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In vitro inhibition of human cytomegalovirus replication in human foreskin fibroblasts and endothelial cells by ascorbic acid 2-phosphate

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Abstract

Antiviral activity of L-ascorbic acid-2-phosphate (ASC-2P), a long-acting derivative of L-ascorbic acid, against several human cytomegalovirus (CMV) strains was examined in cultures of human foreskin fibroblasts (HFF) and endothelial cells (EC). ASC-2P at concentrations ranging from 0.2 to 2 mM had no effect on the number of cells expressing 72 kDa CMV immediate early antigen (IEA) while it inhibited expression of 68 kDa late antigen (LA) in infected cultures of both cell types (30% and 55% reduction for EC and HFF, respectively). In HFF cells, virus yield was reduced up to 4-fold, when ASC-2P was added after CMV infection. Antiviral effects were significantly increased in cultures pretreated with ASC-2P. In HFF and EC pretreated for three subcultures (18 days) with 0.2 mM ASC-2P, a significant reduction of cells expressing IEA (75% and 80% reduction in EC and HFF, respectively) and LA (92% and 90% reduction for EC and HFF, respectively) was observed. Pretreatment for three subcultures with ASC-2P inhibited virus yield 50- to 100- fold in EC and 100- to 1000-fold in HFF. The continuous presence of ASC-2P was not required for its antiviral activity. A significantly higher reduction of virus replication with ganciclovir and foscarnet was obtained in ASC-2P pretreated cells than in untreated controls. The

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results showed that ASC-2P provides L-ascorbic acid with long-lasting antiviral activity against CMV. ASC-2P may be of benefit for the adjunctive treatment of CMV infection.

Keywords: Human cytomegalovirus; L-ascorbic acid 2-phosphate; Ganciclovir; Foscarnet

1. Introduction

Human cytomegalovirus (CMV) was recognized as a major cause of morbidity and mortality among individuals undergoing immunosuppressive therapy, as in bone marrow, cardiac and renal transplant patients and in those with acquired immunodeficiency syndrome (Fiala et al., 1975; Stagno et al., 1983; Quinnan et al., 1984). Two anti-CMV compounds have recently been approved for treatment of patients with CMV disease, ganciclovir (GCV) (Collaborative DHPG Treatment Study Group, 1986; Laskin et al., 1987) and phosphonoformic acid (PFA, foscarnet) (Singer et al., 1985; Walmsley et al., 1988). However, prolonged use of ganciclovir is often associated with serious side effects such as anaemia, neutropenia and irreversible testicular damage (Felsenstein et al., 1985; Erice et al., 1987; Collaborative DHPG Treatment Study Group, 1986; Laskin et al., 1987; Hecht et al., 1988) while treatment with PFA may result in nephrotoxicity and hypocalcaemia (Walmsley et al., 1988; Youle et al., 1988). Moreover, concomitant with the increased use of both GCV and PFA, there has been an increased emergence of drug-resistant CMV strains (Stanat et al., 1991; Knox et al., 1991). It seems therefore still imperative to find novel chemotherapeutic agents active against CMV replication especially those with a different mechanism of action.

L-ascorbic acid (ASC; vitamin C) was shown to have antiviral activity against a broad spectrum of RNA and DNA viruses in vitro (Murata et al., 1972; Schwerdt and Schwerdt, 1975; Bisell et al., 1980; Blakeslee et al., 1985; Harakeh et al., 1990) and in vivo (Klenner, 1971; Murata, 1975a). In vivo, oral and intravenous administration of ASC is said to have produced clinical improvements in patients with influenza, hepatitis, and herpes virus infections, including Epstein-Barr virus infection. Clinical improvement was claimed in AIDS patients who voluntarily ingested high doses of ASC (Cathcart, 1984). In spite of these important activities of ASC, its effects on virus replication have not yet been clarified. ASC is very unstable in solution (Peterkofsky, 1972) which may account for difficulties especially when long-term effects on cell and/or virus metabolism should be observed. The low stability of ASC may be also the main obstacle in clinical use of the drug.

