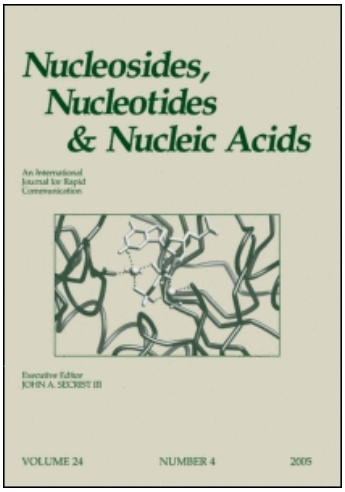

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EARLY THERAPY WITH VALACICLOVIR OR FAMCICLOVIR REDUCES BUT DOES NOT ABROGATE HERPES SIMPLEX VIRUS NEURONAL LATENCY

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"Dedicated to the memory of Dr. Gertrude B. Elion"

ABSTRACT

Mice were infected via the ear pinna using a recombinant strain of HSV-1 expressing the β-gal gene under the LAT promoter. Mice were treated continuously with valaciclovir or famciclovir, from 1 day before or 1 day after virus inoculation for 10 days. Ipsilateral and contralateral trigeminal and cervical ganglia were later assessed by co-cultivation or for X-Gal-positive or LAT-positive neurons. Latency was markedly reduced by early therapy, however, a basal level of HSV-1-positive neurons was detected in all mice.

INTRODUCTION

It is well-established that chemotherapy using conventional nucleoside analogues such as acyclovir does not reduce or eliminate established foci of latent infection in experimental animal models [1-4]. In a particularly exhaustive study, it was reported that ACV failed to eliminate HSV from rabbits infected via the cornea despite continuous intensive therapy during experimental reactivations of HSV-1 [5].

We have previously reported the effects of the oral prodrugs valaciclovir (VACV) and famciclovir (FCV) on the pathogenesis of HSV-1 and HSV-2 in the murine ear pinna infection model [6,7]. Our results confirmed that there was no effect on latent infections that had been established for several months. However, when therapy was initiated within two or three days of virus inoculation, in several papers we demonstrated that both FCV and VACV appeared to

reduce the establishment of latency when this was tested by explant co-cultivation of the dorsal root or trigeminal ganglia [8,9]. Furthermore, we obtained evidence for a superiority of FCV over VACV in this model. We showed, however, that when more sensitive methods were applied, e.g. disaggregation of ganglia followed by infectious center assay or *in situ* hybridization, small numbers of virus-positive neurons were observed in all ganglia even when therapy commenced as early as one day p.i. [10]. In the present study this work is extended such that continuous therapy is commenced before virus inoculation and continued throughout the acute phase of virus infection. Mice were infected with a recombinant virus containing a β-galactosidase (β-gal) reporter gene under the LAT promoter; X-Gal staining and *in situ* hybridization for latency-associated transcripts (LATs) are used to detect virus-containing neurons.

RESULTS AND DISCUSSION

Effects of Chemotherapy on Clinical Signs During Acute Infection The pathogenesis of the recombinant virus, LβA following ear inoculation at 10^5 p.f.u./mouse was similar to the parental strain, SC16. All mice developed clinical signs (redness and paralysis of the ear pinna) and 65% of LβA-inoculated mice died during the period 5-8 days p.i. Mice inoculated with SC16 showed similar clinical signs and 30% died during the acute phase of the infection (**FIG. 1**). Addition of FCV or VACV to the drinking water at 1mg/ml from one day before, or one day after LβA inoculation up to day 10 p.i., completely prevented mortality and no mice died in any of the treatment groups (**FIG. 1**). The degree of swelling in the virus-inoculated ear pinna induced by infection with LβA was similar to that induced by SC16 (**FIG. 2**). The swelling reaction was markedly reduced in groups of mice treated with either drug from the day before virus inoculation, but little effect was observed in the groups where the start of treatment was delayed until 1 day p.i. There was no significant difference between the effects of the two antiviral compounds on the parameters of acute infection.

