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Enhanced *in vitro* Reactivation of Herpes Simplex Virus Type 2 from Latently Infected Guinea-pig Neural Tissues by 5-Azacytidine

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SUMMARY

5-Azacytidine (5-AZC) reduces cytosine methylation in DNA and has been reported to activate quiescent virus genes. Treatment of explant cultures of latently herpes simplex virus type 2 (HSV-2)-infected guinea-pig dorsal root ganglia and spinal cords *in vitro* with 5-AZC significantly enhanced the rate of HSV recovery. Both the number of isolates from ganglia ($P < 0.001$) and the rate of recovery ($P < 0.001$) were significantly increased with the addition of 50 μM -5-AZC to explant cultures. Increased virus recovery appeared to be due to the induction of reactivation of latent virus, rather than an increase in replication, since 5-AZC inhibited HSV replication. These data support a role for methylation in HSV latency and reactivation.

DNA methylation appears to be a fundamental regulatory mechanism governing gene activity and differentiation (Jones & Taylor, 1980). 5-Cytosine methylation of CpG sequences correlates with transcriptional suppression, whereas hypomethylation correlates with transcriptional activation, in some but not all genes (Pollack *et al.*, 1980; Naveh-Manly & Cedar, 1981). The methylation status of several virus genomes has been related to their expression. Within the herpesvirus family, lymphoid cell lines persistently infected with either herpesvirus saimiri (Desrosiers *et al.*, 1979) or herpes simplex virus type 1 (HSV-1) (Youssoufian *et al.*, 1982) contain methylated virus DNA during non-productive states and unmethylated virus DNA in cells actively producing virus. Among adenoviruses, a similar inverse correlation between the levels of DNA methylation and the extent of mRNA expression has been reported (Sutter & Doerfler, 1980). There is, however, conflicting evidence as to whether HSV DNA is extensively methylated *in vivo* (Dressler *et al.*, 1987).

The cytosine analogue, 5-azacytidine (5-AZC), has a nitrogen atom replacing the carbon atom at position 5 on the pyrimidine ring and therefore is thought not to accept a methyl group (Jones & Taylor, 1980). The extent of demethylation due to 5-AZC, however, exceeds the amount incorporated, which may relate to the inhibition of the DNA methyltransferase by the drug (Jones, 1985). Once demethylation occurs, it is inherited clonally and some genes activated by demethylation are transcribed in progeny cells (Hsiao *et al.*, 1984; Compere & Palmiter, 1981).

Induction of transcriptionally silent virus genes following reduction of methylation by 5-AZC has been reported. Two cell lines transfected with transcriptionally inactive HSV thymidine kinase (TK) genes produced TK transcripts in high yields after induction with 5-AZC (Christy & Scangos, 1982; Clough *et al.*, 1982). Similarly, treatment with 5-AZC induced Epstein-Barr virus early antigens in latently infected human lymphoid cells (Ben-Sasson *et al.*, 1981). Furthermore, an increase in hepatitis B virus core transcripts (Korba *et al.*, 1985) as well as expression of retrovirus type C and type A sequences (Hsiao *et al.*, 1986; Lasneret *et al.*, 1983) and genomes (Jaenisch *et al.*, 1985) have been demonstrated after 5-AZC treatment. We therefore hypothesized that methylation of the HSV genome might be involved in the induction

or maintenance of the latent state by down-regulating viral gene expression and that drug-induced hypomethylation might, therefore, enhance reactivation of latent HSV-2. To explore this possibility, we studied *in vitro* the effect of treatment with 5-AZC on reactivation of HSV from latently infected guinea-pig neural tissues.

Thirty-seven adult guinea-pigs (Hartley strain obtained from Charles River Breeding Laboratories, Wilmington, Mass., U.S.A. or Strain 2 obtained from Children's Hospital Research Foundation, Cincinnati, Ohio) were inoculated with 10^5 to 10^6 p.f.u. of HSV-2 MS (ATCCV-540) strain either by introduction of virus-containing media into the vagina (Stanberry *et al.*, 1986) of female animals ($n = 30$) or by cutaneous inoculation following abrasion with a 27-gauge needle on the medial thigh (Bernstein & Stanberry, 1986) in male animals ($n = 7$). All animals had recovered from virologically confirmed acute HSV infection when sacrificed 40 to 60 days after inoculation. The lumbosacral dorsal root ganglia of all animals and the spinal cords of 15 animals were then minced separately and explanted separately onto confluent rabbit kidney monolayers in flasks 25 cm² (Stanberry *et al.*, 1986). The minced tissues from the dorsal root ganglia of each animal were initially divided into eight flasks. Four cultures were randomly assigned to receive no drug, while two each received 10 or 50 μM -5-AZC which was added to the culture medium (Eagle's Basal Medium, 5% heat-inactivated foetal bovine serum, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 50 $\mu\text{g}/\text{ml}$ gentamicin, 2 $\mu\text{g}/\text{ml}$ amphotericin B, 2 mM-L-glutamine). After initial evaluation had revealed that 50 μM -5-AZC was more effective than 10 μM -5-AZC, subsequent samples from each animal were divided into four flasks, two receiving 50 μM -5-AZC and two receiving medium alone. Cultures were treated with drug for the first 3 days and then all cultures were re-fed every 3 days with drug-free medium for up to 28 days post-explantation. Virus was detected by the production of typical cytopathic effect, being distinguished from indigenous caviid herpesvirus by passage in either human foreskin fibroblast or HEp-2 cells (Hsiung *et al.*, 1976). Selected isolates were confirmed to be HSV by reaction with an HSV monoclonal antibody (Syva, Palo Alto, Ca., U.S.A.).

