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The effect of acyclovir on the acute and latent murine gammaherpesvirus-68 infection of mice

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Summary

Mice inoculated intranasally with murine gammaherpesvirus-68 were used to evaluate the efficacy of acyclovir (ACV) in the treatment of acute and latent infections. Effectiveness was measured by infectious virus assay of the lung (site of active replication) and infectious centre assay of spleen cells (site of latency). Intraperitoneal administration of ACV at 6-h intervals starting soon after inoculation was more effective in reducing infectious virus in the lung than was treatment with 12-hourly injections commencing 3 days post-infection. Further, ACV treatment during acute infection resulted in an approximately 10-fold reduction in the number of infectious centres in the spleen as compared to placebo-treated animals. However, once latency was established, ACV treatment was not effective in reducing the number of infectious centres in the spleen. This is the first report demonstrating that ACV can be used to minimize the replication of murine gammaherpesvirus in mice at the site of primary infection, resulting in a reduction in the number of latently infected spleen lymphocytes.

Introduction

Murine herpesvirus-68 (MHV-68), a naturally occurring murid herpesvirus isolated from small rodents in Czechoslovakia, has been shown to be pathogenic in laboratory mice (Blaskovic *et al.*, 1980). Based on the structure of the viral genome and limited sequence analysis, MHV-68 has been shown to be closely related to Epstein–Barr virus (EBV) and herpesvirus saimiri (HVS) (Efstathiou *et al.*, 1990a,b). Primary infection of Balb/c mice has shown the lung to be the main tissue productively infected by MHV-68, with virus present in alveolar epithelium and mononuclear cells (Sunil-Chandra *et al.*, 1992a). We have established that *in vivo* the virus persists in spleen B-lymphocytes in a latent form analogous to EBV and related gamma-1 herpesviruses (Sunil-Chandra *et al.*, 1992b).

Amongst the various antiherpetic agents studied, acyclovir (ACV) has been reported to be an effective and extremely selective agent for several herpesviruses both *in vitro* and *in vivo* (Elion *et al.*, 1977; Schaeffer *et al.*, 1978; Field *et al.*, 1979; Miller and Miller, 1980, 1982). Oral and intraperitoneal administration of ACV was effective in controlling the acute viral replication that followed experimental herpes simplex virus type 1 (HSV-1) infection in mice (Field and De Clercq, 1981). These studies revealed that prompt treatment with ACV could prevent the establishment of latency, but once HSV-1 latency was established the virus could not be eliminated despite continuous therapy. In addition, ACV could be used to suppress the active phase of primary or recurrent HSV infection, thus preventing the establishment of latency in previously uninfected cells (Field and De Clercq, 1981).

ACV has been reported to arrest the replication of EBV *in vitro* (Pagano *et al.*, 1983), and inhibits virus shedding in patients with acute IM and in elderly patients carrying the virus (Miller *et al.*, 1973; Yao *et al.*, 1989a,b). Furthermore, ACV can inhibit EBV replication in superinfected Raji or EBV virus producer lymphoblastoid cell lines (Datta *et al.*, 1980; Colby *et al.*, 1981). These studies have shown that ACV can be used to distinguish between latent and chronic persistence and is therefore an important tool in studying the mechanisms of persistence of herpesviruses. Apart from the effective and widespread clinical use of ACV in controlling productive alphaherpesvirus infections (Falcon, 1983; Fiddian *et al.*, 1983; Mindel and Sutherland, 1983), clinical trials of ACV in the treatment of EBV-associated disease have had limited success (Pagano *et al.*, 1983). For example, oral hairy leukoplakia in AIDS patients can be treated effectively by ACV, but the drug has no beneficial effect on EBV-associated lymphoproliferative disorders (Resnick *et al.*, 1988; Zutter *et al.*, 1988; Pirsch *et al.*, 1989). Due to a lack of suitable animal models to study gammaherpesvirus infections it has become difficult to develop a rational approach for the antiviral chemotherapy of EBV-associated diseases.

