ID_{50} calculated as described previously.

The activity of five antiviral agents were tested in three separate assays on different days. On each occasion as a control, the ID_{50} of both ACG and BVDU were measured to confirm the validity of the experiment. The ID_{50} of both of these agents measured in rabbit kidney

cells is described in the literature. The ID_{50} for ACG is between 0.07 μg/mL and 0.11 μg/mL and the ID_{50} for BVDU is between 0.007 and 0.009 μg/mL.

Table 5. Antiviral Activity of Nucleoside Analogues against HSV-I (JLJ)

Nucleoside Analogue	ID_{50} μg/mL
ACG	0.08
I-ACG	0.1
Br-ACG	0.9
BVDU	0.007
IVDU	0.009

Summary

From these results it appeared that all of the agents considered for radiolabelling had antiviral activity. BVDU and IVDU had considerably more activity than I-ACG and Br-ACG. This could be explained by either a higher affinity of the halogenated vinyl deoxyuridines for viral TK or by a higher affinity of their triphosphate forms for the virally specified DNA polymerase. Unfortunately, in the literature there are no studies to clarify this. However, if the greater antiviral activity of halogenated vinyl deoxyuridines (lower ID_{50}) is even partially due to a greater affinity for viral TK, then the intracellular concentration of these nucleoside analogues should be quantitatively higher than that of the ACG derivatives.

For further experimentation IVDU was used as the drug of choice. The reasons for this selection were:

1. The very high antiviral activity suggested that the agent must be very actively concentrated by viral TK within the cell.

2. The Iodine radiolabel would be more firmly bound into this molecule and less susceptible for exchange than in I-ACG (L. Wiebe, personal communication).

3. Methods for preparation of large quantities of both cold and "hot" IVDU (with a variety of radiolabels) had just been developed in the Faculty of Pharmacy (114).

B. QUANTITATION OF [^{131}I]-IVDU UPTAKE BY HSV-I INFECTED CELLS IN VITRO

The nucleoside analogue IVDU appeared to be the most promising agent available for this study. When it became available, the [^{131}I] radiolabelled compound was used to quantify the uptake of this compound into HSV-I (TK +ve), HSV-I (TK -ve) and uninfected rabbit kidney cells in vitro. The aim of this study was to quantify the selective and non-selective trapping in infected cells, to determine the time frame of the intracellular trapping, and to determine the dose response curve of the selective trapping of the drug.

1) Time-response curve of cellular uptake of [^{131}I]-IVDU.

Confluent rabbit kidney cells were either infected with 6 x10^{6} plaque forming units (pfu) of HSV (TK +ve) or were mock infected. After 7 h of incubation, 0.017 μCi [^{131}I]-IVDU were added to give a final concentration of 0.00276 μg/mL. Quantitation of both the intracellular and extracellular radiolabelled IVDU were determined at 20 sec, 30 min, 1, 2, 4 and 9 h, as described in Chapter II.

Four infected flasks and three mock infected flasks were harvested at each time point. The results of the time-response curve are shown in Figure 6. The amount of radiolabelled IVDU in mock infected cells

remains low whereas the supernatant concentration remains high. In contrast, the radiolabelled IVDU in the supernatant fluid of HSV (TK +ve) infected cells decreases with time and the intracellular concentration increases proportionately.

2) Effect of increasing titers of infecting virus on [^{131}I]-IVDU uptake.

Seven dilutions of stock HSV (TK +ve) and HSV (TK -ve) were made into 0.2 mL aliquots and were used to infect the cells. Three flasks were infected with HSV (TK +ve), two flasks were infected with HSV (TK -ve) and two flasks were mock infected. After 7 h 0.0226 µCi of [^{131}I]-IVDU was added to each flask to give a final concentration of 0.000858 µg/mL. All cells were harvested after 4 h of incubation and the intra and extracellular radiolabelled IVDU was quantified as described in Chapter II. The mean and range of values of the cellular uptake of [^{131}I]-IVDU at each infected dose are shown in Figure 7. The quantity of [^{131}I]-IVDU taken up by HSV (TK +ve) cells increased with increasing titers of infecting virus. HSV (TK -ve) showed insignificant uptake even at high doses of infecting virus.

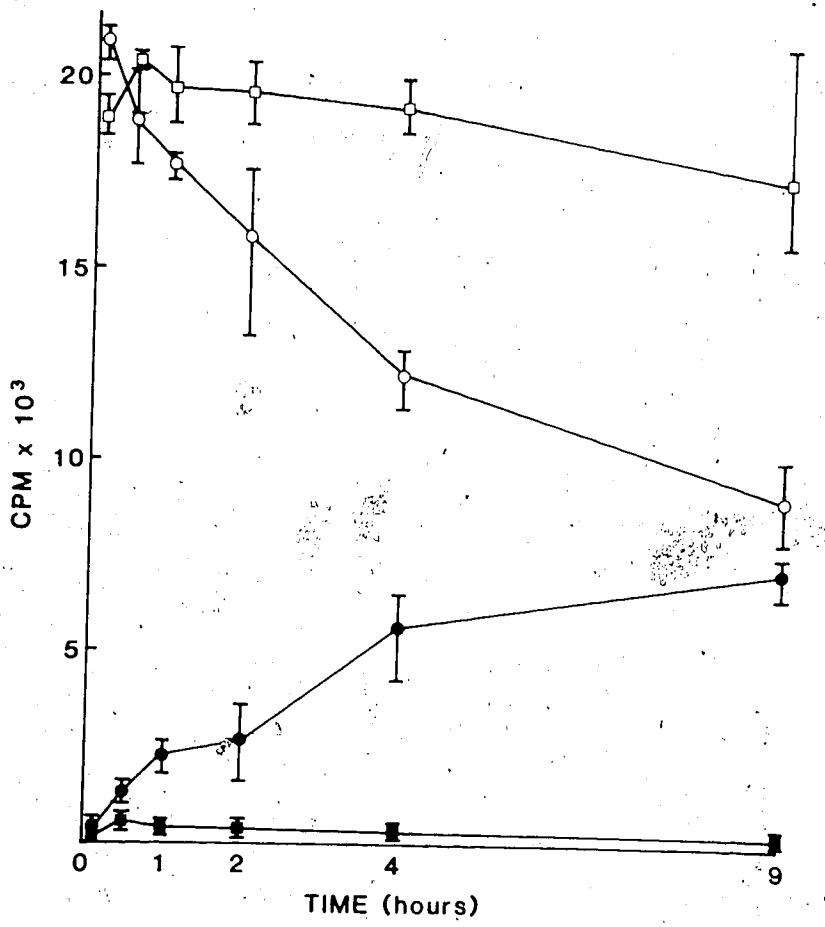

Figure 6

Time-response curve of [^{131}I]-IVDU uptake in HSV (TK +ve) and mock infected rabbit kidney cells. Intracellular [^{131}I]-IVDU in HSV infected cells (●). Extracellular [^{131}I]-IVDU in the medium overlay of HSV infected cells (○). Intracellular [^{131}I]-IVDU in mock infected cells (■). Extracellular [^{131}I]-IVDU in the medium overlay of mock infected cells (□).