A phosphate derivative of ASC L-ascorbic acid 2-phosphate (ASC-2P) (Nomura et al., 1969) was shown to retain activities of ASC such as stimulation of cell growth, DNA replication and collagen synthesis in cultured cells of different origin (Hata et al., 1986; Hata and Senoo, 1989; Saika et al., 1992). Moreover, ASC-2P was shown to have antiscorbutic activity under experimental conditions (el-Naggar and Lovell, 1991). ASC-2P is very stable in solution, especially in neutral pH and 37°C since it becomes sensitive to oxidation only after dephosphorylation to ASC (Hata and Senoo, 1989). For example, the activity was retained for over 1 month when the derivative was dissolved in culture medium and kept at 5°C, and for 1 week, at 37°C.

In a preliminary communication we reported that ASC-2P may inhibit replication of CMV in dermal fibroblast cells (Gümbel et al., 1994). Herein we describe the effects of

ASC-2P on replication of several CMV strains in human foreskin fibroblasts and human endothelial cells which are known to be a common site of CMV infections in tissues, regardless of the organ involved (Grefte et al., 1993; Francis et al., 1989; Bournerias et al., 1989; Min et al., 1987; Myerson et al., 1984; Toorkey and Carrigan, 1989).

2. Materials and Methods

2.1. Cell culture

Human foreskin fibroblasts (HFF) were grown in Eagle's minimal essential medium (EMEM) supplemented with 10% FBS. Cultures of human endothelial cells (EC) were established from an umbilical vein as described previously (Jaffe et al., 1973). The cells were grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS) and 20 ng/ml basic fibroblast growth factor (Boehringer, Mannheim, Germany) in polystyrene culture flasks precoated with human fibronectin (2.5 $\mu g/cm^2$). The cells were subcultured at 5-day intervals and used in 2 to 8 passages in these experiments. To ensure the purity of the EC cultures, flow-cytometric analyses were carried out using an antibody against factor VIII von Willebrand-related antigen (Dakopatts, Glostrup, Denmark) as the primary marker (Scholz et al., 1992). Only EC cultures staining positive for more than 95% were used for experiments.

2.2. Viruses

CMV laboratory strains including AD169, Towne and Davis were purchased from American Type Culture Collection (Rockville, MA, USA). The viruses were propagated in HFF maintained in EMEM supplemented with 4% FBS. Virus titre was determined by examination of immediate early antigen forming units (I.E.F.U.) produced in maintenance medium as described previously (Braun and Schacherer, 1988). In our hands this method has a sensitivity comparable to that of plaque forming units assay. Briefly, a medium of infected cultures at 5-fold dilutions was incubated with confluent HFF monolayers in 96-well plates. 24-hours after infection immunoperoxidase staining of cells using monoclonal antibody (MAb 9921) (DuPont, Bad Homburg, Germany) directed against 72 kDa immediate early antigen of HCMV was performed as described previously (Cinatl et al., 1994). Stained nuclei were counted microscopically and virus titre expressed as numbers of I.E.F.U. per ml.

2.3. Compounds

ASC-2P was a kind gift from Takeda Pharmaceutical Co. (Osaka, Japan). Sodium salt of L-ascorbic acid (ASC) was obtained from Sigma (Deisenhofen, Germany). Both ASC-2P and ASC were dissolved in IMDM at a concentration of 10 mM and sterilized by filtration. The ASC-2P solution was kept at 4°C for up to 3 weeks before use in the experiments. ASC was used fresh after its preparation. Ganciclovir (GCV) and PFA were prepared fresh in distilled water.

2.4. Assay for CMV antigen expression

Inhibitory effects of ASC-2P on the expression of CMV-induced IEA and late antigen (LA) were determined by immunoperoxidase staining of the cells. EC and HFF cultures were infected with different CMV strains at multiplicity of infection (m.o.i.) of 10 and 0.1 I.E.F.U. per cell, respectively. After 90 min incubation period, HFF and EC cultures were washed 3 times with phosphate buffered saline, and a medium containing 4% FBS without or with ASC-2P was added. Infected cells were incubated for 1 or 6 days for determination IE and LA expression, respectively. To perform immunoperoxidase staining HFF and EC were fixed with a 1:1 mixture of acetone/methanol either directly in culture flasks or after trypsinization on cytocentrifuge slides. Monoclonal antibodies directed against 72 kDa IEA and against 68 kDa LA (9920) were obtained from DuPont. Immunoperoxidase staining was performed as described previously (Cinatl et al., 1994). The number of antigen-positive cells was determined microscopically by the examination of at least 500 cells.