HSV was recovered earlier and from a greater percentage of drug-treated dorsal root ganglia explant cultures than from untreated control explant cultures (Fig. 1*a*). By day 14, HSV had been recovered from 11 of 46 (24%) cultures treated with 10 μM -5-AZC and 26 of 78 (33%) cultures treated with 50 μM -5-AZC, compared to 10 of 106 (9%) controls ($P < 0.02$ for control versus 10 μM -5-AZC and $P < 0.001$ for control versus 50 μM -5-AZC, by Chi-square analysis). By day 28, HSV had been detected in 50% of the cultures treated with 50 μM -5-AZC compared to 18% of control cultures ($P < 0.001$). Survival analysis (Breslow, 1970) demonstrated a significant difference between the untreated and the 50 μM -5-AZC groups ($P < 0.001$) but no significant difference between the untreated and the 10 μM -5-AZC groups (in which HSV had been detected in 24% of the cultures) over the 28 day period of observation ($P = 0.22$). Treatment with 50 μM -5-AZC significantly reactivated virus at a higher rate than 10 μM -5-AZC ($P = 0.03$, by survival analysis). Combining the results from the two drug-treated groups also revealed a highly significant difference compared to the control group ($P < 0.001$, by survival analysis).

Drug-treated spinal cord cultures also had a higher cumulative percent of virus recovery over time, compared to untreated controls (Fig. 1*b*). Significant differences were observed both on day 14, when 16 of 30 (53%) cultures treated with 50 μM -5-AZC and 15 of 56 (27%) control cultures reactivated virus ($P < 0.02$) (58% of the 10 μM -5-AZC-treated cultures also reactivated), and day 28 (70% for 50 μM -5-AZC and 34% viral recovery for controls) ($P < 0.002$); 62% of the 10 μM -5-AZC-treated cultures also reactivated. By survival analysis there was also a significant difference for these groups over the 28 day period of observation ($P = 0.05$).

In order to evaluate whether the effects of 5-AZC could be due to enhancement of virus replication after reactivation, rather than by enhancing the induction of virus reactivation, we examined the effects of 5-AZC on productive HSV replication *in vitro* (Fig. 2). No evidence for enhanced replication was found. After 48 h, titres were 0.3 \log_{10} lower in the cultures treated with 10 μM -5-AZC ($P < 0.01$) and 3.5 \log_{10} lower in those treated with 50 μM -5-AZC ($P < 0.001$) when compared to untreated controls (paired *t*-test). This may have been due to cellular toxicity, since treatment for more than 3 days, or with concentrations of 5-AZC exceeding 50 μM ,

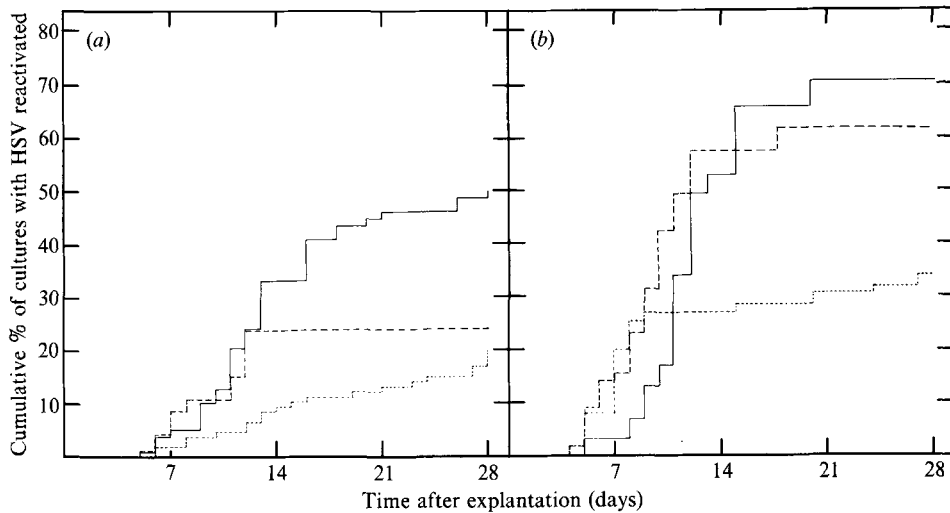


Fig. 1. Recovery of HSV-2 from (a) dorsal root ganglia cultures and (b) spinal cord cultures. Explant cultures, co-cultivated on primary rabbit kidney cell monolayers, were treated with 0 (···), 10 (---) or 50 (—) μM -5-AZC-containing medium for 3 days.