Infectious Virus in the Ear Pinna Consistent with the results for acute clinical signs, the virus titres detected in ear pinnae for SC16 and LβA on day 5 p.i. were similar (5.7 Log_{10}). Virus titres in the ear pinnae were reduced by 1-2 Log_{10} in LβA -infected, treated mice. Virus was detected less consistently in the left trigeminal ganglion and 2/3 ganglia from untreated SC16-inoculated and 2/3 trigeminal ganglia from recombinant-inoculated mice were positive for virus. However, no infectious virus was detected in any of 6 mice treated with FCV and only 1/6 mice treated with VACV (**TABLE 1**).

Detection of Latently Infected Ganglia by Means of Explant Co-cultivation Groups of mice were euthanased on day 40, 106 and 300 p.i. and their left and right trigeminal and third cervical

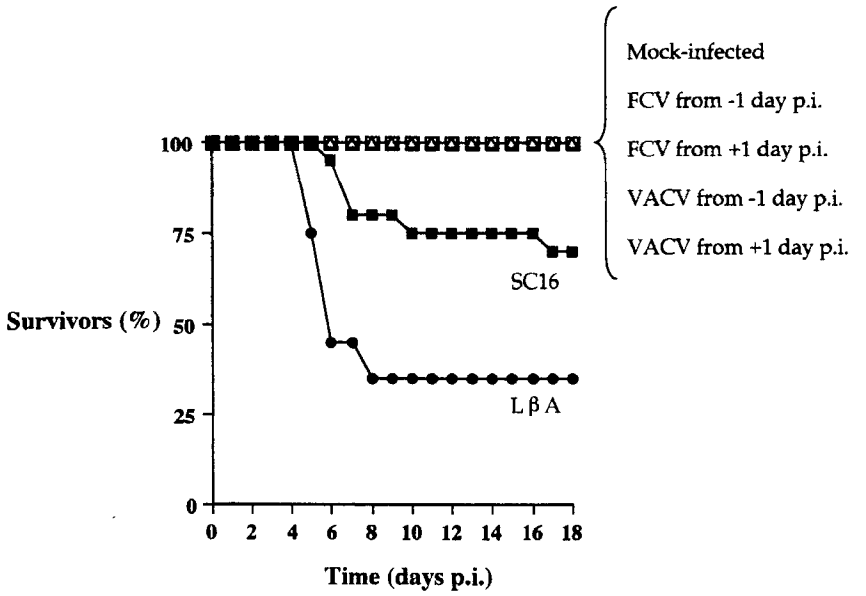

Figure 1. Percentage survival of BALB/c female mice following inoculation of 10^5 p.f.u. of wild type SC16 or the recombinant virus LβA into the left ear pinna. LβA-infected mice were treated with either FCV or VACV at 1 mg/ml in the drinking water from one day before (-1) or one day after (+1) virus inoculation. Therapy was continued until the end of day 10 p.i. Mortality was assessed daily from day 0 to 18 p.i. using groups of 20 mice.