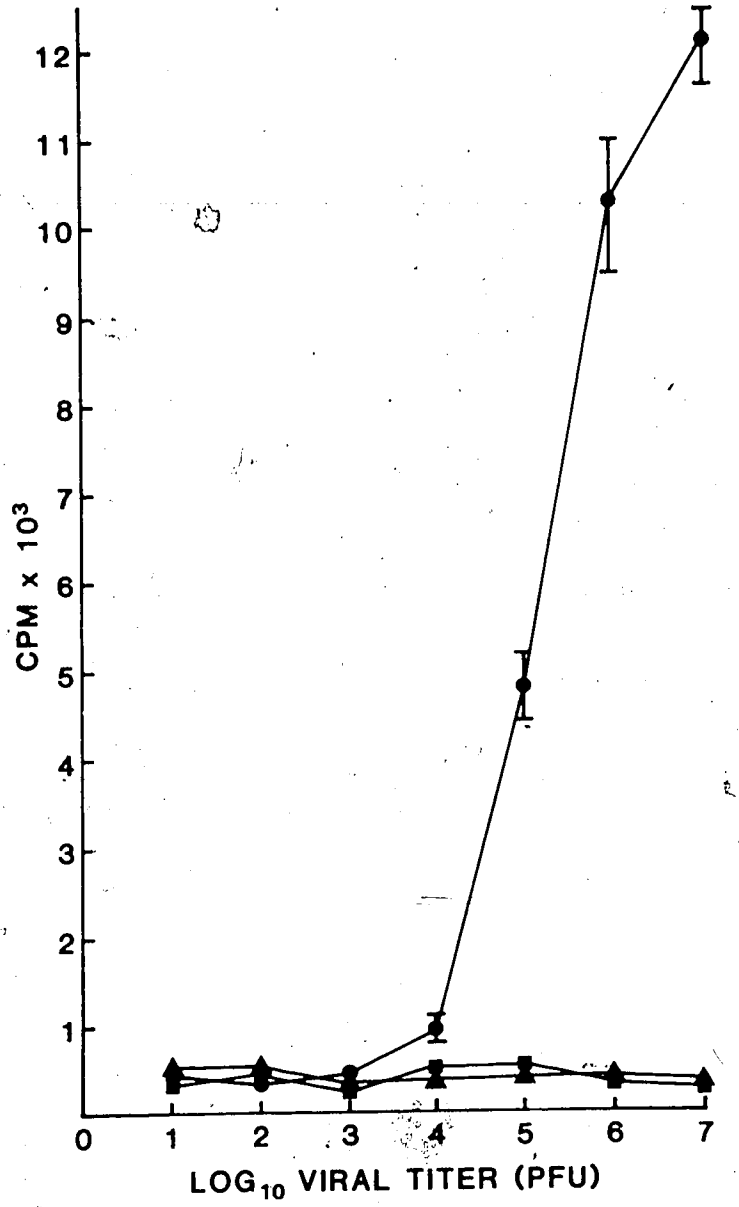

Figure 7

Effect of increasing the titer of infecting virus on the intracellular selective trapping of [^{131}I]-IVDU in HSV (TK +ve) (●), HSV (TK -ve) (▲) and mock (■) infected cells.

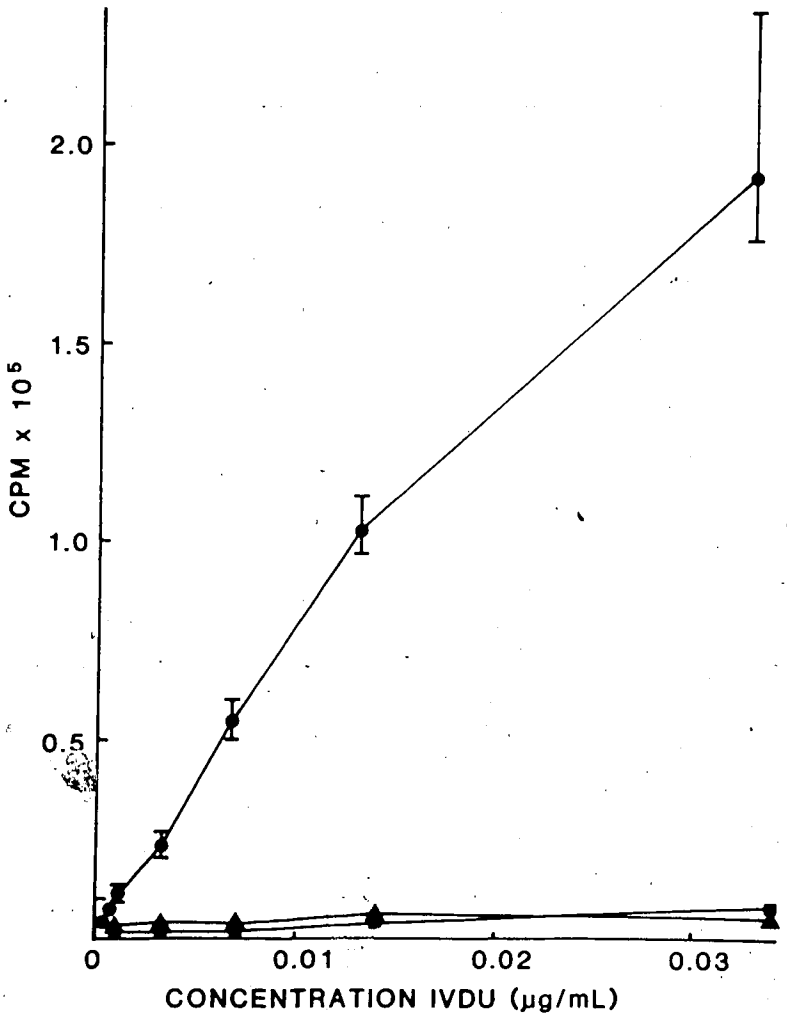

Figure 8

Effect of increasing concentrations of [^{131}I]-IVDU in the medium on the selective intracellular trapping in HSV (TK +ve) (●), HSV (TK -ve) (▲) and mock (■) infected cells.

