The pathogenicity of thymidine kinase-deficient mutants of herpes simplex virus in mice

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SUMMARY

The pathogenicity for mice of two mutants of herpes simplex virus (type 1 and type 2), which fail to induce thymidine kinase, were compared with their respective parent strains. The mutants were much less virulent than the parents following either intracerebral or peripheral inoculation. The replication of the virus at the site of inoculation and its progression into the nervous system were studied. Following a very large inoculum in the ear, the type 1 mutant was found to establish a latent infection in the cervical dorsal root ganglia. Mice inoculated intracerebrally with small doses of the mutant viruses were solidly immune to challenge with lethal doses of the parent strain.

INTRODUCTION

There is a rapidly accumulating literature concerning the anatomy and strategy of the herpes simplex genome and much interesting information has been obtained from cell culture studies on its expression and the nature of proteins specified in infected cells *in vitro*. However, it is very difficult to relate such biochemical information to the complex relationships that this group of viruses have with their natural hosts; in particular, herpes simplex virus has a predilection for specific tissues *in vivo* and has the ability to remain quiescent in latently infected cells thereby dodging its eradication by the immune reactions of the host. It is not known what properties of the virus are concerned in such complex cellular interactions, but it is possible that virus functions which are of seemingly little value in tissue culture systems may have important roles *in vivo*. For example, herpes simplex virus is known to induce virus-specific thymidine kinase (TK) activity, and this is associated with a serologically virus-specific protein (Klemperer *et al.* 1967, Thouless & Wildy, 1975). However, virus mutants (TK⁻) deficient in the production of this enzyme have been found which replicate efficiently in standard cell cultures, though not in serum-starved cells (Jamieson, Gentry & Subak-Sharpe, 1974). It has been suggested that cells infected in the natural disease such as epidermal and neural cells may have low levels of thymidilate metabolism and in these cells the virus may benefit from this apparently unnecessary enzyme.

In this study two (TK⁻) mutants of herpes simplex virus were compared with their parental strains to see if pathogenicity for mice could be correlated with the lack of this specific biochemical function in the virus.

MATERIALS AND METHODS

Cell line

BHK 21 Cl3 cells were grown in supplemented Eagle's medium containing v/v 10 % tryptose phosphate broth and 10 % calf serum (ETC) (Vantsis & Wildy, 1962).

Virus strains

The following strains of herpes simplex virus were used in this work: type 1 Cl (101) and its TK⁻ mutant B2006 subsequently referred to as Cl (101) TK⁻ (Dubbs & Kit, 1964) and type 2 Bry and its TK⁻ mutant Bry TK⁻ (Thouless, 1972). Virus infectivity was assayed by plaque counts in BHK cells using the suspension method of Russell (1962).

Virus growth in cell cultures

Petri dishes (6 cm diameter) containing confluent monolayers of BHK cells were infected at a multiplicity of 0·1 or 10 p.f.u./cell in a volume of 0·5 ml ETC. After 1 h adsorption at 37 °C the cell sheet was washed with phosphate buffered saline, and 5 ml ETC was added to each dish. At intervals after inoculation, 2 dishes of cells were sampled independently. The cells were suspended in the medium using a glass scraper and the whole stored at − 70 °C. The samples were then thawed, and virus was liberated from cells by ultrasonic vibration; released virus was assayed in BHK cells.

Serum-starved BHK cells

Stationary cultures of BHK cells were obtained using the method of serum-starvation described by Jamieson *et al.* (1974). Dishes were seeded with 10^6 cells in normal medium. After 24 h this medium was removed, the cells washed and then ETC containing 0·5 % calf serum was added. The cells were used after 6 days in the low serum medium.

Mice

Three week old BALB/c female mice were obtained from Bantin and Kingman (Grimston, Aldbrough, Hull) and used when they were 3 weeks or 4 weeks old. CBA female mice came from the inbred colony maintained in the Department of Pathology, Cambridge. Nude mice (athymic) were bred in the Department of Pathology; they had BALB/c background and were originally obtained from Bamholtguara Ltd, Ry, Denmark.