In this paper, we study the effect of ACV on acute and latent MHV-68 infections of Balb/c mice.

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Results

Effect of ACV on the growth of MHV-68 in vitro

To determine whether ACV is effective against MHV-68 *in vitro*, a plaque reduction assay was performed on BHK cells. The results clearly indicate that the virus was sensitive to ACV and had an ED_{50} of 0.2 $\mu g\,ml^{-1}$.

ACV treatment of mice during acute infection with MHV-68

Fifty female BALB/c mice aged 3–4 weeks were inoculated intranasally with 4×10^5 pfu MHV-68. 25 mice were given ACV by intraperitoneal injections (70 $mg\,kg^{-1}\,day^{-1}$) at 12-h intervals for 10 days, starting on day 3 post-infection. The other 25 mice were placebo-treated. Three mice were killed on days 3, 5, 7 and 10 post-infection, and tissues were removed from both ACV-treated and placebo-treated groups. The spleen cells from each mouse were separated into adherent cell, B-lymphocyte and T-lymphocyte-enriched fractions and assayed by co-cultivation to determine the number of infectious centres. In the lung, the infectious virus titre was determined by direct tissue homogenization plaque assay. The results (Table 1) show that the number of infectious centres in the spleen was 10-fold lower in mice treated with ACV than in placebo-treated animals. However, this protocol of ACV treatment was less effective at reducing virus in the lung, with only a 1.3 log_{10} pfu difference seen at day 10 post-infection when compared to placebo-treated mice (Table 2). It appears that ACV therapy from day 3 onwards may have little effect on lung virus titres.

In order to obtain efficient clearance of infectious virus from the lungs, a separate experiment was carried out with another batch of mice infected with MHV-68. 25 of these mice were treated intraperitoneally with ACV (70 $mg\,kg^{-1}\,day^{-1}$), starting 6 h post-infection, at 6-h intervals for 8 days. Three mice were sacrificed on each of days 3, 5, 7 and 10 post-infection, and lungs were removed for the assay for infectious virus. Spleens were removed from these mice and the number of infectious centres per spleen was determined at days 3 and 5. The results (Table 3) show a 100- to 1000-fold reduction of infectious virus in lungs sampled at 7 days post-infection. Further, a 10-fold reduction in the number of infectious centres in the spleen was obtained at 5 days post-infection using this treatment regime (Table 4).

The effect of ACV treatment during latent infection

Five of 10 clinically normal MHV-68 infected mice at 35 days post-infection were treated with ACV for 5 days. The drug was administered orally *ad lib* in drinking water at a concentration of 1 $mg\,ml^{-1}$. The average daily consumption for a mouse was 3.2 $ml\,day^{-1}$, and hence the rate of consumption was 160 $mg\,kg^{-1}\,day^{-1}$. The other five mice were placebo-treated and used as control animals. In order to determine whether the virus persists as latent

Table 1. Effect of ACV treatment of mice on the recovery of MHV-68 from spleen cells by co-cultivation

		ACV-treated mice				Placebo-treated mice			
			Infectious centres per 10^7 spleen white cells				Infectious centres per 10^7 spleen white cells		
Days p.i.	Mouse no.	Total no. spleen white cells ($\times 10^7$)*	B	T	PA	Total no. spleen white cells ($\times 10^7$)*	B	T	PA
3†	1		–	–	–	2	0	0	0
	2		–	–	–	3	0	0	0
	3		–	–	–	2	0	0	0
5	1	3	0	0	0	1	0	0	0
	2	3	0	0	0	2.5	0	0	0
	3	4	0	0	0	3	0	0	0
7	1	2.5	2	0	2	2.5	36	0	2
	2	3	9	2	0	4	53	0	10
	3	1.2	0	0	0	3	42	0	8
10	1	3.1	36	0	7	4.3	254	14	65
	2	3.4	74	1	16	2.5	1096	1	432
	3	3.1	176	4	58	5.5	558	4	196

