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Characterization of latent infections in mice inoculated with herpes simplex virus which is clinically resistant to acyclovir

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Mice were inoculated into the ear pinna with herpes simplex virus (HSV) using a strain which is resistant to acyclovir (ACV) chemotherapy. The original inoculum was resistant to ACV because it contained a proportion of thymidine kinase-defective (TK^-) virions. This had been obtained previously by passage of an HSV type 1 strain in mice undergoing suboptimal therapy. The cervical dorsal root ganglia were subsequently explanted from the infected mice and the presence of latent virus therein revealed by reactivation in vitro. These explant cultures yielded both TK^+ and TK^- viruses on reactivation. The establishment of latent infections was not affected by chemotherapy during the acute infection. One TK^- ganglion isolate when studied in detail was found to be attenuated and thus resembled previously examined TK^- strains which had been selected in vitro for ACV-resistance.

herpes simplex virus; acyclovir; latency; resistance; chemotherapy

Introduction

In a previous study [4] it was shown that passage of herpes simplex virus (HSV) in mice undergoing treatment with acyclovir (ACV) resulted in the emergence of a population of virus which was resistant to chemotherapy. It has become clear that a similar pattern of events can occur in the immunocompromised patient undergoing ACV therapy [1,2,16]. More recently Dekker et al. [3] reported on the sensitivity to ACV of a large number of clinical specimens obtained from early human trials with the drug. Several of the isolates (obtained from immunocompetent patients) were found to be mixtures of resistant together with sensitive virions. Both in the mouse experiments and in these human examples the basis for drug-resistance was principally due to an increased proportion of thymidine kinase-defective (TK^-) virions in the virus population.

We felt that it was important to investigate the nature of latent infections establish-

ed in mice inoculated with such resistant virus since this can help to predict patterns of latency and recurrence in man should resistant infections be encountered.

Several previous studies by ourselves [6,8] and others [11,14,15] have shown that TK-defective viruses (which had been selected in vitro for resistance to one of several different thymidine analogues) had a greatly reduced ability to infect neural tissue in vivo and to establish or reactivate from latent infections. A disadvantage with tissue culture systems for the selection of resistant mutants is that silent mutations can pass unnoticed in vitro if they affect only those functions which are essential to the virus when it invades the living host. This was a further reason to extend our previous pathogenicity studies in order to explore the latency potential of virus strains which had been selected for drug-resistance in vivo.

Materials and Methods

Virus infection

Three- to four-week-old BALB/c female mice were infected by inoculating 10^5 PFU virus suspended in 20 µl of Eagle's minimal essential medium (MEM) into the skin of the left ear pinna [5]. The progress of the infection was measured by testing homogenates of ear tissue for virus titre and the clinical severity tested by measuring increased ear thickness of groups of mice. These techniques and the characteristics of infection so produced have been described previously in detail [5,10].

The virus inocula used in the present study were originally derived from the HSV type 1 isolate, SC16 [9]. SC16 was passaged in mice undergoing suboptimal oral therapy with ACV by including 0.25 mg/ml in the drinking water. Isolates were obtained from the ears of these mice at the height of the acute infection and were numbered according to the passage level [4]. The inocula used in the present study were obtained from the second and the fourth passage and were termed SC16 MP2 and SC16 MP4.

Nucleoside therapy

Acyclovir (ACV) was a gift from Dr. P. Collins, The Wellcome Foundation Co., Beckenham, U.K. The powder was dissolved in water and diluted to give 0.25 mg/ml (or 1.0 mg/ml) in the drinking water. Mice consumed approximately 3 ml/day drinking water giving an average intake of 167 ± 56 mg/kg per day.

No distaste for the drug was observed and this method of treatment was found to be a highly effective method of treatment in a previous study using the same parental virus strain, SC16 [7].

For determination of the LD_{50} by intracerebral (i.c.) inoculation, 20 µl of dilutions of virus suspension were inoculated into groups of 3-wk-old anaesthetized mice. The LD_{50} was calculated using the Spearman–Kärber method from the numbers of mice dying between the second and fourteenth day after inoculation [8].