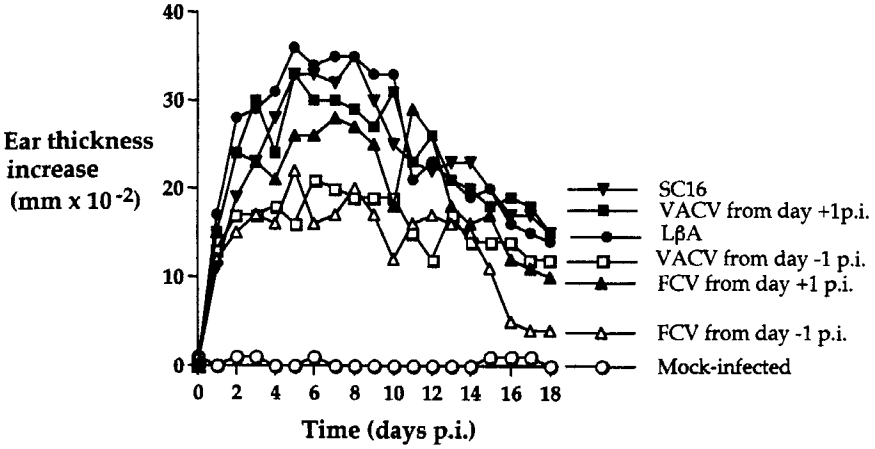

Figure 2. Effect of antiviral therapy on virus-induced inflammation in the ear pinnae measured by means of ear thickness. LβA-infected mice were treated with either FCV or VACV at 1 mg/ml in the drinking water from one day before (-1) or one day after (+1) virus inoculation. Therapy was continued until the end of day 10 p.i. Eight mice were measured at each time point.

Table 1 Infectious Virus Titres on Day 5 p.i.

Group/Tissue	Left Ear Pinna	[d] Left TG	[e] BS
SC16 controls	[a] 5.7 ± 0.1 [b] (3/3)	1.4 ± 0.9 (2/3)	4.2 ± 0.6 (3/3)
LβA controls	5.7 ± 0.1 (3/3)	1.7 ± 1.2 (2/3)	4.7 ± 0.3 (3/3)
LβA + VACV from day +1 p.i.	3.6 ± 0.1 (3/3)	0.9 ± 0.8 (1/3)	2.5 ± 0.5 (3/3)
LβA + VACV from day −1 p.i.	3.6 ± 0.7 (3/3)	- (0/3)	2.4 ± 0.5 (3/3)
LβA + FCV from day +1 p.i.	4.4 ± 0.1 (3/3)	- (0/3)	3.6 ± 0.4 (3/3)
LβA + FCV from day −1 p.i.	[c] - (0/3)	- (0/3)	- (0/3)
Mock-infected	- (0/3)	- (0/3)	- (0/3)

a. Log_{10} values/tissue/group ± standard deviation

b. Number in brackets = number of mice positive/number of mice tested

c. - = below the level of detection for infectious virus i.e. <3 p.f.u./tissue (<0.5 Log_{10})

d. TG = trigeminal ganglia

e. BS = brain stem

ganglia were explanted and maintained in culture for 5 days. Ganglia were then homogenized and tested for the presence of infectious virus. The results (**TABLE 2**) show that a total of 43 from 60 ganglia (72%) from untreated LβA-infected mice were positive for virus compared with 37/60 (62%) for SC16. However, none of 240 ganglia from treated mice were positive for virus by this test.

Detection of Latently Infected Neurons by Means of In Situ Hybridization for LAT Ganglia were removed and sections prepared for *in situ* hybridisation on days 38 and 90 p.i. The *in situ* method proved to be a more sensitive indicator for latency and all ganglia examined (a total of 86 individual ganglia) contained at least 5 positive neurons per section examined. For left trigeminal ganglia the number was 92 and 100 positive neurons per section for LβA and 93 and

Assessment of latency using three different methods at 1,3 and 9 months post inoculation