3) Dose-response curve of [^{131}I]-IVDU uptake.

Known dilutions of [^{131}I]-IVDU were made and added to the flasks 7 h after infection with either 6 x 10^6 pfu of HSV (TK +ve) or HSV (TK -ve) or mock infection. Concentrations were deliberately chosen to cover a broad range around the doses known to cause a 50% reduction in pfu (ID_{50}). The ID_{50} for the JLJ strain used in these experiments was 0.008 µg/mL. The mean and range of counts recovered in the cellular fraction of the HSV (TK +ve), HSV (TK -ve) and mock-infected cells are depicted in Figure 8. The uptake of [^{131}I]-IVDU increased proportionately to the amount of drug added. This relationship was only seen in HSV (TK +ve) infected cells and occurred even above the ID_{50}.

Summary

In the first series of experiments the efficacy of several nucleoside analogues as antiviral agents was measured. The two most potent agents were BVDU and IVDU. In the second series of experiments a quantitation of radiolabelled IVDU uptake was made as a guide both to the feasibility and to the optimal conditions for in vivo studies with animal models. These results show that the selective uptake of [^{131}I]-IVDU is dependent on the infecting dose of the virus. Since the experiments were completed in under 12 h, it was assumed that the time was inadequate for a second phase of viral infection to occur. As shown in Figure 7, an infecting dose of 10^4 pfu for a 25 cm^2 flask containing 5 x 10^5 cells is required before significant uptake can be detected. Therefore in an animal model at least 1 in 50 cells at any time would have to be infected before selective concentration of [^{131}I]-IVDU could conceivably be detected by isotope scanning. The titer of HSV measured in brain biopsy specimens from HSV encephalitis patients is not well

documented. One study reported that in 11/18 brain biopsies the virus concentration was $>10^4$ Tissue Culture Infective $Dose_{50}$ ($TCID_{50}$)/g brain (47). A number of factors could affect the titer of virus from brain biopsy. The most important is probably the proximity of the biopsy to the most active site of infection. Since there is extensive histological destruction of HSV infected brain tissue, it is likely that the titers of virus at the site of infection will be adequate to allow selective concentration of the gamma labelled nucleoside analogue.

Two other important factors in developing a brain scan for HSV encephalitis are to determine the optimal time and a dosage of isotope administration. These results confirm that the uptake increases directly with the concentration of isotope used and time the cells are exposed to the isotope. From these studies, significant nucleoside concentration was detected by 1 h which confirms that the uptake is rapid enough to permit the development of an in vivo scan even with nucleosides undergoing relatively fast excretion or metabolism.

The intracellular trapping of [^{131}I]-IVDU continued to increase in almost a linear fashion even at concentrations of IVDU considerably higher than the ID_{50}. The isotope was trapped even when the nucleoside concentration was sufficient to inhibit virus replication suggesting that a high concentration of isotope could be used in developing a radioisotope scan. The results in this study lend support to the concept that [^{131}I]-IVDU might be a suitable nucleoside analogue to use in the development of a selective brain scan for HSV encephalitis.

CHAPTER V TISSUE DISTRIBUTION AND PHARMACOKINETICS OF IVDU IN RABBITS

The third stage of this research entailed an initial evaluation of the tissue distribution in a small number of rabbits of a bolus dose of radiolabelled IVDU. The tissue distribution was measured both in uninfected rabbits and in rabbits with fulminant herpes encephalitis.

A. TISSUE DISTRIBUTION

Six weanling rabbits (wt. approx. 1 kg) from one litter were used. Four rabbits were infected with an intracerebral injection of 10^6 pfu of HSV-I (JLJ). Two rabbits were left uninfected. After four days, when the infected rabbits were convulsing, all six rabbits were given into the marginal vein of the left ear a bolus of 20 μCi of [^{125}I]-IVDU at a dose of 1 ng/kg intravenously. At 1 h and 6 h, one uninfected and 2 infected rabbits were sacrificed by intracardiac injection of a cardioplegic solution (4 M KCl). Prior to this a 5 mL blood sample was drawn by cardiac aspiration.

In all rabbits a detailed dissection was performed and multiple samples (at least 3) from each organ were weighed, placed in a scintillation vial and their radioactivity counted in a Beckman 8000 gamma counter. To allow a comparison between different animals the ratio of the tissue to blood radioactivity was calculated for each organ. The results are shown in Tables 6 and 7. The average tissue to blood ratio and the range of values are given.

In the uninfected animal, only the kidney had a higher level of radioactivity than the blood. This was presumably due to a renal route

of excretion. This high blood level was disconcerting as it signified high "background" activity which could obscure any selective trapping in the brain of an infected animal. The brain of the uninfected rabbit had very little uptake of isotope in contrast to the brains of infected rabbits. In any given organ, as shown by a narrow range of values, there was little variation in counts, confirming the reproducibility of the assay. In Table 7, six hours after the drug administration, the high blood level has persisted and the activity per g of tissue was higher than for either the kidney and liver. A small but definite difference was still detectable between the brains of infected and uninfected animals, but this would be undetectable in a brain scan due to the high levels remaining in the blood.

Summary

The tissue distribution study described, was performed in a few animals to determine in vivo the feasibility of using radiolabelled IVDU as a scanning agent. In view of the limited numbers of animals involved, the results should be treated with caution. However, the demonstration of relatively high ratios of radioisotope in the blood to other organs is of major importance in our attempt to use IVDU as a scanning agent. Furthermore, the relative amount of radioisotope in the blood was still higher after six hours, suggesting that delaying the scan until the background counts decreased might not be of value. The exact blood levels achieved and the duration of detectable isotope in the blood were subsequently studied. The isotope did appear to be somewhat concentrated within the herpes infected brain tissue in comparison to the uninfected brain tissue, however, this effect was at best limited, and unfortunately appeared to be highest when the blood

Table 6. Tissue Distribution of [^{125}I]-IVDU One Hour After Injection. Tissue to Blood Ratio (dpm/g tissue:dpm/g blood)

Tissue	Uninfected Animal	Infected Animals
Blood	1.000	1.000
Brain	0.090 ± 0.029	0.418 ± 0.087
Kidney	1.295 ± 0.259	1.675 ± 0.445
Liver	0.685 ± 0.175	1.326 ± 0.147
Thyroid	0.768 ± 0.152	0.798 ± 0.016
Muscle	0.326 ± 0.052	0.630 ± 0.193

Table 7. Tissue Distribution of [^{125}I]-IVDU Six Hours After Injection. Tissue to Blood Ratio (dpm/g tissue:dpm/g blood)

Tissue	Uninfected Animal	Infected Animals
Blood	1.000	1.000
Brain	0.046 ± 0.005	0.083 ± 0.0
Kidney	0.914 ± 0.020	0.935 ± 0.117
Liver	0.505 ± 0.031	0.445 ± 0.003
Thyroid	0.661 ± 0.067	0.669 ± 0.017
Muscle	0.630 ± 0.009	0.167 ± 0.024

levels were peaking. From these preliminary results it appeared that the high blood level of radiolabel might present a major problem and so detailed blood kinetic studies appeared warranted.