B: B-cell-enriched fraction (Ig+ spleen cells).
T: T-cell-enriched fraction [spleen cells not adherent to anti-IgG (H+L) coated plates].
PA: plastic adherent cells (non-specifically adherent to uncoated plates).
* On the basis of counts of the total number of spleen white cells, of plastic non-adherent cells and subsequently of cells not adherent to antibody-coated plates (T-cell-enriched fraction), approximately 1/3 of the total number of spleen cells were B, T or PA cells.
† ACV treatment of mice started at day 3 post-infection at 70 $mg\,kg^{-1}\,day^{-1}$ as two 12-hourly intraperitoneal injections.

Table 2. Effect of ACV treatment of mice on infectious virus titres in lung tissue during acute infection

		Infectious virus titre (log_{10} pfu $lung^{-1}$)	
Days post-infection	Mouse no.	ACV-treated mice	Placebo-treated mice
3*	1	–	5.7
	2	–	5.7
	3	–	5.8
5	1	5.3	6.3
	2	5.2	5.6
	3	5.9	6.2
7	1	4.2	5.0
	2	4.4	5.9
	3	4.7	5.9
10	1	1.0	2.3
	2	1.0	1.3
	3	<0	2.6

* ACV treatment of mice started at 3 days post-infection at 70 mg kg^{-1} day^{-1} given as two 12-hourly intraperitoneal injections.
– ACV treated mice were not assayed on the day of treatment (day 3 post-infection).

Table 3. Infectious virus titre in lung tissue following intensive ACV treatment

		Infectious virus titre (log_{10} pfu $lung^{-1}$)	
Days post-infection	Mouse no.	Placebo-treated	ACV-treated*
3	1	5.8	4.0
	2	5.8	4.8
	3	6.2	5.0
5	1	6.7	6.3
	2	6.4	5.9
	3	6.5	6.2
7	1	8.2	4.7
	2	6.6	4.4
	3	7.3	4.6
10	1	5.8	2.5
	2	4.0	2.3
	3	3.9	<2.0

* ACV treatment started 6 h after infection and involved 6-hourly intraperitoneal injections (0.1 ml) of ACV (70 mg ACV per kg weight) per mouse with an average weight of 20 g, and continued for 8 days.

Table 4. Number of infectious centres in spleens of infected mice following intensive ACV treatment

Days post-infection	Mouse no.	Cells per spleen	Infectious centres per spleen (ACV-treated*)	Cells per spleen	Infectious centres per spleen (placebo-treated)
3	1	3.3×10^7	0	4.3×10^7	0
	2	5.3×10^7	0	3.3×10^7	0
	3	5.9×10^7	0	3.5×10^7	0
5	1	3.8×10^7	4	5.5×10^7	62
	2	7.3×10^7	6	5.9×10^7	30
	3	4.7×10^7	6	7.0×10^7	52

* ACV treatment started 6 h after infection and involved 6-hourly i.p. injections of (0.1 ml) of ACV (70 mg ACV per kg weight) per mouse with an average weight of 20 g, and continued for 8 days.

Table 5. Effect of ACV treatment of mice on the recovery of MHV-68 from spleen cells assayed by co-cultivation*

	Infectious centres per 10^7 spleen white cells**					
	ACV-treated mice†			Placebo-treated mice		
Mouse no.	B	T	PA	B	T	PA
1	119	0	10	101	1	4
2	35	0	2	35	1	2
3	53	0	1	71	1	6
4	127	1	5	81	0	6
5	34	0	4	48	0	6