Table 2		Conventional co-cultivation			In situ hybridization		β-gal^{+ve} neurons	
		day 40	day 106	day 300	day 38	day 90	day 38	day 92
SC16	LTG	[a] 4/5	[a] 3/5	[a] 2/5	[a] 5/5 [b] 93 ± 28	[a] 5/5 [b] 86 ± 20	NA	NA
No	LCIII	4/5	4/5	5/5	5/5 29 ± 8	5/5 37 ± 11	NA	NA
therapy	RTG	3/5	2/5	0/5	5/5 47 ± 5	ND	NA	NA
	RCIII	3/5	2/5	5/5	ND	ND	NA	NA
LβA	LTG	4/5	4/5	5/5	5/5 92 ± 31	5/5 100 ± 34	[a] 5/5 [b] 12 ± 6	[a] 5/5 [b] 15 ± 8
No	LCIII	4/5	4/5	5/5	5/5 28 ± 5	5/5 25 ± 10	5/5 2 ± 2	5/5 6 ± 5
therapy	RTG	3/5	3/5	0/5	5/5 46 ± 4	ND	0	0
	RCIII	3/5	3/5	5/5	ND	ND	ND	ND
VACV	LTG	0/5	0/5	0/5	3/3 51 ± 10	ND	0/5	4/5 11 ± 5
from day +1	LCIII	0/5	0/5	0/5	3/3 16 ± 1	ND	0/5	0/5
p.i.	RTG	0/5	0/5	0/5	3/3 28 ± 3	ND	0/5	0/5
	RCIII	0/5	0/5	0/5	ND	ND	ND	ND
VACV	LTG	0/5	0/5	0/5	3/3 28 ± 4	ND	0/5	0/5
from day -1	LCIII	0/5	0/5	0/5	3/3 18 ± 5	ND	0/5	0/5
p.i.	RTG	0/5	0/5	0/5	3/3 16 ± 5	ND	0/5	0/5
	RCIII	0/5	0/5	0/5	ND	ND	ND	ND
FCV	LTG	0/5	0/5	0/5	3/3 28 ± 6	ND	0/5	3/5 3 ± 3
from day +1	LCIII	0/5	0/5	0/5	3/3 14 ± 3	ND	0/5	0/5
p.i.	RTG	0/5	0/5	0/5	3/3 13 ± 5	ND	0/5	0/5
	RCIII	0/5	0/5	0/5	ND	ND	ND	ND
FCV	LTG	0/5	0/5	0/5	3/3 21 ± 6	ND	0/5	0/5
from day -1	LCIII	0/5	0/5	0/5	3/3 19 ± 6	ND	0/5	0/5
p.i.	RTG	0/5	0/5	0/5	3/3 5 ± 4	ND	0/5	0/5
	RCIII	0/5	0/5	0/5	ND	ND	ND	ND

a. Number of mice positive from group of 5 or 3 tested for latency.
b. Number of positive neurons per section (n=30; non-consecutive); mean ± SD
LTG = left trigeminal ganglia; RTG = right trigeminal ganglia
LCIII = left cervical dorsal root ganglia; RCIII = right cervical dorsal root ganglia
ND = not done; NA = not applicable

86 for SC16 on days 38 and 90 respectively (**TABLE 2**). Therapy with either compound appeared to reduce the number of LAT-positive neurons by 30-50% and there appeared to be little difference between ganglia from mice where therapy had commenced before or after virus inoculation.

Detection of Latently Infected Neurons by Means of X-Gal Staining Relatively few positive cells were detected by means of the reporter gene. All left trigeminal and left third cervical ganglia from untreated mice were found to contain a small number of positive staining neurons. The numbers were 12 and 2 respectively which correspond to 13% and 7% the number of LAT-positive neurons on day 38 p.i. and similar ratios on day 92 (15% and 24% respectively). Among the treated mice tested the only ganglia in which blue staining neurons were detected came from mice where treatment had been delayed to 1 day p.i. and 3/5 and 4/5 left trigeminal ganglia were positive by this test on day 92 p.i. (**TABLE 2**).

Water bottles were checked at least twice a day and the volume consumed by each group was measured in order to calculate the dose of drug which corresponded to 100-150 mg/Kg/24 h for all groups. There was no evidence for a reduction in consumption of drinking water by infected mice at any stage. We have shown previously that therapy by means of drinking water at 1 mg/ml is superior to oral gavage twice or three times a day in this model [11,12]. Our previous experience suggests that therapy by means of the drinking water is the most effective way to administer FCV or VACV to mice, although we have noted that the majority of fluid intake occurs during the night.