B. BLOOD KINETICS

Three weanling rabbits (wt. approx. 1 kg) were injected with a bolus injection of 268 μCi of [^{125}I]-IVDU contained in 2 μg of IVDU. The injection was made into a left ear vein and all subsequent blood samples were withdrawn from the right ear. The blood samples were placed into a heparinised counting vial of known weight, weighed and then counted in a gamma counter. The average and range of the dpms per g whole blood from the three animals were calculated and this is plotted against time in Figure 9.

From this elimination curve it can be seen that after an ini high level five minutes after the injection, the blood level dro rapidly probably due to tissue redistribution. Then a slower phase of isotope elimination occurs. One day after the injection, approximately 5% of the initial dose is still detectable in the blood. Levels this high would make an in vivo scan, which relies on detecting relatively small differences in brain tissue, particularly difficult. The relatively high levels of radioisotope in the blood 24 h after injection was surprising since BVDU is cleared from the plasma within 4 h. However, recent studies have shown it is metabolized to bromovinyl uracil which peaks in the plasma about 2 h after dosing and remains detectable for 24 h (115). These studies were done in immunosuppressed patients, but if the metabolic pathways of IVDU and BVDU are similar and if the metabolism of these nucleoside analogues in man and rabbits is similar, this observation may account for the persistent high levels of radioactivity in the blood for >24 h.

Another possibility is that the Iodine may have been cleaved off the vinyl deoxyuridine and is persisting in the blood in a free state.

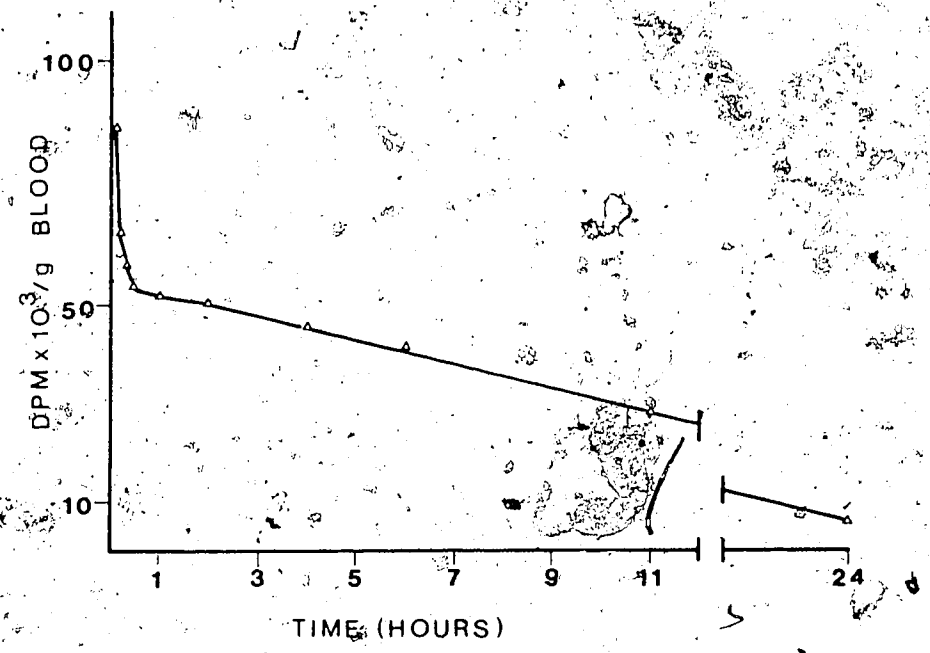

Figure 9

Elimination curve of radioactivity from whole blood.

This problem is being looked at by analysing the plasma from these animals using high pressure liquid chromatography.

CHAPTER VI CONCLUSION

This research ha vered three distinct areas. The first area involved the est shment of animal models of herpes encephalitis suitable for radioisotope brain scanning and tissue distribution studies. The second area involved evaluation of several nucleoside analogues for antiviral activity and then quantitation of the uptake of IVDU into herpes infected cells. The third area involved preliminary *in vivo* tissue distribution and pharmacokinetic studies of a bolus injection of radiolabelled IVDU.

Seven distinct variables were examined in an attempt to establish a good animal model of herpes encephalitis. A weanling rabbit model of herpes encephalitis was eventually developed in which an intracerebral inoculation of 10^6 pfu of HSV-I (JLJ) induced reproducibly a clinically distinct illness after 5 days and subsequently death. This model appeared appropriate for scanning in view of the large brain and relative localization of infection. A mouse model of encephalitis resulting from intracerebral inoculation of HSV-I (TK +ve, JLJ strain) or HSV-I (TK -ve, B2006 strain) was also developed and appeared suitable for both tissue distribution studies and investigations of the specificity of the radiolabel uptake in encephalitis.

In cell culture, the antiviral activity of several nucleoside analogues was evaluated and then a quantitation of cellular uptake into herpes infected cells was performed for the most promising agent (IVDU). This uptake was dependent upon the quantity of virus present, the duration of infection and the concentration of radiolabelled agent to

which the cells were exposed. These results suggested that an in vivo scan was feasible.

In the rabbit model, a preliminary study of the tissue distribution of radioactivity following an infusion of radiolabelled IVDU has shown that the radiolabel is higher in the brains of animals with herpes encephalitis than in uninfected animals. However a persistent high blood level of radioactivity would mask this difference in a brain scan.

The current research being undertaken in the Faculty of Pharmacy on this project involves a determination of the identity of the persistent radioactivity in the blood. Once this is understood, perhaps either by chemical modification or additional drug administration the clearance of the radioactivity can be accelerated from the blood making the differential uptake into a herpes infected brain apparent. At this point the prospect of a clinical trial could become a reality.

REFERENCES

1. Fenner, F.
The classification and nomenclature of viruses. Summary of results of meetings of the International Committee on Taxonomy of Viruses in Madrid, September 1975.
Virology 71:371-378 (1976).