B: Ig+ spleen cells (B-cell-enriched fraction).
T: spleen cells not adherent to anti-IgG (H+L) coated plates (T-cell-enriched fraction).
PA: plastic adherent cells (non-specifically adherent to uncoated plates).
* Co-cultivation of spleen cell subsets started at 40 days post-infection in ACV-treated or untreated mice.
** Mean number of total spleen white cells per spleen was $6 \pm 0.7 \times 10^7$. On the basis of counts of the total number of spleen white cells, of plastic non-adherent cells and subsequently of cells not adherent to antibody-coated plates (T-cell-enriched fraction), approximately 1/3 of the total number of spleen cells were B, T or PA cells.
† At 35 days post-infection mice were treated with ACV in drinking water (concentration of 1 mg ACV ml^{-1}) for 5 days.

virus in the B-cell-enriched spleen cell population, both ACV-treated and placebo-treated mice were killed, and spleen cells were separated into adherent, B-lymphocyte and T-lymphocyte-enriched fractions and assayed for recovery of infectious centres. The results (Table 5) show that the number of infectious centres obtained from treated mice did not differ from that obtained from placebo-treated mice. This indicates that ACV has no effect on MHV-68, which persists mainly in the B-cell fraction at 35 days post-infection, and suggests a latent as opposed to a chronic infection in this cell population.

A separate experiment was carried out with a group of 30 mice to determine the effects of prolonged ACV treatment during latent MHV-68 infection. Mice were infected with the virus and ACV treatment was given to 15 of them at 10 days post-infection. The drug was administered orally *ad lib* in drinking water that contained 1 mg ml^{-1} ACV, and the treatment was continued for 90 days. Three mice from each of the ACV-treated and placebo-treated groups were killed at 25, 50 and 100 days post-infection. Spleens from each of the mice killed at 25 and 50 days post-infection were assayed for infectious centres. The results clearly indicate that ACV cannot cure the latent infection in spleens (Table 6). It has been shown previously (Sunil-Chandra *et al.*, 1992a) that a low level of virus can be recovered from the lungs of latently infected mice only by homogenization and assay of co-cultivated lung tissue. The direct homogenization and assay of lung without co-cultivation with BHK cells has consistently failed to recover infectious virus from latently infected mice (Sunil-Chandra *et al.*, 1992a). In order to determine whether this low-level persistence of virus in the lungs of recovered animals is due to chronic low-level replication or to the presence of latently infected cells, lungs removed from these mice at 50 and 100 days post-infection were co-cultivated for 5 days with BHK cells, homogenized and assayed for the recovery of infectious virus. One-third of the mice treated with ACV and 2/3 of the placebo-treated mice had 10^3 plaque-forming units (pfu) 'amplified' infectious virus at 50 days post-infection, and

Table 6. Effects of prolonged ACV treatment during latent infection

		ACV-treated*		Placebo-treated	
Days p.i.	Mouse no.	Spleen cells	Infectious centres per spleen	Spleen cells	Infectious centres per spleen
25	1	1×10^8	230	1×10^8	590
	2	7.5×10^7	560	9×10^7	450
	3	9.4×10^7	420	8.5×10^7	640
50	1	8.5×10^7	550	9×10^7	470
	2	1.1×10^8	590	1×10^8	460
	3	1.1×10^8	480	1×10^8	587

* ACV treatment started at 10 days post-infection, with 1 mg ml^{-1} ACV in drinking water for 40 days.

'amplified' infectious virus at 50 days post-infection, and 1/3 of the mice from both groups had 10^3pfu 'amplified' infectious virus at 100 days post-infection. These results suggest that there are no differences in the 'amplified' infectious virus titres obtained from the lungs of ACV-treated and placebo-treated mice, indicating that the likely mode of MHV-68 persistence in the lung is that of latency rather than low-level chronic replication. However, rather marginal effects of drug treatment on the lung in the acute phase indicate that more effective drugs are needed to confirm this observation.