Intradermal inoculation and virus growth in the mouse ear

20 μl of virus suspension were inoculated into the left pinna of anaesthetized 3-week-old mice (Hill, Field & Blyth, 1975). In some experiments u.v.-irradiated virus (survival 10^{-8}) was used as a control inoculum. To study the replication of virus in the ear, the left pinnae were cut off from groups of 3 mice, the tissue from each mouse was then minced with scissors and ground in 1 ml ETC in small glass grinders. The suspensions were then diluted for independent assay using BHK cells.

Intracerebral (i.c.) inoculation and virus growth in mouse brain

20 μl of virus suspension were inoculated into the left cerebral hemisphere of 3-week-old mice. The LD50 was determined by the Spearman-Kärber method (Finney, 1952) from the number of mice dying between the 2nd and the 14th day after inoculation. To monitor virus replication after intracerebral inoculation, the whole brain was removed from 2 mice at each time. Each brain was homogenized in a 5 ml glass homogenizer containing 1 ml ETC. The debris was removed by centrifuging at 250 g for 10 min; the supernatant fluid was then diluted for virus estimation.

Reactivation of latent virus from dorsal root ganglia

The 2nd, 3rd and 4th cervical dorsal root ganglia were dissected from mice into 0·5 ml ETC. The ganglia were maintained at 30 °C in this medium in closed bottles for 5 days, then ground up and titrated for the presence of infectious virus as described above.

Examination of tissue sections by immunofluorescent microscopy

Frozen sections of ganglion tissue were cut using a Cambridge cryostat and the sections were fixed for 30 min. in cold acetone and stained by the indirect fluorescent antibody technique (Coons & Kaplan, 1950), using hyperimmune rabbit anti-herpes simplex type 1 serum (Watson *et al.* 1966) and fluorescein isothiocyanate conjugated to anti-rabbit IgG (Wellcome Reagents Ltd). The sections were examined on a Zeiss Ultraphot II fluorescent microscope using incident illumination.

RESULTS

Growth of virus in BHK cells

The growth of the herpes simplex virus type 1 mutant, Cl (101) TK$^-$ and the type 2 mutant Bry TK$^-$ were compared with their respective parent strains in actively growing monolayer cultures of BHK cells at 37 °C. With an input multiplicity of 10 p.f.u./cell, each mutant grew with similar kinetics to its parent strain and achieved approximately the same yield of virus (Fig. 1). The growth of Cl (101) TK$^-$ was also investigated using the lower input multiplicity of 0·1 p.f.u./cell in order to compare the ability of the virus to spread from cell to cell and should also have been more similar to the *in vivo* infection. Again no significant difference between the growth of the two viruses was observed (Fig. 1*a*).

Jamieson *et al.* (1974) reported that two TK$^-$ mutants of herpes simplex virus showed greatly reduced yields when grown in stationary cultures of BHK cells obtained by serum starvation. Using similar serum-starved cultures, we found the yields of our mutants, Cl (101) TK$^-$ and Bry TK$^-$ were reduced 7·5-fold and 40-fold respectively compared with their parent strains following an input multiplicity of 1·0 p.f.u./cell (Table 1).