When treatment with either VACV or FCV was commenced 1 day before or 1 day after virus was inoculated into the ear pinna, none of the ganglia reactivated virus when they were tested by explant co-cultivation. This is consistent with previous publications by ourselves and others for early therapy [3,9,10,13,14].

When more-sensitive tests were employed, the ganglia obtained from mice from the same experimental groups were all found to contain neurons in which HSV could be detected although the number of positive neurons were significantly fewer than seen in ganglia from untreated controls. However, there does not appear to be a simple relationship between the minimum number of neurons scoring positive by *in situ* hybridization and the positive outcome of explant cultivation. For example, five ganglia with a mean of 28 LAT-positive neurons per section and 5 with a mean of 29 both came from groups in which 5/5 mice scored positive by explant culture two days later. By contrast other groups with 28, 28, 28 and 51 mean LAT-positive neurons from groups that had been treated with FCV or VACV gave 0/12 when tested by co-cultivation. This suggests that some HSV-positive neurons from drug-treated animals which contain LATs may differ from those obtained from untreated controls in the other respects, most probably in the number of HSV DNA genome copies per cell [15].

The neurons of latently infected mice have previously been shown to express β-gal under the LAT promoter for many months after virus inoculation [16]. This was confirmed in the present study by the detection of β-gal positive ganglionic neurons at 92 days p.i. However, fewer (approx. 10%) neurons scored positive for β-gal than by the LAT *in situ* method at all time points tested thus the latter technique appeared to be a more sensitive test for latency although the detection of the reporter gene product is much quicker and less labour intensive. Furthermore, all ganglia from treated mice were negative for latency as judged by the 5-day explant culture test. Further studies and a wider range of animal models may be required to determine which of the three methods for quantifying latency following chemotherapy correlates best with the more functional tests such as spontaneous or induced reactivation *in vivo*.

The implication from these results is that, while early therapy may reduce the number of latently-infected neurons, it is extremely difficult to completely prevent the establishment of latent foci under ideal experimental conditions. This may be the result of direct uptake of virus into peripheral nerve endings from the inoculum and virus released into the skin due to incomplete inhibition of virus replication *in vivo*. It may be even more difficult to prevent the establishment of latency when highly efficient methods of virus inoculation are employed such as the application of high titre virus to the cornea of the eye [17].

It is interesting to compare our data on the protective effects of therapy with those recently published by LeBlanc et al [17]. These workers observed no difference between the effects of FCV and VACV on the quantity of HSV DNA in latently infected trigeminal ganglia. However, there were no survivors in the groups not receiving therapy so no ganglia were available to compare with those obtained from treated mice. The experiment involved high titre virus (10^6 p.f.u./mouse) applied bilaterally direct to the cornea that resulted in rapid and high mortality (100%) by day 5 p.i. without therapy and some mortality despite therapy. Furthermore, therapy was discontinued on day 7 p.i. at which time virus reached peak titre in the brains of treated animals. This method of inoculation would favour direct uptake of virus into the axons and transfer to the ganglionic neurons prior to initiation of therapy (which commenced from day 1 p.i.) and the establishment of unamplified latency as described by Simmons et al [18]. Therefore, the effects of therapy in this ocular model would be expected to be less, and potential differences between the two drugs may have been obscured.

Generally, the results from animal models appear to be consistent with clinical data from several human trials. For example, in a multicenter study reported by Peacock et al [19], 45 patients with primary genital HSV-2 infections, who had had lesions for <7 days, were given 5mg/kg acyclovir (ACV) or placebo intravenously 3 times/day for 5 days. No differences were seen between the placebo and ACV-treated groups for the proportion of patients with a recurrence, the time to recurrence or, the mean monthly incidence of recurrences subsequently.