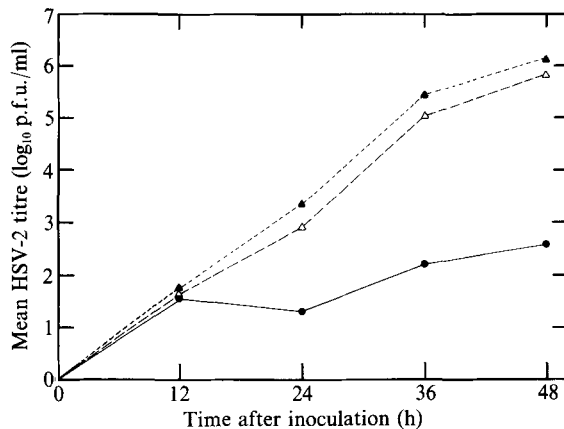


Fig. 2. Effects of 5-AZC on acute HSV-2 replication *in vitro*. Rabbit kidney cell monolayers were incubated in the presence of 0 (\blacktriangle), 10 (\triangle) or 50 (\bullet) μM -5-AZC for 24 h and then inoculated with HSV-2 MS strain (m.o.i. 0.001). After a 1 h adsorption period at 37 °C, medium containing 0, 10 or 50 μM -5-AZC, respectively, was re-added. One ml of medium was removed and replaced every 12 h. The removed medium was centrifuged at 1000 *g* for 15 min at 4 °C to remove cells and 0.2 ml of supernatant was titrated for HSV on rabbit kidney cells with a 0.75% methylcellulose overlay. Plaques were counted after crystal violet staining 3 days later. Each point represents the mean of triplicate determinations.

produced toxic effects with a granular, ruffled appearance, followed by detachment of the rabbit kidney cell monolayer.

In this study we have shown that treatment of latently infected guinea-pig neural tissue *in vitro* with either 10 or 50 μM -5-AZC enhanced HSV recovery and that 50 μM was the more effective concentration. Use of 5-AZC at 50 μM in explant cultures increased the number of animals that could be identified as containing latently infected dorsal root ganglia from 34% to 74% ($P < 0.01$) and increased the identification of animals with latently infected spinal cords from 44% to 75% ($P < 0.08$). Since 5-AZC inhibited productive virus replication, the increased recovery

from drug-treated cultures would appear to reflect enhanced viral reactivation, presumably due to inhibition of cytosine methylation (Jones & Taylor, 1980). Recently, Whitby *et al.* (1988) also reported that several hypomethylating agents including dimethylsulphoxide, L-ethionine and 5-AZC increased the incidence of HSV-1 reactivation *in vitro* from latently infected mouse cervical ganglia.

Hypomethylation of DNA by 5-AZC may occur by its incorporation into DNA in place of cytidine or by inhibition of the DNA methyltransferase by covalent linkage to the enzyme or release of a modified inactive enzyme (Taylor & Jones, 1982). DNA synthesis in the neuron could occur when the cell is undergoing repair synthesis or mitosis (Sanes & Okun, 1972). The newly synthesized hypomethylated DNA may then allow immediate activation of parental quiescent genes, such as latent viral sequences or cellular genes that are necessary for reactivation or completion of the viral lytic cycle. This latter hypothesis could explain how 5-AZC induces reactivation even if the latent viral genome is not methylated (Dressler *et al.*, 1987). Demethylation could occur *in vivo* when a neuron is undergoing repair synthesis or in response to hormonal signals (Hill, 1984). Reactivation of HSV *in vivo* has been reported following trauma to the peripheral site (Harbour *et al.*, 1983) or nerve damage (Price & Schmitz, 1978). Alternatively, after mitosis of the hemimethylated sites, the under-methylated DNA may reactivate previously silent genes. This implies either that there is an over-riding of the cell cycle block in sensory neurons or that another replicating cell also harbours latent virus genome.

These data support the hypothesis that hypo- or demethylation is involved during HSV reactivation although, since the effects of 5-AZC are not limited to hypomethylation alone (Reichman & Penman, 1973), it cannot be stated with certainty that this is its only mechanism of virus reactivation. The experience with other demethylating agents, however, provides corroborating evidence for a role of hypomethylation (Whitby *et al.*, 1988; Bernstein & Kappes, 1988). It is also intriguing to speculate whether the treatment of patients with 5-AZC for sickle cell anaemia (Ley *et al.*, 1983), various leukaemias (Mompalmer *et al.*, 1984) and solid tumours (van Groeningen *et al.*, 1986) might induce reactivation *in vivo* of established latent herpesvirus infections.

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