Discussion

ACV has been widely used in the treatment of HSV infections in man. This antiviral compound is also effective *in vitro* and *in vivo* against the gammaherpesvirus EBV (Yao *et al.*, 1989a,b; Pagano *et al.*, 1983). In contrast, another gammaherpesvirus, herpesvirus saimiri, is not sensitive to ACV, indicating differences in viral TK or polymerase activity within this family of herpesviruses (Honess *et al.*, 1982; Kit, 1985). It was therefore of interest to examine the sensitivity of MHV-68 to this compound, particularly as the genome structure of this virus is more closely related to HVS and other members of the gamma-2 herpesvirus subfamily (Efstathiou *et al.*, 1990a,b). MHV-68 was sensitive to ACV *in vitro* with an ED_{50} in BHK cells of $0.2\,\mu g\,ml^{-1}$.

The ability of ACV to reduce MHV-68 replication in the lungs of mice was disappointing. When the compound was administered from 3 days post-infection, relatively little difference was observed in the virus titres recovered from ACV-treated and placebo-treated mice. In this study, a 12-h interval between ACV injections almost certainly produced sub-optimal concentrations of the drug in the lung, and this might have influenced the clearance of virus from this organ. To overcome this and to increase the chance of restricting viral replication in the lung, ACV treatment was undertaken every 6 h, starting 6 h post-infection. In this instance, a clear reduction in infectious virus titres was observed by day 7 in the treated groups, although there was no difference observed at early times post-infection. These data contrast with studies on HSV-1-infected mice (Field *et al.*, 1984). Such animals were fully protected from disease, provided that the therapy commenced at the same time as virus inoculations; no protection was observed when treatment started on day 3 post-infection or later. These studies also highlight the effectiveness of oral dosing via drinking water to deliver the compound.

In contrast to the effect of ACV on MHV-68 replication in the lung, treatment of mice during a primary infection dramatically reduced the number of infectious centres in the spleen. This indicates that ACV chemotherapy of MHV-68-infected mice early in the acute infection could be used to reduce the establishment of latent infection in B-lymphocytes. However, ACV had no effect on the number of infectious centres that could be recovered from spleen B-cells obtained from latently infected mice 40 days post-infection. Furthermore, a prolonged treatment of ACV had no effect on the number of infectious centres recovered from spleens at 25 and 50 days post-infection. This provides strong evidence that MHV-68 establishes latent infection of B-cells *in vivo* (Sunil-Chandra *et al.*, 1992b), and that ACV is unable to 'cure' a latent infection. This observation is supported by experiments in which ACV failed to eliminate a latent MHV-68 infection in NSO myeloma cells (murine B cell line) *in vitro* (Sunil-Chandra *et al.*, 1993). These findings on the failure of this compound to eliminate a latent MHV-68 infection are consistent with observations of the persistence of HSV latency in the face of continued ACV therapy (Field and De Clercq, 1981).

Although the disease associations of B-cells latently infected following MHV-68 infection have not been studied in detail, in man B-cells latently infected with EBV are associated with lymphoproliferative disorders or lymphomas in transplant patients, AIDS patients and patients with Burkitt's lymphoma. In a long-term study, we have observed a lymphoproliferative disease in 9% of mice latently infected with MHV-68, 50% of which were judged to have high-grade lymphomas (N.P. Sunil-Chandra and A.A. Nash, unpublished observation). We are currently investigating the transforming properties of this virus in mice and the use of antiviral chemotherapy in the control of lymphoproliferative disease. Studies on the chemotherapy of MHV-68-infected mice early in the primary infection may therefore provide useful information for devising strategies to prevent the establishment of EBV latency, with the aim of reducing the incidence of lymphoma.

The similarity in the biological properties of MHV-68 and EBV suggests that this new murine model of gammaherpesvirus infection may be of value in testing new-generation antiherpes drugs with activity against EBV. In particular, it will be important to know whether treatment during a primary infection can influence the number of latently infected B-cells and hence lymphoproliferative disease.

Materials and Experimental procedures

Mice

Unless otherwise stated, all the animal experiments were carried out using female Balb/c mice obtained from Bantin and Kingman (Grimston, Aldbrough, Hull, UK) and infected at age 3–4 weeks.