2. Buchman, T.G., Roizman, B., Adams, G., Stover, B.H.
Restriction endonuclease fingerprinting of herpes simplex virus DNA: a novel epidemiological tool applied to a nosocomial outbreak.
J. Infect. Dis. 138:488-498 (1978).

3. Dowdle, W.R., Nahmias, A.J., Harwell, R.W., Pauls, F.P.
Association of antigenic type of herpesvirus hominis with site of viral recovery.
J. Immunol. 99:974-980 (1967).

4. Lerner, A.M.
In: Principles of Internal Medicine. Isselbacher, K.J., Adams, R.D., Braunwald, E., Petersdorf, R.G., Wilson, J.D. Eds.
McGraw-Hill, New York p. 847-852 (1980).

5. Wolontis, S., Jeansson, S.
Correlation of herpes simplex virus types 1 and 2 with clinical features of infection.
J. Infect. Dis. 135:28-33 (1977).

6. Young, S.K., Rowe, N.H., Buchanan, R.A.
A clinical study for the control of facial mucocutaneous herpes virus infections. 1. Characterisation of natural history in a professional school population.
Oral Surg, Oral Med, Oral Path. 41:498-507 (1976).

7. Baringer, J.R.
Herpes-simplex virus infection of nervous tissue in animals and man.
Progr. Med. Virol. 20:1-26 (1975).

8. Klein, R.J.
Pathogenetic mechanisms of recurrent herpes-simplex virus infections.
Arch. Virol. 51:1-13 (1976).

9. Hill, T.J., Blyth, W.A.
An alternative theory of herpes-simplex recurrence and a possible role of prostaglandins.
Lancet. 1:397-398 (1976).

10. Blyth, W.A., Hill, T.J., Field, H.J., Harbour, D.A.
Reactivation of herpes simplex infection by ultraviolet light and possible involvement of prostaglandins
J. gen. Virol. 33:547-550 (1976).

11. Buchman, T.G., Simpson, T., Nosal, C., Roizman, B., Nahmias, A.J.
The structure of herpes simplex virus DNA and its application to molecular epidemiology.
Ann. N.Y. Acad. Sci. 354:279-290 (1980).

12. Warren, K.G., Brown, S.M., Wroblewska, Z., Gilden, D., Koprowski, H., Subak-Sharpe, J.
Isolation of latent herpes simplex virus from the superior cervical and vagus ganglions of human beings.
N. Engl. J. Med. 298:1068-1069 (1978).

13. Whitley, R.J., Lakeman, A.D., Nahmias, A., Roizman, B.
DNA restriction-enzyme analysis of herpes simplex virus isolates obtained from patients with encephalitis.
N.Engl. J. Med. 307:1060-1062 (1982).

14. Smith, M.G., Lennette, E.H., Reames, H.R.
Isolation of the virus of herpes simplex and the demonstration of intranuclear inclusions in a case of acute encephalitis.
[redacted]thol. 17:55-68 (1941).

15. [redacted] Harter, D.H.
[redacted] simplex virus encephalitis.
[redacted] 247:337 (1982).

16. Alford, C.A., Dolin, R., Hirsch, M.S., Karchmer, A.W., Whitley, R.J.
Herpes simplex encephalitis and clinical trial design.
Lancet. 1:1013 (1982).

17. Barza, M., Pauker, S.G.
The decision to biopsy, treat, or wait in suspected herpes encephalitis.
Ann. Intern. Med. 92:641-649 (1980).

18. Whitley, R.J., Soong, S-J., Linneman, C., Liu, C., Pazin, G., Alford, C.A. and the National Institute of Allergy and Infectious Diseases (NIAID) Collaborative Antiviral Study Group.
Herpes Simplex Encephalitis: Clinical Assessment.
JAMA 247:317-320 (1982).

19. Whitley, R.J., Soong, S.J., Dolin, R., Galasso, G.J., Ch'ien, L.T., Alford, C.A. and the NIAID Collaborative Antiviral Study Group.
Adenine arabinoside therapy of biopsy-proved herpes simplex encephalitis.
N. Engl. J. Med. 297:289-294 (1977).

20. Oommen, K.J., Johnson, P.C., Ray, C.G.
Herpes simplex type 2 virus encephalitis presenting as psychosis.
Am. J. Med. 73:445-448 (1982).

21. Whitley, R.J., Nahmias, A.J., Soong, S-J., Galasso, G.G., Fleming, C.L., Alford, C.A. and the NIAID study group with assistance of Connor, J., Bryson, Y., Linnemann, C.
Vidarabine therapy of neonatal herpes simplex virus infection.
Pediatrics 66:495-501 (1980).

22. Whitley, R.J., Soong, S-J, Dolin, R., Betts, R., Linnemann, C., Alford, C.A. and the NIAID Study Group.
Early vidarabine therapy to control the complications of herpes zoster in immunosuppressed patients.
N. Engl. J. Med. 307:971-975 (1982).

23. DeClercq, E., Descamps, J., Verhelst, G., Walker, R.T., Jones, A.S., Torrence, P.F., Shugar, D.
Comparative efficacy of antiherpes drugs against different strains of herpes simplex virus.
J. Infect. Dis. 141:563-574 (1980).

24. Nahmias, A.J., Visintine, A.M.
Neonatal herpes simplex infection. In: Infections of the Fetus and Newborn Infant. Philadelphia.
W.B. Saunders, 1976, p. 156-190.

25. Leider, W., Magoffin, R.L., Lennette, E.H., Leonards, L.N.R.
Herpes-simplex-virus encephalitis: its possible association with reactivated latent infection.
N. Engl. J. Med. 273:341-347 (1965).

26. Baringer, J.R.
Recovery of herpes simplex virus from human sacral ganglions.
N. Engl. J. Med. 291:828-830 (1974).

27. Davis, L.E., Johnson, R.T.
An explanation for the localization of herpes simplex encephalitis?
Ann. Neurol. 5:2-5 (1979).

28. Dinn, J.J.
Distribution of herpes simplex virus in acute necrotising encephalitis.
J. Pathol. 127:135-138 (1979).

29. Dinn, J.J.
Transolfactory spread of virus in herpes simplex encephalitis.
Brit. Med. J. 281:1392 (1980).

30. Goodpasture, E.W., Teague, O.
Transmission of the virus of herpes febrilis along nerves in experimentally infected rabbits.
J. Med. Res. 44:139-184 (1923).

31. Nahmias, A.J., Dowdle, W.R.
Antigenic and biologic differences in herpes virus hominis.
Prog. Med. Virol. 10:110-159 (1968).