2.5. Virus yield assay

HFF and EC cultures in polystyrene culture flasks were infected with 10 I.E.F.U. and 0.02 I.E.F.U. per cell, respectively. After 90 min incubation period, HFF and EC cultures were washed 3 times with phosphate buffered saline and a medium containing 4% FBS without or with ASC-2P was added. The maintenance medium of EC was changed 6 days after infection, while HFF cells were incubated without medium change. Virus yield was determined 12 days after virus infection. For this purpose cultures were frozen and thawed twice to liberate the cell associated virus and the medium was clarified by centrifugation ($600 \times g$ for 10 min.). Virus yield was determined as I.E.F.U./ml after titration in HFF monolayers (see above).

2.6. Cytotoxicity assay

Cytotoxicity measurements were based on the effects of ASC, ASC-2P, GCV and PFA on HFF and EC growth. For this purpose, the cells were seeded at a density of 2×10^4 cells per cm^2 in culture medium containing different concentrations of ASC-2P or ASC. The cells were counted using a hemocytometer five days after seeding and cell viability was determined using trypan blue exclusion.

3. Results

3.1. Effect of ASC-2P on CMV antigen expression and virus yield

In HFF control (untreated) cultures infected with AD169 at an m.o.i. of 0.1, about 90% of the cells expressed LA 6 days after infection, while in EC control cultures, 40% of the cells expressed LA after inoculation with an m.o.i. of 10 (Fig. 1). Treatment with 0.1 mM ASC-2P had no significant effect on the number of cells expressing LA in both

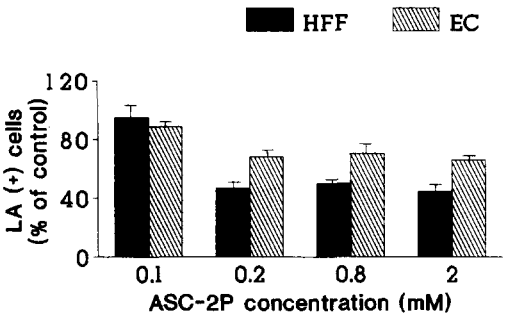

Fig. 1. Effects of ASC-2P on the number of HFF and EC expressing CMV LA. ASC-2P at different concentrations was added to a maintenance medium after infection with the AD169 strain. Results are mean ± SD of triplicate culture from three independent experiments.

cell types. The number of HFF and EC expressing LA was reduced by 55% and 30%, respectively, at a concentration of 0.2 mM ASC-2P. Increasing the concentration of ASC-2P did not result in a significant increase of anti-CMV activity. The inhibition of LA expression in EC was similar for all CMV strains used (data not shown). ASC-2P at concentrations ranging from 0.1 mM to 2 mM had no effect on the number of HFF and EC cells expressing IEA infected with different CMV laboratory strains (data not shown).

Virus yield in HFF infected with AD169 at an m.o.i. of 0.02 was reduced up to 4 times with ASC-2P concentrations ranging from 0.2 to 1.6 mM (Table 1).

3.2. Effect of EC pretreatment with ASC-2P on antigen production and virus yield

To study the effects of cell pretreatment with ASC-2P on CMV antigen expression, HFF and EC cultures were incubated for different time intervals (1 to 5 subcultures) with 0.2 mM concentration of the drug. The antiviral activity against AD169 increased with increasing times of pretreatment in EC and HFF (Fig. 2). ASC-2P pretreatment resulted in the inhibition of numbers of HFF and EC cells expressing both IEA and LA. In comparison to control cultures, 80% and 75% reductions of HFF and EC expressing IEA, respectively, were observed after 3 subcultures (18 days) of the cells in medium with 0.2 mM ASC-2P. The LA production, was reduced by 90% and 92% in HFF and EC cells, respectively. The incubation of EC and HFF for more than three passages in a medium containing 0.2 mM of ASC-2P did not result in a further increase of anti-CMV activity. Similar antiviral effects as for AD169 strain were found for Towne and Davis strains (data not shown).

The antiviral activity of ASC-2P on virus yields were tested in HFF and EC cultures pretreated for 3 subcultures with a 0.2 mM concentration of the drug. Twelve days after infection virus yields were 50- to 100-fold lower in the ASC-2P pretreated cultures than in the control EC cultures (Table 2). In HFF virus yield was about 100- to 1000-fold lower in ASC-2P treated cells than in control cultures.