Inoculation of the mouse ear

Clinical patterns. A relatively large inoculum (10^6 p.f.u./mouse) of Cl (101) was required to produce overt signs of disease. All mice inoculated with such doses
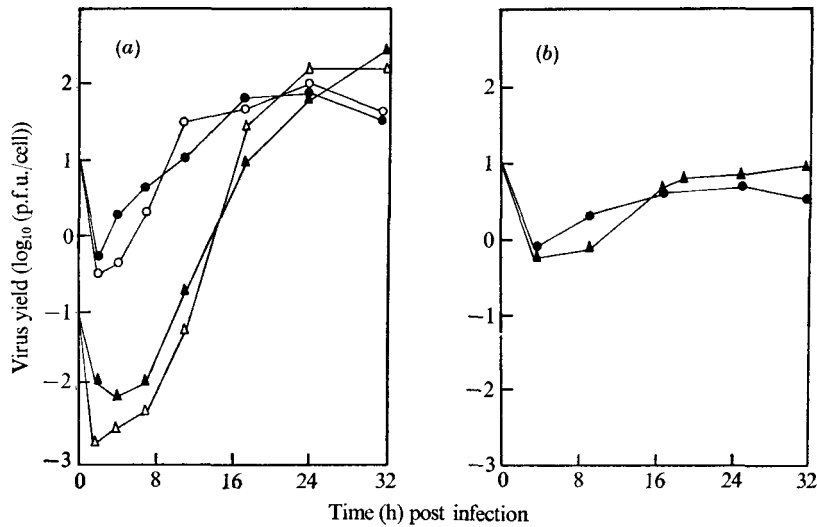
Fig. 1. Multiplication of wild type and TK⁻ strains of virus in actively growing
BHK C21 cells. (a) Type 1 virus Cl (101) m.o.i. 10 p.f.u./cell = ●, 0·1 p.f.u./cell
= ▲; Cl (101) TK⁻ m.o.i. 10 p.f.u./cell = ○, 0·1 p.f.u./cell = △. (b) Type 2 virus
Bry m.o.i. 10 p.f.u./cell = ●, Bry TK⁻ 10 p.f.u./cell = ▲.

Table 1. *Virus yields obtained from actively growing or serum-starved monolayer
cultures of BHK cells following infection with* 1 *p.f.u./cell wild type or mutant virus
strains*

Time (h) after infection ...	Medium containing 10 % calf serum		Medium containing 0·5 % calf serum	
	2	48	2	48
Virus strain				
Cl (101)	$2·6 \times 10^4$*	$2·6 \times 10^7$	$2·1 \times 10^4$	$2·1 \times 10^7$
Cl (101) TK⁻	$1·7 \times 10^4$	$4·2 \times 10^7$	$2·9 \times 10^4$	$2·7 \times 10^6$
Bry	$1·7 \times 10^4$	$3·7 \times 10^6$	$1·1 \times 10^4$	$1·1 \times 10^6$
Bry TK⁻	$3·0 \times 10^4$	$1·0 \times 10^7$	$1·8 \times 10^3$	$2·7 \times 10^4$

* Values = p.f.u. per 10^6 cells.

developed local erythema within 24 h of infection. On histological examination
the tissue of the inoculated ear taken at this time was found to be densely in-
filtrated with polymorphonuclear leukocytes. However, these local changes were
found when similar large inocula of u.v.-irradiated virus having no residual in-
fectivity were used. Mice inoculated with infectious Cl (101) virus developed
neurological signs; particularly paralysis of the inoculated ear which appeared
5–7 days after inoculation and in most cases resulted in permanent flaccid paraly-
sis (Table 2). No mice inoculated with Cl (101) TK⁻ showed any outward signs of
neurological involvement even after a dose of 10^8 p.f.u. and none died.

Mice were also inoculated in the ear with 10^6 p.f.u. of the type 2 strain, Bry and
its corresponding TK⁻ mutant. In this case the difference in the clinical signs pro-
duced by the viruses was even greater. Mice inoculated with Bry strain showed a

Table 2. *Clinical signs in mice following inoculation with wild type and TK⁻ mutant strains of HSV*

Virus strain ...	Cl (101)		Cl (101) TK⁻	
Dose*	10^7	10^8	10^7	10^8
Erythema of inoculated ear	15/15†	10/10	14/15	26/26
Ear paralysis	7/15	8/10	0/15	0/26
Mortality	1/8	2/6	0/10	0/26
Virus strain ...	Bry		Bry TK⁻	
Dose	2×10^6		2×10^6	
Erythema of inoculated ear	10/10		10/10	
Ear paralysis	10/10		0/10	
Mortality	10/10		0/10	