Thus, the implication of our results is that even intensive antiviral therapy starting within a few hours of exposure is unlikely to completely abrogate latency. However, our results also show a significant reduction in the number of latent foci that are established and imply that there may also be a quantitative reduction in the latent genomes. If this were to occur in man, therapy during the first few days of the infection may significantly reduce the burden of latency and may, in turn reduce the incidence of recurrent disease.

EXPERIMENTAL

Virus Strains HSV-1 strain SC16 has been used previously to study antiviral therapy in the murine model [6]. A recombinant strain of HSV-1 SC16, (LβA) [16] contains the lac-Z reporter gene under the LAT promoter. This virus shows equal sensitivity to antiviral agents in tissue culture when compared with wild type SC16. Following infection at a peripheral site, ganglionic neurons were shown to express β-gal for several months after inoculation [16]. Both viruses were propagated in BHK-21 cells using standard methods.

Mice and Virus Inoculation Mice were inoculated sub-cutaneously in the skin of the left ear pinna using previously published methods [6].

Antiviral Compounds and Treatment Regimen FCV and VACV were supplied by SmithKline Beecham (Brentford, United Kingdom). The compounds were dissolved in tap water at a final concentration of 1 mg/ml and supplied in the drinking water commencing either 24h before, or 24h p.i. The actual dose was estimated daily based on the consumption of drinking water. Treatment was terminated on day 10 p.i.

Clinical Signs Mice were visually assessed each day from day 1 to 18 p.i. using methods that have previously been described in detail [3].

Titration of infectious virus in tissue samples Left ear pinna, left trigeminal ganglia or brain stem samples were collected from 3 mice per treatment group on day 5 p.i. only. Each tissue sample was homogenized and titrated independently for infectious virus by a plaque assay [6].

Detection of Latent Virus in the Ganglia by Explant Culture Five mice per group were tested for the presence of latent virus. The left (ipsilateral) and right (contralateral) trigeminal ganglia and left and right cervical dorsal root ganglia (CIII) were removed on days 40, 106 and 300 p.i. Trigeminal and cervical dorsal root ganglia were placed in 1ml or 0.5ml Eagle's minimum essential medium (EMEM) respectively, in 2ml glass vials with loose lids, and incubated at 37°C for 5 days. The ganglia were then tested for infectious virus [9].

Detection of Latently Infected Neurons by In Situ Hybridization Probes for the detection of LATs were generated by T7 polymerase transcription of Hind III-linearized pSLAT2 with a

digoxigenin (DIG) detection system [20]. Sections from mice inoculated with SC16 (wild type) showed positive cells containing brown stain confined to the nucleus. By contrast, sections of ganglia from LβA-infected mice contained positive staining mainly in the cytoplasm as reported for this strain by Lachmann & Efstathiou, 1997 [16]. The signal distribution and intensity varied among individual neurons and there was some block-to-block variation. However, neurons, which contained signal clearly above the background level, were readily distinguished and recorded as LAT-positive. Every *in situ* hybridization test with the LAT probe included RNase- and DNase-treated sections to control against spurious staining artifacts.

Groups of 5 mice (infected, untreated controls) or 3 mice (infected, treated mice), were sampled on days 38 and 90 p.i. and tested for the presence of LATs. Left and right trigeminal ganglia and left CIII ganglia were tested from all groups on day 38 p.i. On day 90 p.i. only untreated control mice (SC16 and LβA) remained and their left ganglia only were tested.