Reactivation of latent virus

The 2nd, 3rd and 4th cervical dorsal root ganglia (drg) from the left side were explanted and incubated whole for 5 days at 37°C in 0.5 ml MEM supplemented with 1% calf serum. At the end of this time the ganglion tissue, either all three ganglia or individually, were homogenised using a small glass grinder and the presence of infectious virus was revealed by plaque titration in BHK cells. These methods have been described previously [5], and were found to be an efficient method for detecting latent virus infection.

Single plaque isolation

Virus was inoculated onto preformed BHK monolayers in 5-cm Petri dishes and the cultures incubated at 37°C. The medium was MEM with 2% calf serum thickened with carboxy-methyl cellulose. As soon as plaques began to appear (24–48 h after inoculation) plates were chosen which contained no more than one or two well separated plaques and a few infected cells were aspirated by means of a pasteur pipette. These cells were used to inoculate further BHK cells in order to produce a working stock of virus.

Plaque reduction assay

The sensitivity of virus isolates to ACV was measured by determining the 50% effective dose (ED_{50}) drug concentration in a plaque reduction assay using established monolayers of BHK cells; the methods have been described previously [4].

Thymidine kinase determinations

All enzyme determinations were carried out on cell extracts obtained 18 h after inoculation of BU-BHK cells (which are themselves TK^-) always using a virus multiplicity of 5 PFU/cell. The details of the method have been described previously [4].

Results

Establishment of latency, with low passage ACV-resistant virus

The first part of the investigation concerned virus which had previously been passaged twice in mice undergoing ACV therapy. The inoculum (SC16 MP2) had already been shown [4] to comprise approximately 50% TK-defective virions with an overall ED_{50} value for ACV of 0.04 μg/ml. This virus was also partially resistant to chemotherapy when inoculated into mice [4]. Five mice were inoculated with 10^5 PFU of SC16 MP2 into the left ear. ACV was given in the drinking water (0.25 mg/ml) from the time of virus inoculation for 10 days. Six weeks after inoculation the 2nd, 3rd and 4th cervical ganglia were explanted and tested for latent infection following incuba-

tion for 5 days; the ganglia from each mouse were pooled. Four of five mice yielded virus from the ganglia on reactivation in vitro.

The nature of virus reactivated from ganglia

The results of ED_{50} titrations and TK determinations on the reactivated viruses are shown in Table 1a. These results show that one of the four mice yielded a virus (SC16 MP2 drgC) which closely resembled the original parental strain (SC16). The remaining isolates had the characteristics of mixtures comprising a majority of (TK^+) sensitive virus, but also containing some (TK^-) resistant virus. The viruses yielded by 3 of the explant cultures resembled the virus SC16 MP2 used for the inoculum. However, since three ganglia from each mouse were incubated together mixtures may have arisen from viruses reactivated from separate ganglia in the culture tube.

The establishment of latency with ACV-resistant virus that was predominantly TK-defective

Further experiments were carried out using an inoculum obtained from the 4th passage level (SC16 MP4). This virus was resistant to chemotherapy in mice and when tested in vitro its ED_{50} was 0.4 μg/ml. It appeared to comprise approximately 95%

TABLE 1

Thymidine kinase induction and ACV sensitivity of viruses reisolated from the ganglia of latently infected mice

Virus strain	TK (% SC16)[a]	ED_{50} μg/ml[b] ACV	% plaques at 0.1 μg/ml ACV
a.			
Parental sensitive strain SC16	100	0.02	⩽1
Inoculum (SC16 MP2)	55	0.04	21
MP2-drg A	71	0.03	13
MP2-drg B	70	0.06	23
MP2-drg C	98	0.02	⩽1
MP2-drg D	78	0.05	16
b.			
Inoculum (MP4)	6	0.4	100
MP4-drg 1 CIII[c]	4	1.0	83
MP4-drg 1 CIV	4	2.0	100
MP4-drg 4 CIV	10	1.0	93
MP4-drg 10 CIII	4	1.0	78

[a] Enzyme assays performed on cell extracts obtained 18 h p.i. using a multiplicity of infection of 5 PFU/cell.