* Dose in p.f.u./ear inoculated in 0·02 ml.
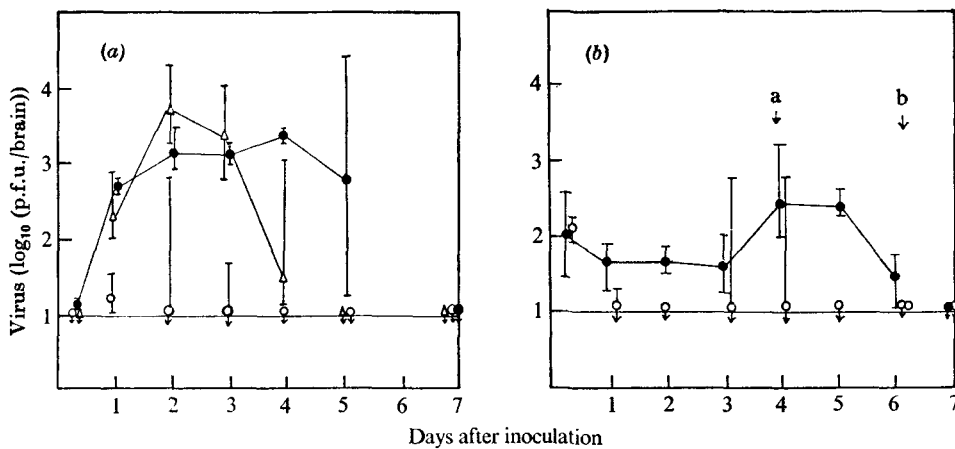† numerator = number mice responding; denominator = total in group.

Fig. 2. Multiplication of virus in the inoculated ears of 4-wk-old BALB/c mice with wild type and TK⁻ strains of herpes simplex virus. Points are geometric means obtained from 3 mice; bars represent ranges. Points with arrow (↓) indicate that mean fell below the sensitivity of the assay. (*a*) Cl (101) inoculum 10^4 p.f.u./mouse = ●, Cl (101) TK⁻ inoculum 10^4 p.f.u./mouse = ○, Cl (101) TK⁻ inoculum 10^5 p.f.u./mouse = △. (*b*) Bry inoculum 10^4 p.f.u./mouse = ●, Bry TK⁻ inoculum 10^4 p.f.u./mouse = ○, (a) first erythema observed, (b) first deaths occur.

range of neurological signs: ear paralysis, limb paralysis, circling etc. and all the mice subsequently died. No signs of abnormality were observed in any mice inoculated with Bry TK⁻ apart from the usual local erythema of the inoculated ear (Table 2).

Local virus multiplication in the inoculated ear. The growth of the viruses in the inoculated ear was compared using small inocula. As shown in Fig. 2*a*, the input inoculum of 10^4 p.f.u., strain Cl (101) replicated rapidly, achieved a 300-fold yield of infectious virus by 2 days after inoculation and maintained this level until day 4. In contrast, a similar inoculum of Cl (101) TK⁻ produced a transient small increase 1 day after inoculation, but little infectivity thereafter. By increasing the inoculum of Cl (101) TK⁻ to 10^5 p.f.u., substantial growth of the mutant strain was noted, but the titres declined sharply 3–4 days after inoculation (Fig. 2*a*). Mice infected

Table 3. *LD 50 determined in 3-week-old mice following intracerebral inoculation*