Virus

Murine herpesvirus-68 (MHV-68) was obtained from Professor Blaskovic, Slovakia (Blaskovic *et al.*, 1980) and the clone G2.4 was isolated in this department (Efstathiou *et al.*, 1990a). Virus working stocks were grown in BHK cells, and virus preparations were stored at –70 °C (Sunil-Chandra *et al.*, 1992a).

Intranasal inoculation of mice with virus

Mice were inoculated intranasally (i.n.) with 4×10^5 pfu of MHV-68. A volume of 40 μl was administered to lightly anaesthetized mice.

Separation of spleen lymphocyte subsets and adherent cells

Spleen B-cells, T-lymphocytes and adherent cells were enriched by panning on antibody-coated plates as described previously (Sunil-Chandra *et al.*, 1992b). Sheep anti-mouse IgG(H+L)-affinity purified antibody (Sera Lab Ltd, Sussex, England) was used to coat 60-mm tissue-culture-grade plastic Petri dishes (Becton Dickinson Labware, UK) for the positive selection of B-cells.

Plaque reduction assay for acyclovir

The concentration of ACV active against MHV-68-infected BHK cells was determined by the method described by Field and Darby (1980). Briefly, MHV-68 was diluted to give 200 pfu $(0.2\,ml)^{-1}$ and was allowed to adsorb for 1 h on 2×10^6 BHK cells per 60-mm plastic dish. These cells were cultured in 5 ml of Glasgow minimal essential medium (GMEM) containing various concentrations of acyclovir and 10% new-born calf serum. The percentage plaque reduction was calculated (by extrapolation) as a percentage of the value for control cultures incubated without acyclovir. The ACV concentration required for a 50% plaque reduction was considered an effective dose (ED_{50} value).

Acyclovir therapy of MHV-68-infected mice

Acyclovir [9-(2-hydroxy ethoxy methyl)-guanine] was received as a dry powder, and was a gift from Dr P. Collins (Wellcome Foundation, Beckenham, Kent). The protocols described by Field *et al.* (1979) and Field (1982) for the treatment of HSV infection of mice were followed in order to study the effect of ACV on MHV-68 replication in mice. For oral therapy, the drug was dissolved in drinking water to give a final concentration of $1\,mg\,ml^{-1}$. The cages contained groups of five mice provided with acyclovir in 250 ml of drinking water in a graduated bottle. Intraperitoneal (i.p.) administration of acyclovir was by injection of 0.2 ml ($3.5\,mg\,ml^{-1}$) of ACV in sterile distilled water twice daily at 12-h intervals for 10 days, or 0.1 ml ($3.5\,mg\,ml^{-1}$) of ACV in sterile distilled water at 6-h intervals daily for 8 days, to give 70 mg of the drug per kg body weight daily. Control animals were placebo-treated using phosphate-buffered saline.

Assay for infectious virus in lung tissues

Three mice were killed at appropriate time-points using a lethal intraperitoneal injection of Euthatal [pentobarbitone Sodium B.P. (Vet.)]. Lungs were removed and stored at –70 °C until required. Lungs were homogenized separately in 2 ml ETC_{10} (Eagles minimal essential medium supplemented with 10% new-born calf serum and 10% tryptose phosphate broth). The presence of infectious virus was determined by plaque assay on BHK cells. This was achieved by adsorbing three to five 10-fold dilutions (10^{-1}–10^{-5}) of each tissue homogenate with 2×10^6 BHK cells at 37 °C for 1 h and then incubating at 37 °C in a 5% CO_2 atmosphere for 4 days. Results were recorded as the number of plaque-forming units (pfu) per lung at each time-point.

Assay for infectious centres

Red cells were removed from spleen cell suspensions by water lysis, and either whole spleen white cells or enriched spleen cell subsets (separated on plastic dishes) were co-cultivated with permissive BHK cells to determine the number of infectious centres per spleen, as described previously (Sunil-Chandra *et al.*, 1992a,b).

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