32. Hammer, S.M., Buchman, T.G., D'Angelo, L.J., Karchmer, A.W., Roizman, B., Hirsch, M.S.
Temporal cluster of herpes simplex encephalitis: investigation by restriction endonuclease cleavage of viral DNA.
J. Infect. Dis. 141:436-440 (1980).

33. Pereira, L., Cassai, E., Honess, R.W., Roizman, B., Terni, M., Nahmias, A.
Variability in the structural polypeptides of herpes simplex virus 1 strains: potential application in molecular epidemiology.
Infect. Immunity. 13:211-220 (1976).

34. Longson, M., Bailey, A.S., Klapper, P.
Herpes encephalitis.
Recent Adv Clin Virol 2:147-157 (1980).

35. Hirsch, M.S.
Discussion in: Case Records of the Massachusetts General Hospital (case 44-1979).
N. Engl. J. Med. 301:987-994 (1979).

36. Klapper, P.E., Laing, I., Longson, M.
Rapid non-invasive diagnosis of herpes encephalitis.
Lancet. 2:607-608 (1981).

37. Upton, A., Gumpert, J.
Electroencephalography in diagnosis of herpes-simplex encephalitis.
Lancet. 1:650-652 (1970).

38. Illis, L.S., Taylor, F.M.
The electroencephalogram in herpes-simplex encephalitis.
Lancet. 1:718-721 (1972).

39. Pexman, J.H.W.
The angiographic and brain scan features of acute herpes simplex encephalitis.
Br. J. Radiol. 47:179-184 (1974).

40. Kim, E.E., DeLand, F.H., Montebello, J.
Sensitivity of radionucleotide brain scan and computed tomography in early detection of viral meningoencephalitis.
Radiology 132:425-429 (1979).

41. Greenberg, S., Taber, L., Septimus, E., Kohl, S., Puck, J., Byran, R.
Computerized tomography in brain biopsy-proven herpes simplex encephalitis. Early Normal Results.
Arch. Neurol. 38:58-59 (1981).

42. Davis, J.M., Davis, K.R., Kleinman, G.M., Kirchner, H.S., Taveras, J.M.
Computed tomography of herpes simplex encephalitis with clinico-pathological correlation.
Radiology 129:409-417 (1978).

43. Landry, M.L., Booss, J., Hsiung, G.D.
Duration of vidarabine therapy in biopsy-negative herpes simplex encephalitis.
JAMA 247:332-334 (1982).

44. Johnson, K.P., Rosenthal, M.S., Lerner, P.I.
Herpes simplex encephalitis: the course in five virologically proven cases.
Arch. Neurol. 27:103-108 (1972).

45. Adams, J.H., Urquhart, G.E.D.
Early diagnosis of herpes encephalitis.
N. Engl. J. Med. 297:1288 (1977).

46. Levine, D.P., Lauter, C.B., Lerner, A.M.
Simultaneous serum and CSF antibodies in herpes simplex virus encephalitis.
JAMA 240:356-360 (1978).

47. Nahmias, A.J., Whitley, R.J., Visintine, A.N., Takei, Y., Alford, C.A. and the NIAID Collaborative Antiviral Study Group.
Herpes Simplex Virus Encephalitis: Laboratory evaluations and their diagnostic significance.
J. Infect. Dis. 145:829-836 (1982).

48. York, G.K.
Herpes simplex encephalitis: Biopsy or treatment?
Ann. Int. Med. 93:506 (1980).

49. Kaufman, H.H., Catalano, L.W.
Diagnostic brain biopsy: a series of 50 cases and a review.
Neurosurgery 4:129-136 (1979).

50. Whitley, R.J., Soong, S-J., Hirsch, M.S., Karchmer, A.W., Dolin, R., Galasso, G., Dunnick, J.K., Alford, C.A. and the NIAID Collaborative Antiviral Study Group.
Herpes simplex encephalitis: Vidarabine Therapy and Diagnostic Problems.
N. Engl. J. Med. 304:313-318 (1981).

51. Cheng, Y-C., Domin, B.A., Sharma, R.A., Bobek, M.
Antiviral action and cellular toxicity of four thymidine analogues: 5 ethyl-,5 vinyl-,5 propyl-, and 5-allyl-2'-deoxyuridine.
Antimicrob. Agents Chemother. 10:119-122 (1976).

52. Hilty, M.D., Thomas, D.C., Haynes, R.E., Cramblett, H.G.
Thymidine kinase activity in the cerebrospinal fluid of rabbits with herpesvirus hominis encephalitis.
Ped. Res. 9:143-146 (1975).

53. Hilty, M.D., Merchant, V., Haynes, R.E.
Thymidine kinase and herpes simplex encepahlitis.
Ped. Res. 9:383 (1975).

54. Chen, A.B., Ben-Porat, T., Whitley, R.J., Kaplan, A.S.
Purification and characterization of proteins excreted by cells infected with herpes simplex virus and their use in diagnosis.
Virology 91:234-242 (1978).

55. Coleman, R.M., Bailey, P., Whitley, R., Nahmias, A.
ELISA for detection of herpes simplex virus (HSV) antigens in the cerebrospinal fluid (CSF) of patients with encephalitis (Abstracts 5-6) In: Abstracts of 82nd Annual Meeting of American Society for Microbiology, Washington, D.C. 1982.

56. Dayan, A.D., Stokes, M.I.
Rapid diagnosis of encephalitis by immunofluorescent examination of cerebrospinal fluid cells.
Lancet. 1:177-179 (1973).

57. Longson, M., Liversedge, L.A., Wilkinson, I.M.S.
Diagnosis of viral encephalitis.
Lancet. 1:371 (1973).

58. Tourtellotte, W.
On cerebrospinal fluid immunoglobulin G (IgG) quotients in multiple sclerosis and other diseases. A review and a new formula to estimate the amount of IgG synthesized per day by the central nervous system.
J. neurol. Sci. 10:279-304 (1970).

59. Boston Interhospital Virus Study Group and NIAID Antiviral Study Group.
Failure of high dose 5-iodo-2'-deoxyuridine in the therapy of herpes simplex virus encephalitis: Evidence of unacceptable toxicity.
N. Engl. J. Med. 292:599-603 (1975).

60. Wenman, W.M., Brunton, J.L., Lank, B.A., Ronald, A.R.
Herpes simplex encephalitis: poor outcome despite adenine arabinoside therapy.
Can. Med. Assoc. J. 126:819-821 (1982).

61. Nadel, A.M.
Vidarabine therapy for herpes simplex encephalitis.
The development of an unusual tremor during treatment.
Arch. Neurol. 38:384-385 (1981).