Table 1
Comparison of the anti-HCMV activity of ASC-2P, GCV and PFA in HFF and effect of the pretreatment of HFF with ASC-2P on the activity of GCV and PFA

Compound	Drug concentration (GCV, PFA in μM and ASC-2P in mM)	Log virus titre [a] (I.E.F.U./ml)	
		without ASC-2P pretreatment	with ASC-2P [b]
ASC-2P	0	5.93 ± 0.029 [c]	–
	0.1	5.81 ± 0.053	
	0.2	5.37 ± 0.032	–
	0.4	5.32 ± 0.041	–
	0.8	5.33 ± 0.036	–
	1.6	5.34 ± 0.047	–
GCV	0	6.08 ± 0.043	3.18 ± 0.026
	5	5.64 ± 0.026	1.52 ± 0.031
	10	4.04 ± 0.033	0
	40	1.97 ± 0.048	0
	80	0	0
PFA	0	5.98 ± 0.041	3.41 ± 0.037
	100	5.32 ± 0.028	2.32 ± 0.028
	200	3.97 ± 0.029	1.54 ± 0.021
	400	1.63 ± 0.036	0
	600	0	0

[a] Virus titre in culture medium was determined 12 days after infection with an m.o.i. of 0.2 I. E. F. U. per cell; [b] HFF cells were grown for 3 subcultures (21 days) in medium containing 0.2 mM ASC-2P before use in the antiviral assay; [c] Results represent mean value (± SD) of triplicate cultures from two independent experiments

3.3. Long-lasting antiviral activity of ASC-2P

To study whether antiviral activity in ASC-2P treated cells may have a long-lasting character, HFF and EC were grown for 3 subcultures in a medium with 0.2 mM ASC-2P and used after 1 and 3 further subcultures in medium without ASC-2P in antiviral assays. The results demonstrated that continuous ASC-2P treatment is not required to

Table 2
Effect of ASC-2P pretreatment on virus yield of different HCMV strains

CMV strain	Log I.E.F.U./ml			
	without ASC-2P		with ASC-2P [a]	
	HFF	EC	HFF	EC
AD 169	6.11 ± 0.041 [b]	3.81 ± 0.074	3.11 ± 0.028	1.64 ± 0.073
Towne	6.0 ± 0.033	2.85 ± 0.066	3.32 ± 0.043	1.15 ± 0.047
Davis	6.04 ± 0.056	3.18 ± 0.082	3.98 ± 0.042	1.51 ± 0.085

[a] HFF and EC were grown for 3 subcultures in medium with 0.2 mM ASC-2P before virus infection. The maintenance medium contained 0.2 mM ASC-2P.
[b] Results represent mean values (± SD) of quadruplicate culture from four independent experiments. The maintenance medium contained 0.2 mM ASC-2P.

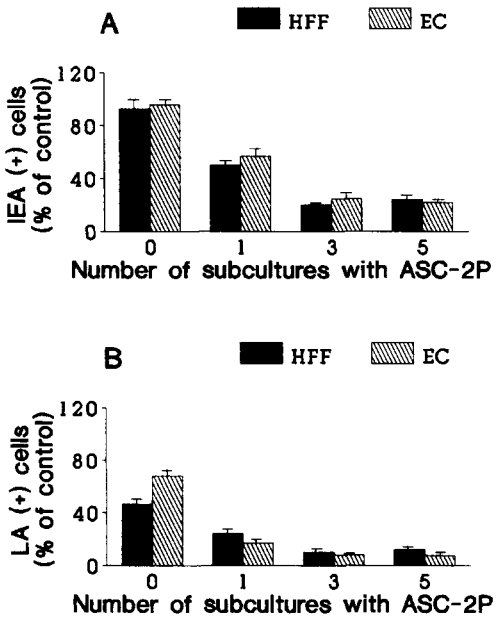

Fig. 2. Effects of pretreatment with ASC-2P on numbers of HFF and EC expressing CMV IEA (A) and LA (B). EC were infected with the AD169 strain without pretreatment or after pretreatment for 1 (6 days), 3 (18 days) and 5 (30 days) subcultures with 0.2 mM ASC-2P. Maintenance medium of pretreated cultures contained 0.2 mM ASC-2P. Results are mean ±SD of triplicate culture from three independent experiments.