[b] ED_{50} measured by plaque reduction assay in BHK cell monolayers.

[c] Roman numerals refer to 2nd, 3rd or 4th cervical ganglia.

TK-deficient virus. Ten mice were inoculated in the ear with this virus and all were treated as before with 0.25 μg/ml ACV in the drinking water.

The nature of virus reactivated from mice inoculated with predominantly TK-defective virus

After 6 wk, these mice were killed and the left ganglia (2nd, 3rd and 4th cervical) explanted. On this occasion each of the three ganglia from the ten mice were incubated individually. In this way 4 ganglion virus isolates were obtained from three mice which yielded virus positive ganglia. The characteristics of these isolates are shown in Table 1b. As in the experiment described above, the viruses isolated from the latently infected ganglia bore a marked resemblance to the inoculum viruses. They showed similar TK levels but were all slightly more resistant to ACV when measured in vitro.

To check the viruses for homogeneity single plaques were picked for two of the isolates, and used to prepare virus stocks for further analysis. The results obtained from these 'plaque purified' strains (Table 2) were striking. The 4 single plaque isolates obtained from SC16 MP4 drg1 CIII, which itself induced a low but significant level of TK activity, all resembled the original ganglion isolate. In contrast the plaque purified strains obtained from SC16 MP4 drg4 CIV yielded 3 low TK-inducing strains and one with a value of 42% of the parental strain, SC16. The slightly higher TK induced by SC16 MP4 drg4 CIV could thus be explained by the presence of a significant proportion of TK^+ virus. The value of 42% TK obtained for the single plaque isolate was unsurprising since previous work had shown that homogeneous virus populations were not always obtained following one round of single plaque isolation and the isolate was subsequently shown to be a mixture comprising approximately 50% TK^+ and TK^- virus.

TABLE 2

TK induction of single plaque derived strains obtained from viruses reisolated from latently infected mice

Virus isolate	TK (% SC16)
SC16 MP4 drg1 CIII	
Single plaque 'clones':	
C1	2.3
C2	2.1
C3	2.0
C4	2.0
SC16 MP4 drg4 CIV	
Single plaque 'clones':	
C1	2.7
C2	42
C3	2.2
C4	2.3

An investigation of the virulence of a TK-defective strain reactivated from a ganglion

The next question to be pursued was whether a TK-defective virus isolate which had reactivated from a ganglion would be attenuated in mice as had been previously shown for such TK-defective strains selected in vitro. The plaque purified virus obtained from the ganglion (SC16 MP4 drg1 CIII clone 1) was inoculated into the ears of further mice using the standard dose of 10^5 PFU. Mice were untreated or given ACV in the drinking water (1 mg/ml). For comparison the parental strain (SC16) was similarly inoculated. The results of ear thickness measurement (a measure of clinical severity) and virus titres in ear tissue measured at days 3 and 5 after inoculation are shown in Fig. 1 and Table 3. It may be seen that there was little difference between the replication of the resistant and sensitive strain in the absence of chemotherapy but the mutant strain produced less inflammation as measured by increased ear thickness. Mice were subsequently tested for the presence of latent infections in the dorsal root ganglia. None of ten mice tested yielded virus following attempted reactivation in vitro.

A further animal test was carried out on MP4 drg1 CIII clone 1. Mice were inoculated i.c. and the LD_{50} determined. The result obtained was 1.3×10^2 PFU/LD_{50} compared with 7 PFU/LD_{50} for the parental strain SC16. So by these animal tests, establishment or reactivation of latent infections and 'neuro-virulence' following i.c. inoculation, the TK-defective strain isolated from the mouse ganglion was attenuated and did not differ from other TK-defective viruses derived from the same parental strain, SC16 that had been previously isolated from tissue cultures.