62. Whitley, R.J., Soong, S-J., Alford, C.A., Hirsch, M.S., Karchmer, A.W., Dolin, R., Galasso, G.
Herpes encephalitis: Letter.
N. Engl. J. Med. 304:1237 (1981).

63. Kern, E.R., Richards, J.T., Overall, J.C., Glasgow, L.A.
Alteration of mortality and pathogenesis of three experimental herpesvirus hominis infections of mice with adenine arabinoside 5'-monophosphate, adenine arabinoside and phosphonoacetic acid.
Antimicrob. Agents Chemother. 13:53-60 (1978).

64. Gill, M.J., Aronyk, K., Devine, G., Larke, R.B., Goldsand, G., Miedzinski, L., Tyrrell, D.L.J.
Ara-AMP treatment of biopsy proved herpes encephalitis. In Medical Virology III. Proceedings of International Symposium on Medical Virology. (Los Angeles, Oct. 1983). Elsevier Biomedical 1984.

65. Park, N-H., Pavan-Langston, D., McLean, S.L., Albert, D.M.
Therapy of experimental herpes simplex encephalitis with acyclovir in mice.
Antimicrob. Agents Chemother. 15:775-779 (1979).

66. Park, N-H., Pavan-Langston, D., DeClercq, E.
Efficacy of (E)-5-(2-Bromovinyl)-2'-deoxyuridine in the treatment of experimental herpes simplex virus encephalitis in mice.
Antiviral Res. 3:7-15 (1983).

67. Doerr, R., Vochting, K.
Etudes sur le virus de l'herpes febrile.
Revue generale d'ophthalmologie 10:409-421 (1920).

68. Baringer, J.R., Griffith, J.F.
Experimental herpes simplex encephalitis:
Early neuropathologic changes.
J. Neuropath Exp. Neurol. 29:89-104 (1970).

69. Narang, H.K., Codd, A.A.
Progression of herpes encephalitis in rabbits following the intraocular injection of Type 1 virus.
Neuropath. Appl. Neurobiol. 4:457-470 (1978).

70. Narang, H.K., Codd, A.A.
The pathogenesis and pathway into the central nervous system after intraocular infection of herpes simplex virus type 1 in rabbits.
Neuropath. Appl. Neurobiol. 4:137-150 (1978).

71. Saito, Y., Price, R.W., Rottenberg, D.A., Fox, J.J., Su, T.L., Watanabe, K.A., Philips, F.S.
Quantitative autoradiographic mapping of herpes simplex virus encephalitis with a radiolabelled antiviral drug.
Science 217:1151-1153 (1982).

72. Kern, E.R., Overall, J.C., Glasgow, L.A.
Herpesvirus hominis infection in newborn mice. I. An experimental model and therapy with iododeoxyuridine.
J. Infect. Dis. 128:290-299 (1973).

73. DeClercq, E., Luczak, M.
Intranasal challenge of mice with herpes simplex virus: An experimental model for evaluation of the efficacy of antiviral drugs.
J. Infect. Dis. 133 Suppl: A226-236 (1976).

74. Tomlinson, A.H., MacCallum, F.O.
The effect of Iodo-deoxyuridine on herpes simplex virus encephalitis in animals and man.
Ann. N.Y. Acad. Sci. 173:20-28 (1970).

75. Schabel, F.M.
The antiviral activity of 9- -D-arabinofuranosyladenine (Ara-A).
Chemotherapy 13:321-338 (1968).

76. Tokumaru, T.
The protective effect of different immunoglobulins against herpetic encephalitis and skin infection in guinea pigs.
Arch. fur die Gesamte Virusforschung 22:332-348 (1967).

77. Biron, K.K., Noblin, J.E., DeMiranda, P., Elion, G.B.
Uptake, distribution, and anabolism of acyclovir in herpes simplex virus-infected mice.
Antimicrob. Agents Chemother. 21:44-50 (1982).

78. Knotts, F.B., Cook, M.L., Stevens, J.G.
Pathogenesis of herpetic encephalitis in mice after ophthalmic inoculation.
J. Infect. Dis. 130:16-27 (1974).

79. [illegible]t, S., Dubbs, D.R.
[illegible]quisition of thymidine kinase activity by herpes simplex infected [illegible]use fibroblast cells.
Biochem. Biophys. Res. Commun. 11:55-59 (1963).

80. Klemperer, H.G., Haynes, G.R., Shedden, W.I.H., Watson, D.H.
A virus-specific thymidine kinase in BHK21 cells infected with herpes simplex virus.
Virol. 31:120-128 (1967).

81. Elion, G.B., Furman, P.A., Fyfe, J.A., DeMiranda, P., Beauchamp, L., Schaeffer, H.J.
Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxy-methyl) guanine.
Proc. Natl. Acad. Sci. USA 74:5716-5720 (1977).

82. Fyfe, J.A., Keller, P.M., Furman, P.A., Miller, R.L., Elion, G.B.
Thymidine kinase from herpes simplex virus phosphorylates the new antiviral compound, 9-(2-hydroxyethoxymethyl) guanine.
J. Biol. Chem. 253:8721-8727 (1978).

83. Miller, W.H., Miller, R.L.
Phosphorylation of Acyclovir (Acycloguanosine) Monophosphate by GMP kinase.
J. Biol. Chem. 255:7204-7207 (1980).

84. Furman, P.A., St. Clair, M.H., Fyfe, J.A., Rideout, J.L., Keller, P.M., Elion, G.B.
Inhibition of herpes simplex virus-induced DNA polymerase activity and viral DNA replication by 9-(2-hydroxyethoxymethyl) guanine and its triphosphate.
J. Virol. 32:72-77 (1979).

85. Lamb, R.A., Etkind, P.R., Choppin, P.W.
Evidence for a Ninth Influenza Viral Polypeptide.
Virology 91:60-78 (1978).

86. Field, H.J., Larder, B.A., Darby, G.
Isolation and characterization of acyclovir-resistant strains of herpes simplex virus.
Am. J. Med. Acyclovir Symposium 369-371 (1982).

87. Dutt, M.K., Johnston, I.D.A.
Computed tomography and EEG in herpes simplex encephalitis. Their value in diagnosis and prognosis.
Arch. Neurol. 39:99-102 (1982).

88. Finter, N.B.
Dye uptake. Methods for assessing viral cytopathogenicity and their application to Interferon assays.
J. gen. Virol. 5:419-427 (1969).

89. McLaren, C., Sibrack, C.D., Barry, D.W.
Spectrum of sensitivity to acyclovir of herpes simplex virus clinical isolates.
Am. J. Med. Acyclovir Symposium 376-379 (1982).