Detection of Latency by Means of β-gal Activity. Ganglia (left and right trigeminal and left CIII), were fixed in 2% paraformaldehyde-0.2% glutaraldehyde in PBS for 1h on ice. Samples were then transferred to detergent solution (0.01% sodium deoxycholate, 0.02% Nonidet P-40, 2 mM $MgCl_2$ in PBS) and incubated on ice for 30 min, after which they were transferred to X-Gal solution (detergent solution containing 4.5 mM potassium ferricyanide, 4.5 mM potassium ferrocyanide, and 1 mg of X-Gal per ml), and incubated at 37°C for 6 h. Following staining, ganglia were rinsed in PBS and clarified by immersion in 20% glycerol in PBS for 2 h at +4°C and then 50% glycerol overnight at 4°C. The next day all samples were placed in 80% glycerol for 2 h and then 100% glycerol at 4°C. Ganglia were mounted under a coverslip and, initially the number of blue cells was determined microscopically in intact ganglia. After the number of blue neurons had been counted, ganglia were embedded in paraffin wax and serial 5 μm sections were cut. The sections were examined under a light microscope, and the number of positive neurons was determined per ganglionic section (approx. 30 random sections were counted for each ganglion). Five mice per group (except SC16-infected, untreated control mice), were sampled on days 38 and 92 p.i. and tested by X-Gal staining.

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REFERENCES

1. **W. A. Blyth, D. A. Harbour & T. J. Hill,** *J. Gen. Virol* (1980) **48,** 417-419.
2. **G. Darby & H. J. Field,** *Pharmacol. & Therapeut* (1984) **23,** 217-251.

3. **H. J. Field, S. E. Bell, G. B. Elion, A. A. Nash & P. Wildy,** *Antimicrob. Agents Chemother* (1979) **15**, 554-561.

4. **R. J. Klein, E. DeStefano, A. E. Friedman-Kein & E. Brady,** *Antimicrob. Agents Chemother* (1981) **19**, 937-939.

5. **A. B. Nesburn, D. E. Willey & M. D. Trousdale,** *Proc. Soc. Exp. Biol. Med* (1983) **172**, 316-323.

6. **H. J. Field, D. Tewari, D. Sutton & A. M. Thackray,** *Antimicrob. Agents Chemother* (1995) **39**, 1114-1119.

7. **A. M. Thackray & H. J. Field,** *Antimicrob. Agents Chemother* (1996) **40**, 846-851.

8. **H. J. Field & A. M. Thackray,** *Int. Antivir. News* (1996) **4**, 23-27.

9. **A. M. Thackray & H. J. Field,** *J. Infect. Dis* (1996) **173**, 291-299.

10. **A. M. Thackray & H. J. Field,** *Antimicrob. Agents Chemother* (1998) **42**, 1555-1562.

11. **H. J. Field & A. M. Thackray,** *Antivir. Chem. Chemother* (1997) **8(1)**, 59-66.

12. **H. J. Field & A. M. Thackray,** *Antivir. Chem. Chemother* (1995) **6(4)**, 210-216.

13. **H. J. Field & E. De Clercq,** *J. Gen. Virol* (1981) **56**, 259-265.

14. **R. J. Klein, A. E. Friedman-Kein & E. DeStefano,** *Antimicrob. Agents Chemother* (1979) **15**, 723-729.

15. **N. M. Sawtell, D. K. Poon, C. S. Tansky & R. L. Thompson,** *J. Virol* (1998) **72**, 5343-5350.

16. **R. H. Lachmann & S. Efstathiou,** *J. Virol* (1997) **71**, 3197-3207.

17. **R. A. LeBlanc, L. Pesnicak, M. Godleski & S. E. Straus,** *J. Infect. Dis* (1999) **180**, 594 599.

18. **A. Simmons, B. Slobedman, P. Speck, J. Arthur & S. Efstathiou,** *J. Gen. Virol* (1992) **73**, 1287-1291.

19. **J. E. Peacock Jr, L. G. Kaplowitz, P. F. Sparling, D. T. Durack, J. W. Gnann Jr, R. J. Whitley, M. Lovett, Y. J. Bryson, R. J. Klein, A. E. Friedman-Kein et al,** *Am. J. Med* (1988) **85**, 301-306.

20. **J. Arthur, S. Efstathiou & A. Simmons,** *J. Gen. Virol* (1993) **74**, 1363-1370.