Virus strain	Mouse strain	Log$_{10}$ LD 50/0·02 ml and standard error	p.f.u./LD 50
Cl (101)	BALB/c	0·0, 0·2	1
Cl (101) TK$^-$	BALB/c	2·3, 0·2	200
Cl (101)	CBA	< −0·5	< 0·3
Cl (101) TK$^-$	CBA	0·4, 0·2	3
Bry	BALB/c	0·07, 0·2	1
Bry TK$^-$	BALB/c	> 5·0	> 10^5

with 10^4 p.f.u. of the type 2 strain Bry (Fig. 2*b*) showed a slow rise in virus titre reaching a maximum on the 4th day after inoculation. The TK$^-$ mutant was not found to replicate consistently following this dose. After the first day virus was isolated from only 2 mice; 1/3 mice on each of days 3 and 4 after infection.

Evidence of centripetal spread of virus. The cervical dorsal root ganglia were removed from 3 mice 5 days after inoculation with 10^7 p.f.u. of strain Cl (101); these were found to contain about 10 p.f.u./ganglion of infectious virus by titration, and revealed antigen-containing cells in cryostat sections stained by the indirect immunofluorescent method. Up to 5 foci of fluorescent cells were observed in each ganglion section. These foci comprised both individual cells and small groups of cells in which neurons and satellite cells were identified. No virus was isolated from ganglia taken from a similar number of mice infected with Cl (101) TK$^-$ nor were antigen-containing cells observed in cryostat sections. Ganglia were also explanted from such mice 2–3 months later and cultured to reactivate latent virus. All of 7 mice originally inoculated in the left ear with 10^7 p.f.u. strain Cl (101) were found to harbour latent virus in the left (but not right) cervical ganglia. On the other hand only 5/12 of the mice inoculated in the ear with 10^8 p.f.u. of strain Cl (101) TK$^-$ yielded reactivated virus from the ganglia. Two of these isolates were tested and shown still to fail to induce the thymidine kinase enzyme. Mice originally inoculated with Bry could not be tested for latency as there were no survivors with these doses. However, virus could not be reactivated by explantation of ganglia from 11 mice previously inoculated with 10^6 p.f.u. of Bry TK$^-$.

Intracerebral inoculation of mice

Clinical patterns. Considerable differences in the virulence of the parent and TK$^-$ mutant viruses were observed following their inoculation into the brains of mice. For the type 1 viruses, the LD 50 determined in 3-week-old BALB/c mice, differed by about 200 fold (Table 3) and in the case of the type 2 viruses, the difference was about 10^5. CBA mice were found to be generally more susceptible to the type 1 viruses, but the difference between the virulence of the parent and mutant Cl (101) TK$^-$ was still apparent. The clinical signs produced in mice following the inoculation of the type 2 virus Bry, differed from those produced by type I virus. Cl (101)-infected mice became hunched with ruffled fur, and death rapidly followed the appearance of these signs. In contrast, the signs in Bry-inoculated mice
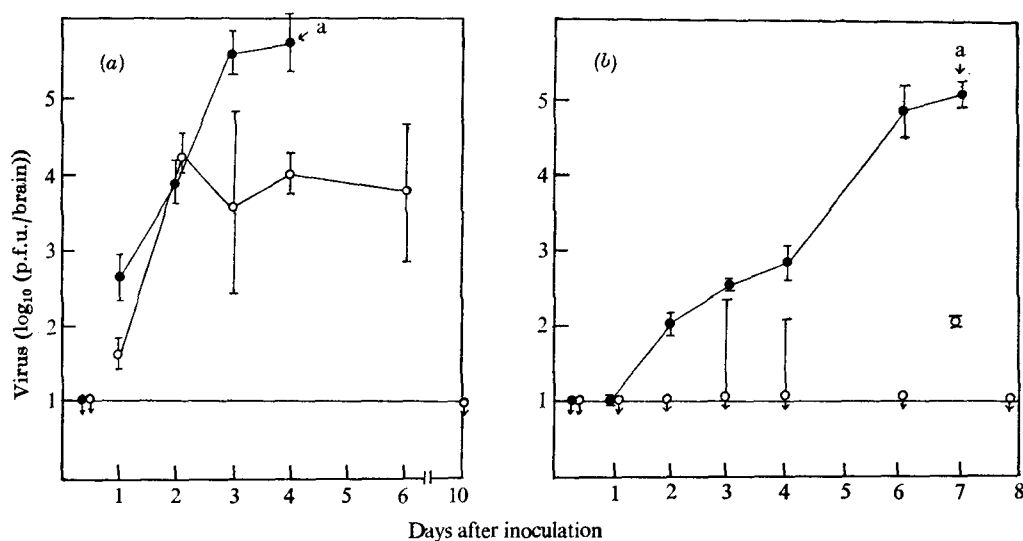
Fig. 3. Multiplication of virus in the brains of 3-wk-old BALB/c mice following inoculation with 10 p.f.u. wild type or TK$^-$ strains of virus. Points are geometric means obtained from 2 mice; bars represent ranges. Points with arrow (↓) indicate that mean fell below sensitivity of the assay. (*a*) Cl (101) = ●, Cl (101) TK$^-$ = ○; (*b*) Bry = ●, Bry TK$^-$ = ○. a = first deaths occur.