inhibit the expression of LA in treated cells (Fig. 3). However, antiviral effects decreased in successive passages of ASC-2P pretreated HFF and EC cells in a medium without ASC-2P. After 1 subculture (6 days) without ASC-2P, LA expression in HFF and EC, compared to control cultures, was 32% and 78% lower, respectively. After 3

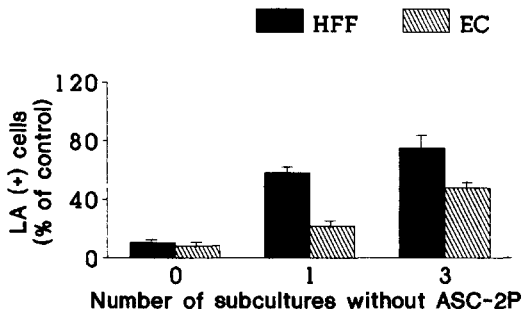

Fig. 3. Long-lasting antiviral activity in HFF and EC pretreated for 3 subcultures (18 days) with 0.2 mM ASC-2P against CMV (AD169 strain). Numbers of EC expressing IEA (A) and LA (B) were determined in pretreated cultures without passaging or after 1 (6 days) or 3 (18 days) subcultures in medium without ASC-2P. Infected cultures were incubated in maintenance medium without ASC-2P. Results are mean ±SD of triplicate culture from three independent experiments.

Table 3
Cytotoxic effects of ASC-2P and ASC on HFF and EC cultures after 5 days of pretreatment

Drug concentration (mM)	Number of cells per $cm^2 \times 10^{-4}$			
	ASC-2P		ASC	
	HFF	EC	HFF	EC
0.2	10.8 ± 1.01 [a] (138) [b]	9.2 ± 0.94 (115)	9.92 ± 0.90 (124)	9.6 ± 0.87 (120)
1	10.24 ± 0.78 (128)	10.4 ± 1.2 (130)	3.44 ± 0.56 (43)	7.4 ± 0.94 (93)
2	9.84 ± 0.88 (123)	10.0 ± 1.1 (25)	0.72 ± 0.11 (9)	0
10	9.36 ± 0.91 (117)	9.6 ± 0.93 (120)	0	0

[a] Results represent mean values (± SD) of triplicate culture from two independent experiments. [b] Values in parenthesis represent a percentage of viable cells in untreated control cultures.

subcultures (18 days) without ASC-2P, the production of LA in HFF and EC, in comparison to untreated cells, was reduced by 25% and 50%, respectively.

In pretreated HFF, virus yield was still significantly lower after 3 subcultures without ASC-2P (data not shown).

3.4. Comparison of the antiviral activities of GCV, PFA, and ASC-2P and the effects of GCV and PFA in HFF cells treated with ASC-2P on the replication of the AD 169 strain

In HFF cells, virus yield was reduced in a dose-dependent manner with both GCV and PFA. Virus replication was completely inhibited by GCV and PFA at concentrations

Table 4
Cytotoxic effects of GCV and PFA on HFF cultures after pretreatment with ASC-2P

Drug	Concentration (μM)	HFF control		ASC-2P pretreated HFF [a]	
		Number of cells per $cm^2 \times 10^{-4}$	% of untreated control cultures	Number of cells per $cm^2 \times 10^{-4}$	% of untreated control cultures
GCV	50	7.96 ± 0.40 [b]	99	7.60 ± 0.40	95
	100	6.48 ± 0.32	81	6.96 ± 0.32	87
	200	6.0 ± 0.32	75	5.92 ± 1.04	74
	500	5.04 ± 0.48	63	5.28 ± 1.12	66
	1000	3.92 ± 0.24	49	4.16 ± 0.56	52
	2000	3.28 ± 0.24	41	3.04 ± 0.48	38
PFA	200	8.0 ± 0.48	99	7.60 ± 0.96	95
	500	6.96 ± 0.64	87	7.76 ± 0.64	97
	1000	4.72 ± 0.40	59	6.72 ± 0.48	84
	2000	3.60 ± 0.32	45	5.44 ± 0.72	68
	4000	2.40 ± 0.16	30	4.80 ± 0.40	60