90. Chou, S.M., Cherry, J.D.
Ultrastructure of Cowdry Type A inclusions. I. In human herpes simplex encephalitis.
Neurology 17:575-586 (1967).

91. Gaylord, W.H.
Cellular reaction during virus infections. In: Frontiers in Cytology.
Ed. Palay S.L. New Haven. Yale University Press 1958:460.

92. Baringer, J.R.
Human herpes simplex virus infections.
In Thompson R.A., Green J.R. (eds): Advances in Neurology 1974 Vol. 6, pp. 41-51.

93. Drachman, D.A., Adams, R.D.
Herpes simplex and acute inclusion-body encephalitis.
Arch. Neurol. 7:45-63 (1962).

94. Bergey, G.K., Coyle, P.K., Krumholz, A., Niedermeyer, E.
Herpes Simplex Encephalitis with occipital localization.
Arch. Neurol. 39:312-313 (1982).

95. Dayan, A.D., Gooddy, W., Harrison, M.J.G., Rudge, P.
Brain stem encephalitis caused by herpesvirus hominis.
Brit. Med. J. 4:405-406 (1972).

96. Tucker, B.A., Doekel, R.C., Whitley, R.J., Dismukes, W.E.
Herpes simplex viral encephalitis.: An atypical presentation.
South Med. J. 71:1431-1433 (1978).

97. Morawetz, R.B., Whitley, R.J., Murphy, D.M.
Experience with brain biopsy for suspected herpes encephalitis: A review of forty consecutive cases.
Neurosurgery 12:654-657 (1983).

98. Vanderhaeghen, J.J., Perier, O., Bossaert, Y.
Acute necrotizing encephalitis. Observations of herpes-like particles by electron microscopy.
Path europ 1:29-49 (1966).

99. Dix, R.D., Baringer, J.R., Panitch, H.S., Rosenberg, S.H., Hagedorn, J., Whaley, J.
Recurrent herpes simplex encephalitis: Recovery of virus after ara-A treatment.
Ann. Neurol. 13:196-200 (1983).

100. Davis, L.E., McLaren, L.C.
Relapsing herpes simplex encephalitis following antiviral therapy.
Ann. Neurol. 13:192-195 (1983).

101. Koenig, H., Rabinowitz, S.G., Day, E., Miller, V.
Post-infectious encephalomyelitis after successful treatment of herpes simplex encephalitis with adenine arabinoside.
N. Engl. J. Med. 300:1089-1093 (1979).

102. Olson, L.C., Buescher, E.L., Artenstein, M.S., Parkman, P.D.
Herpesvirus infections of the human central nervous system.
N. Engl. J. Med 277:1271-1277 (1967).

103. Lewis, M.E., Warren, K.G., Jeffrey, V.M., Tyrrell, D.L.J.
Effects of prolonged cultivation in the presence of acyclovir on recovery of latent herpes simplex virus from human trigeminal ganglia.
Antimicrob. Agents Chemother. 23:487-489 (1983).

104. Sequiera, L.W., Carrasco, L.H., Curry, A., Jennings, L.C., Lord, M.A., Sutton, R.N.P.
Detection of herpes-simplex viral genome in brain tissue.
Lancet. 2:609-612 (1979).

105. Fraser, N.W., Lawrence, W.C., Wroblewska, Z., Gilden, D.H., Kroprowski, H.
Herpes simplex type I DNA in human brain tissue.
Proc. Natl. Acad. Sci. USA 78:6461-6465 (1981).

106. Caplan, L.R.
Brain biopsy in herpes simplex encephalitis.
N. Engl. J. Med. 303:700 (1980).

107. Dix, R.D., McKendall, R.R., Barringer, J.R.
Comparative neurovirulence of herpes simplex virus type I strains after peripheral or intracerebral inoculation of Balb/c mice.
Infect. Immun. 40:103-112 (1983).

108. Nolan, D.C., Lauter, C.B., Lerner, A.M.
Idoxuridine in herpes simplex virus (Type I) encephalitis: experience with 29 cases in Michigan, 1966-1971.
Ann. Intern. Med. 78:243-246 (1973).

109. Nolan, D.C., Carruthers, M.M., Lerner, A.M.
Herpesvirus hominis encephalitis in Michigan: report of thirteen cases, including six treated with idoxuridine.
N. Engl. J. Med 282:10-13 (1970).

110. Fishman, R.A.
Steroids in the treatment of brain edema.
N. Engl. J. Med. 306:359-360 (1982).

111. Lopez, C.
Resistance to HSV-I in the mouse is governed by two major, independently segregating Non-H-2 Loci.
Immunogenetics 11:87-92 (1980).

112. Field, H.J., Darby, G., Wildy, P.
Isolation and characterization of acyclovir-resistant mutants of herpes simplex virus.
J. gen Virol. 49:115-124 (1980).

113. Wallis, C., Melnick, J.L.
Photodynamic inactivation of animal viruses: a review.
Photochem. Photobiol. 4:159-170 (1965).

114. Samuel, J., Knaus, E.E., Wiebe, L.I., Tyrrell, D.L.J.
Synthesis of ^{131}I, ^{125}I and ^{82}Br labelled (E)-5-(2-halovinyl)-2'-deoxyuridines.
Int. J. Appl. Radiat. Isotopes (in press 1984).

115. Whitley, R.J., Barton, N.H., Welchel, J., Diethelm, A.G., Curtis, J., Luke, R., Kulkarni, A.S.
Twenty-third conference on antimicrobial agents and chemotherapy: 1983 Las Vegas. Abstract 145.

116. Stocklin, G.
Bromine-77 and Iodine-123 Radiopharmaceuticals.
Int. J. Appl. Radiat. Isotopes 28:131-148 (1977).

117. Schroeder, C.H., Glorioso, G.J., Kirchner, H., Kaerner, H.C.
Neuropathogenicity of Herpes Simplex Virus in Mice. Protection against lethal encephalitis by co-infection with a non-encephalitogenic strain.
J. gen Virol. 64:1973-1984 (1983).

118. Harmenberg, J., Wahren, B., Oberg, B.
Influence of cells and virus multiplicity on the inhibition of Herpesviruses with acyclovir.
Intervirology 14:239-244 (1980).

119. DeClercq, E.
Comparative efficacy of antiherpes drugs in different cell lines.
Antimicrob. Agents Chemother. 21:661-663 (1982).

120. Whitley, R.J.
Treatment of Herpesvirus Infection.
N. Engl. J. Med. 310:654 (1984).

121. Dubbs, D.R., Kit, S.
Mutant strains of herpes simplex deficient in thymidine kinase-inducing activity.
Virology 22:493-502 (1964).