developed more slowly and the mice showed a wide range of neurological signs, particularly limb-paralysis of varying severity. All the mice inoculated with 10 p.f.u. of the wild type virus, Bry, showed signs of neurological involvement though some of these mice recovered from the neurological disease. No mice inoculated i.c. with up to 100 p.f.u. of Cl (101) TK$^-$ showed any outward signs of disease; however, administration of 10^4 p.f.u. of Cl (101) TK$^-$ resulted in rapid death within 4 days. Three clones of Cl (101) TK$^-$ were prepared by two successive single plaque isolations. The cloned strains were inoculated into the brain using 10^4 p.f.u. and were found to have identical characteristics with those of the working stock virus used for the previous experiments. A further clone of the mutant was grown in the presence of bromodeoxyuridine (50 μg/ml) and resembled the original strain in a similar test.

Growth of virus in the brain. Ten p.f.u. were inoculated and the whole brain (including medulla) was removed at intervals after infection from 2 mice at each time. In the case of the type 1 viruses, both wild type and TK$^-$ mutant replicated at similar rates for 2 days after infection (Fig. 3*a*). The wild type virus showed a steady increase of virus titre, with the first death at 4 days after inoculation. The mutant, however, reached a plateau at day 3 which was maintained up to 6 days after inoculation. No further samples were taken until day 10 when no remaining infectious virus could be found. The maximum titre reached in the brains of mice infected with Cl (101) TK$^-$ was approx. 10^4 p.f.u., whereas at the time when first deaths occurred in animals infected with wild type virus, the titres exceeded 10^5 p.f.u. The restriction of replication of the mutant may be crucial for the survival of these animals; it is also consistent with the observation that doses of

Table 4. *Mortality on challenge of previously inoculated mice 2–3 months later with* 100 *LD*50 *wild type virus*

Previous inoculation			Mortality on intracerebral challenge with 100 p.f.u. HSV1 Cl (101) (Number dead/number inoculated)
Virus strain	Dose (p.f.u.)	Route	
Uninoculated control	—	—	6/7 8/8
u.v.-inactivated*⎱ Cl (101) ⎰	10^4	i.c.	10/10
	10^2	i.c.	10/10
Cl (101) TK⁻	10^2	i.c.	0/8
Cl (101) TK⁻	10^1	i.c.	0/8
Cl (101) TK⁻	10^0	i.c.	0/8
Cl (101) TK⁻	10^8	Left ear	0/9
Bry	10^1	i.c. (survivors)	0/3
Bry	10^0	i.c. (survivors)	0/3
Bry TK⁻	10^2	i.c.	2/9
Bry TK⁻	10^1	i.c.	7/9
Bry TK⁻	10^0	i.c.	7/8
			Mortality on intracerebral challenge with 100 p.f.u. Bry
Uninoculated control	—	—	2/2
Bry TK⁻	10^5	i.c.	0/4
Bry TK⁻	10^4	i.c.	0/8
Bry TK⁻	10^3	i.c.	0/7
Bry TK⁻	10^1	i.c.	1/6

* u.v.-irradiated virus (survival 10^{-8}) diluted to give 10^4 and 10^2 erstwhile p.f.u.