[a] HFF cells were grown for 3 subcultures in a medium with 0.2 mM ASC-2P before cytotoxicity testing.
[b] Results represent mean values (± SD) of triplicate culture from two independent experiments.

of 80 μM and 600 μM, respectively. In comparison, virus growth was reduced 4 fold with different concentrations of ASC-2P ranging from 0.2 μM to 1.6 μM (Table 1). The reduction of virus yield in HFF cells treated with GCV, PFA and ASC-2P is shown in Table 1. When HFF were grown for 3 subcultures (35 days) in a medium with 0.2 mM ASC-2P before antiviral susceptibility testing with GCV and PFA, a 100 to 10^4 fold higher reduction of the virus yield was observed in pretreated cells for GCV in comparison to untreated controls. The antiviral activity of PFA increased 40 to 1,000 times in ASC-2P pretreated cells.

3.5. Cytotoxicity assay

The cytotoxicity of ASC-2P for HFF and EC in comparison with that of ASC is shown in Table 3. No toxic effects were observed with ASC-2P concentrations ranging from 0.2 mM to 10 mM. In contrast, ASC at a concentration as high as 2 mM was toxic for HFF and EC and completely destroyed EC and HFF cells after 5 days of treatment with 2 mM and 10 mM of ASC respectively. While the cytotoxicity of DHPG was similar in ASC-2P treated and untreated HFF, a significantly higher number of cells was observed with PFA concentrations ranging from 1,000 μM to 4,000 μM of PFA in ASC-2P pretreated cells in comparison to control HFF (Table 4).

4. Discussion

In the present study we demonstrated that ASC-2P inhibits CMV replication in HFF and EC cultures. It is difficult to maintain the activity of ASC in a solution because ASC is easily oxidized especially at 37°C (Peterkofsky, 1972). ASC-2P is significantly more stable than ASC (Hata and Senoo, 1989). We showed that ASC-2P exerts its antiviral effects in HFF and EC cultures when added only once after virus infection. Contrasting this, to achieve antiviral effects of ASC against CMV and human immunodeficiency virus type 1 (HIV-1) the drug had to be added daily to the culture medium of fibroblast and lymphoid cells (Harakeh et al., 1990; Gümbel et al., 1994). ASC-2P itself may not function directly as active vitamin C but must be dephosphorylated probably on the cell membrane (Hata and Senoo, 1989). A continuous conversion of ASC-2P to ASC may account for the ability of ASC-2P to function as a long-acting vitamin C analog.

ASC-2P does not inhibit completely CMV replication in cultured cells. ASC-2P treatment of HFF and EC resulted in 100- to 1000-fold and 50- to 100-fold reductions of CMV yields, respectively. However, the antiviral activity against different CMV strains was significantly greater in ASC-2P pretreated cultures than in HFF and EC treated after virus infection. The antiviral activity was retained in ASC-2P pretreated cultures even after the omission of the drug from a culture medium. These findings suggest that ASC-2P does not exert its antiviral effects directly on CMV replication but rather induces some cellular mechanisms which account for the inhibition of virus replication. Since both synthesis of IE and L CMV proteins were inhibited it is probable that ASC-2P exerts its antiviral effects at different stages of virus infection. ASC-2P may be of benefit to the adjunctive treatment of human cytomegalovirus (HCMV) infection

since a significantly higher reduction of virus yield was observed in HFF cells pretreated with ASC-2P prior to antiviral susceptibility testing with GCV and PFA. Even after pretreatment for several subcultures, no toxic effects of ASC-2P were observed. In contrast, growth stimulation of EC and HFF was observed after ASC-2P treatment. These results are consistent with those of Hata and Senoo (1989) who had demonstrated that the treatment of dermal fibroblasts with ASC-2P for several weeks significantly stimulated cell growth in comparison to untreated control cultures. An interesting finding was, that a higher antiviral activity of GCV and PFA was observed in ASC-2P pretreated HFF in comparison to untreated control cells without increased cytotoxicity. With high concentrations of PFA, cytoxicity decreased in pretreated HFF suggesting that in vivo, the frequency and intensity of adverse reactions of foscarnet may be reduced by adjunctive ASC-2P therapy.