The effects of chemotherapy on the establishment of latency with ACV-resistant virus

Finally, an experiment was carried out to examine the effects of chemotherapy on

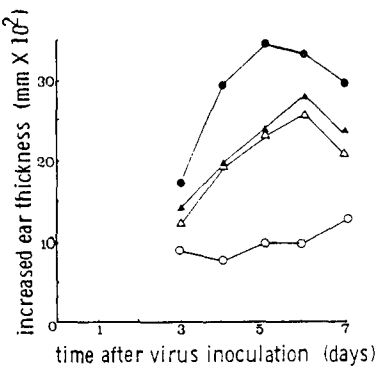

Fig. 1. Inflammation in the ears of mice, with or without chemotherapy, after inoculation with a TK-deficient ganglion isolate compared with its parental strain. ● = SC16 (no chemotherapy); ○ = SC16 (ACV 1 mg/ml in drinking water); ▲ = SC16 MP4 drg1 CIII clone 1 (no chemotherapy); △ = SC16 MP4 drg1 CIII clone (ACV 1 mg/ml in drinking water). 10^5 PFU virus was inoculated into the left ear pinna. Chemotherapy was started at the time of virus inoculation. Ear thickness is the mean value of left–right (uninoculated side) obtained from three mice.

TABLE 3

Virus growth in the ears of mice with or without chemotherapy after inoculation with a TK-defective ganglion isolate compared with its parental strain

Virus inoculum[a]	ACV chemotherapy[b]	Virus titre[c] (log_{10} PFU/ear)	
		Day 3	Day 5
SC16	yes	1.7;0.7	<0.0
SC16	none	4.9;0.9	3.2;0.6
SC16 MP4 drg1 CIII C1	yes	3.7;0.9	3.2;0.6
SC16 MP4 drg1 CIII C1	none	4.6;0.3	3.8;0.5

[a] 10^5 PFU virus was inoculated into the left ear pinna.
[b] Chemotherapy (ACV, 1 mg/ml in drinking water) was started at time of virus inoculation.
[c] Virus titres are geometric mean and standard deviation obtained from 3 mice tested independently at each time.

the establishment and nature of latent infections following inoculation with resistant virus. Thirty mice were inoculated with the virus obtained from the 4th passage in mice, SC16 MP4. Ten mice were untreated, 10 given 0.25 mg/ml and 10 given 1.0 mg/ml in the drinking water for 10 days starting at the time of virus inoculation. After 6 wk, the 2nd, 3rd and 4th left cervical ganglia from all the mice were explanted and incubated separately for 5 days prior to testing for infectious virus; 90 ganglia in all. The ear thickness increases measured at 5 days p.i. (the mean value of the left, -right inoculated ear thickness in mm $\times 10^2$) were untreated 21.3 ± 5.9, 0.25 mg/ml ACV 19.9 ± 4.3 and 1 mg/ml ACV 16.3 ± 3.3. This showed a typical infection was established and that the infection was resistant to the lower chemotherapy although appeared to be slightly reduced by the higher drug concentration. When the ganglia were subsequently tested for the presence of infectious virus the majority of cultures proved negative. In all 5/30 mice yielded virus from the ganglia (Table 4). These

TABLE 4

The effects of chemotherapy on the establishment of latent infections in mice inoculated with ACV-resistant HSV (SC16 MP4)

ACV therapy[a]:	None	0.25 mg/ml	1.0 mg/ml
Proportion[b] of mice yielding latent virus	2/10	2/10	1/10
Virus isolated[c] and (TK induction)	drg5 CIII (77%)[d] drg8 CIII (4%)	drg4 CIII (2%) drg6 CIV (92%)	drg10 CIII (5%)