> 10^3 p.f.u. Cl (101) TK⁻, which would produce correspondingly higher titres of virus in the brain, usually resulted in death of the mouse. Virus isolated at day 3 from the brains of mice inoculated with Cl (101) TK⁻ was passaged 4 times in mouse brain in an effort to isolate virus showing enhanced virulence; no change in the character of the virus was observed. In mice inoculated with Bry, the virus titre increased slowly and thus paralleled the slower development of neurological signs. As with the ear experiment, the isolation of the mutant for Bry TK⁻ from brains was sporadic (observed in 1/2 mice on days 3, 4 and 7 only) (Fig. 3*b*).

Protection after challenge with wild type virus. After the experiments described above a number of mice were collected which were left over from various experiments. These were used to investigate the resistance to challenge with a lethal dose of the wild type strain. Mice previously inoculated with Cl (101) TK⁻ either i.c. (100, 10 or 1 p.f.u.) or into the left ear (10^8 p.f.u.) were challenged with 100 p.f.u. of strain Cl (101) given intracerebrally. Complete protection was observed (Table 4) – no mice showed any specific signs of virus infection. Control (previously uninoculated) mice of similar age or mice inoculated i.c. with u.v.-inactivated Cl (101) were found to be highly susceptible to the same dose of virus. When mice that had received Bry TK⁻ were similarly challenged with Cl (101) some protection was

also observed; when challenged with 100 p.f.u. of the homologous virus the protection was more substantial (Table 4).

Inoculation of nude (athymic) mice

It has been reported that suppression of various aspects of the immune system of the mouse exacerbates the acute infection with herpes simplex virus in mice and predisposes to neurological invasion by the virus. It was of interest to infect nude mice peripherally with the TK$^-$ mutant to see whether the virus was capable of invasion and spread through the nervous system under circumstances highly favourable to the virus.

Four adult male nude mice were inoculated with a large dose (10^8 p.f.u.) of Cl (101) TK$^-$ into the left ear. After 5 days 2 mice were killed and the cervical dorsal root ganglia examined for the presence of virus – none was found. Ten days after infection the 2 remaining mice were killed (one *in extremis*) when large amounts of virus were found in the now ulcerated left ears. In only one of the 2 mice (the animal *in extremis*) very small amounts of infectious virus were found in the 2nd and 4th cervical ganglia and spinal cord. Study of tissue sections of the 3rd cervical ganglion by immuno-fluorescence revealed between 5 and 10 antigen-containing neurons.

DISCUSSION

Our results confirm those of Jamieson *et al.* (1974) who report that TK$^-$ mutants of herpes simplex virus replicate efficiently in actively growing cell cultures though less well in poorly multiplying serum-starved cells. This latter observation led us to speculate that such mutants might be less virulent when tested *in vivo*. We have investigated two TK$^-$ mutants and have shown that this is so; for example, the type 1 and type 2 mutants studied were respectively 100-fold and 10^5-fold less virulent by the intracerebral route. To our knowledge this is the first time this observation has been made with the exception of Marcialis *et al.* (1975) who used an iododeoxyuridine-resistant mutant which presumably also lacks the ability to induce thymidine kinase. It may be that the deficiency of the enzyme thymidine kinase is responsible for the lack of virulence although we cannot exclude the possibility that there are other defects in the virus arising from unrecognized mutations. However, from the work of Klemperer *et al.* (1967) and Thouless & Wildy (1975) at least it can be said that in the case of our mutants the only serologically recognized function missing is that of the TK gene. Aside from this it is well known that some temperature sensitive mutants of herpes simplex virus also have low virulence for mice (Zygraich & Huygelen, 1973; Lofgren *et al.* 1977 and our own unpublished observations).