The mechanism by which ASC-2P inhibits CMV replication is not clear. In earlier studies, ASC caused degradation of single-and double-stranded genomes of RNA and DNA phages (Wong et al., 1974; Murata, 1975b; Murata and Uike, 1976). Site-specific cleavage of phage DNA occurring at unique sites due to redox reactions involving copper and ascorbate was reported (Kazakow et al., 1988). Hydroxyl radicals (OH^-) generated from hydrogen peroxide were implicated as the reactive species mediating cleavage of nucleic acid (Wong et al., 1974; Chiou, 1983; Kazakow et al., 1988). Therefore, one possible mechanism of ASC action on CMV infected cells is that newly synthesized viral DNA and/or RNA becomes susceptible to ascorbate-mediated damage, resulting in reduced viral protein production. It should be also noted that oxidative degradation of ASC is associated with the formation of highly reactive breakdown products including furan-type compounds that form adducts with amino and hydroxyl groups of proteins resulting in site-specific cleavage or cross-linking of protein (Nakanishi et al., 1985; Garland et al., 1986; Ortwert et al., 1988). Such modifications of newly synthesized CMV proteins could disturb their function and thus contribute to antiviral effects of ASC.

On the other hand, antiviral activity of ASC could stem from its ability to function as a free radical scavenger (Rose and Bode, 1993). ASC and other reducing agents such as N-acetyl-L-cysteine or glutathione were shown to suppress HIV-1 replication in lymphocytes and monocytes/macrophages in vitro (Harakeh et al., 1990; Dröge et al., 1992; Ho and Douglas, 1992; Roederer et al., 1992). It has been shown that some of these antioxidants may antagonize stimulatory effects of reactive oxygen intermediates (ROI) on HIV-1 replication. Glutathione added to a culture medium of monkey kidney cells was able to exert a strong antiviral activity against herpes simplex virus (Palamara et al., 1994). We observed that a treatment of EC cultures with buthionine sulfoximine which may deplete in different cell types as much as 90% of intracellular glutathione (Roederer et al., 1992) resulted in significant stimulation of CMV replication and potentiated stimulatory effects of substances such as sodium butyrate or phorbol ester on CMV replication (unpublished results). CMV disease frequently develops in patients with cancer, recipients of organ and bone marrow transplants, and persons with AIDS. Since pathological conditions in such patients are frequently associated with the increased ROI production (Dröge et al., 1992), a role of oxidative stress in CMV infection deserves further studies.

In vivo ASC is said to have produced clinical improvements in patients afflicted with influenza, hepatitis, and herpes virus infection (Klenner, 1971; Murata, 1975a). Clinical improvement was claimed in AIDS patients who voluntarily ingested high doses of ASC (Cathcart, 1985). However, whether ASC directly influences virus replication or whether it may contribute to suppression of virus infection by some indirect mechanisms such as stabilization of immune system was not investigated. The concentrations at which the anti-CMV effect of ASC-2P was seen in this in vitro study correspond to those which are physiologically attainable for ASC in human blood plasma (Frei et al., 1989). We showed that ASC-2P has no toxicity for HFF and EC even when added to a culture medium at high concentrations which were deleterious for the cells when active vitamin C was used. ASC toxicity is minimal in most individuals under most conditions. This might depend upon the vitamin being adequately maintained in the reduced state (Rose and Bode, 1993). However, under experimental conditions the product of ascorbate oxidation, dehydro-L-ascorbic acid (DHAA) was associated with several forms of cell and tissue damage. For example, DHAA destroys the pancreatic beta cells that produce insulin (Patterson, 1950), disrupts erythrocytes (Bianchi and Rose, 1985), inhibits cellular metabolism in surviving fragments of human placenta (Rose et al., 1992) and alters transport properties of brush border membrane vesicles produced from mammalian intestine (Bianchi and Rose, 1986). It is possible that a limited release of ASC from ASC-2P accounts for low DHAA levels and thus for the absence of toxicity at high concentrations in HFF and EC cultures. This feature of ASC-2P is of interest since it could help to avoid complications such as bowel intolerance which limited application of ASC-2P by persons with AIDS (Abrams, 1990). In conclusion ASC-2P provides vitamin C prodrug which, due to its high stability, may provide significant therapeutic advantages over ASC. The character of ASC-2P antiviral response in cultured cells suggests that ASC-2P could be useful both in prevention and treatment of CMV disease.

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