[a] ACV dissolved in drinking water for 10 days from time of virus inoculation.
[b] Number of mice yielding virus following reactivation from explant cultures of cervical dorsal root ganglia one month after inoculation.
[c] Virus isolates from explant cultures, e.g. drg5 CIII refers to the isolate obtained from the 3rd cervical ganglion of mouse number 5.
[d] TK induction expressed as percentage of the parental strain HSV1 SC16.

isolates were distributed among the 3 experimental groups and examples of both TK^+ (drg6 and drg5) and TK^- strains drg10) were represented. The experiment was limited in that a relatively small number of individual virus isolates was obtained presumably reflecting the predominance of TK^- virus in the inoculum SC16 MP4; however the data obtained from this experiment does form a basis on which to make some predictions about the likely characteristics of latent infections established in man.

Discussion

The most significant observation made in the present study was that when mice were infected in a peripheral site, using an inoculum which was clinically resistant to ACV, a latent infection could be established in the peripheral nervous system which, on reactivation, yielded resistant virus. The inocula used were known to comprise a mixture of resistant (TK^-) and sensitive strains. It was therefore of interest that when viruses were reisolated from latently infected ganglia they were also found to be heterogeneous and included strains that appeared to be either predominantly TK^- or TK^+. If we argue that the artificial reactivation by in vitro culture of ganglia explants resembles the natural process of reactivation in man giving rise to the recurrences which characterise human herpes simplex it seems wholly reasonable to argue that these data suggest that latent infection will be established during chemotherapy and that subsequently reactivation will produce mixtures of resistant or sensitive strains. It may be that successive recurrences will become increasingly resistant in the manner revealed by our previous mouse experiments [4] and also encountered in the severely immunocompromised patient [1,2,16]. However, it should also be noted that when an individual TK^- human isolate was examined in detail this strain was less neuropathogenic in mice [12] and similar observations were made on the TK^- ganglion isolate in the present study although this strain appeared to replicate well in the skin of mice. Just how much such strains will contribute to clinical resistance in man is still uncertain. It should be noted, however, that while TK^- strains established latency with difficulty individual isolates were obtained from ganglia which were TK^- suggesting that this enzyme activity is not an absolute requirement for either the establishment of latent infection or reactivation of virus from the resting state in the infected neuron. It will be noted that some TK-defective strains induced measurable enzyme activity; the enzyme assay is extremely sensitive and activity $> 1\%$ wild type was regarded as significant. In the case of the ganglion isolate pursued in the present study (drg1 CIII clone 1) this low activity does not represent residual activity induced by a normal number of enzyme molecules with altered substrate specificity. On the contrary no TK polypeptide was present (B.A. Larder, pers. commun.) and when infected cells were examined by autoradiography following labelling of the mutant-infected cells with tritiated thymidine all infected cells were found to produce low-level graining in the emulsion overlay (Field, unpublished observations). These data would be consistent with the hypothesis of Summers et al. [13] that low TK induction may result from read-through of a premature termination codon giving rise to low levels of normal virus enzyme in the infected cells. In any case this particular mutant did not have a

special ability to establish new latent infections when reinoculated into mice despite it having itself been isolated from latently infected cells. Moreover, the inoculation of mice with the resistant inoculum SC16 MP4 showed that while this strain produced a disease in the skin similar to the wild type strain, comparatively few mice (5/30) had evidence of latent infection. When the parental strain (SC16) was inoculated at the same dose, latent infections were produced in all animals. The low incidence of latency produced by the resistant inoculum probably reflects the fact that approximately 95% of the inoculum consisted of TK-defective viruses.

In conclusion, these data suggest that TK-defective variants of HSV, perhaps present as a small proportion in a mixture of viruses, are likely to have a role in the chemotherapy of recurrent HSV. However, it should not be forgotten that other mechanisms are available to the virus to enable it to accomplish resistance apparently without the accompanying disadvantage to the virus of greatly impaired infectivity in vivo, for example by changed substrate specificity of TK or changes in the HSV DNA polymerase. We should thus remain alert for all kinds of resistant virus emerging in clinical specimens obtained from infections associated with chemotherapy.

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