The experiments (Figs. 2 and 3) designed to investigate the extent of virus multiplication at the site of inoculation (either ear or brain) showed as expected a considerable diminution in virus titre and this was particularly marked with respect to the type 2 mutant strain, Bry TK$^-$, following intracerebral inoculation. However, in inoculated ears a small proportion of the mice, 3 and 4 days after inoculation, gave titres comparable with the wild type. This raised anxiety that there may have been a small proportion of revertants (or wild-type virus) in the

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mutant stocks. Although we did not test the TK activity from those particular yields it is very unlikely that they had the TK character for the following reasons: (1) Experiments not described here employing deliberate mixtures of mutant and wild type virus led us to conclude that excess mutant virus did not interfere with the wild type over a range of proportions. (2) Freshly isolated clones (of mutant stock selected with or without bromodeoxyuridine) behaved identically in mice. (3) Serial passage of mutant-infected mouse brain again showed no change in the rate of virus multiplication. It will be noticed in Fig. 2a that when the inoculum of the type 1 mutant Cl (101) TK⁻ was increased to 10^5 p.f.u., appreciable multiplication was observed although the titre of virus declined on the 3rd and 4th days after inoculation. This leads us to suspect that there may be scattered cells which are able to support efficient replication of the mutant.

No evidence of migration of mutant virus into the nervous system of BALB/c mice was found during the acute phase of the disease, either by titration or by immunofluorescent microscopy. However, after inoculation of 10^8 p.f.u. of Cl (101) TK⁻, virus was subsequently reactivated from dorsal root ganglia of several mice. This technique may be a more sensitive method for detecting the penetration of a few virus particles into the nervous system; but it should be remembered that such large inputs of virus may result in direct uptake of the inoculum into axons (Field & Hill, 1974). It is evident from present experiments that TK⁻ mutant virus may be able to establish a latent state despite this enzyme deficiency. After a high dose of Cl (101) TK⁻ into athymic mice, small amounts of infectious virus were located in the spinal cord and ganglion of one mouse. Thus even under circumstances when the cell-mediated response is impaired and which should be highly favourable to the virus, spread of virus into the nervous system appears very inefficient and again may require sustained high titres of infectious virus at the site of inoculation.

Intracerebral inoculation of small doses or peripheral inoculation of a large dose of Cl (101) TK⁻ was found to protect mice from subsequent challenge with potentially lethal amounts of the wild-type strain. Such protection after herpes simplex virus infection is well documented (e.g. Godfrey, 1972) and is thought to be due to cell-mediated rather than to humoral immune mechanisms (Oakes, 1975). Previous reports, e.g. Price, Katz & Notkins (1975), suggest that such immunity may not prevent a subsequent inoculation into a peripheral site from achieving a new latent infection. Using the present system it would be possible to investigate in detail the influence of such immunizing inoculations on the pattern of local virus replication during a subsequent infection with wild-type virus.

Our present findings support the idea that the ability of the virus to induce thymidine kinase may considerably favour its replication in those cells (particularly epidermal cells) that are involved early in the infection. The same seems to be true also of the cells of the nervous system. This theory is consistent with the suggestion of Jamieson et al. (1974) that such cells, having low levels of thymidilate metabolism may be comparable with serum-starved or resting cells. This would then explain the reduced virulence of the TK⁻ mutants.

We foresee two uses for these TK⁻ mutants. Firstly when compared with neuro-

tropic strains they are valuable in the study of host responses to infection with herpes simplex virus. Secondly they would provide a good starting point for the development of attenuated vaccines where non-neurotropic viruses will be required.

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