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HHV-6 inhibition by two polar compounds

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Summary

Dimethyl sulphoxide and dimethyl formamide, two polar compounds and powerful cell differentiation inducers, inhibit HHV-6 infection when added to HHV-6-infected HSB_2 cultures. This was established by a delay in the time-course of infection and in the development of virus-induced cytopathic effects. Furthermore, viral titration of supernatants showed a significant reduction (3 $\log_{10}$) of the number of infectious particles. Electron microscopy confirmed that viable cells and extracellular virions were present in the cultures containing the polar compounds, while in the non-treated cultures all cells were lysed and no extracellular virus was evident. The mode of action of these compounds is still unclear and warrants further investigation.

HHV-6; Polar compound; Viral inhibition; Dimethyl sulphoxide; Dimethyl formamide

Introduction

Previous reports suggest that polar compounds inhibit in vitro replication of retroviruses (Sato et al., 1971; Viza et al., 1989, 1991) as well as viruses of the herpes family (Yanase and Sugawara, 1989). It was thus tempting to investigate whether dimethyl sulphoxide (DMSO) or dimethyl formamide (DMF) – two polar compounds and powerful cell differentiation inducers – are also capable of inhibiting the human herpes virus-6 (HHV-6) (Salahuddin et al., 1986),

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which may be a co-factor in the course of HIV infection (Carrigan et al., 1990; Levy et al., 1990; Lusso et al., 1989, 1991).

The present studies show that incubation with DMSO or DMF of HSB_2 cells continuously infected with HHV-6 results in a partial viral inhibition. This is shown by a slower course of infection and by a reduced yield of infective viral particles in the culture medium. It thus seems that alteration of the differentiation state of a cell may interfere with viral production, at least as far as certain herpesviruses and certain retroviruses are concerned.

Materials and Methods

Cells

HSB_2, an immature T-cell line (Ablashi et al., 1988b) was used as the infection target as well as for growing the HHV-6. The cells were cultured in RPMI-1640 medium supplemented with 5% foetal calf serum (FCS) without antibiotics. Cells were grown in static suspensions at 37°C in RPMI-1640 with 10% FCS in 5% CO_2.

Viruses

HHV-6 virus GS strain (Salahuddin et al., 1986) was used in these studies. Dilutions (10^{-2}–10^{-3}) of highly concentrated cell-free supernatants were used to infect HSB_2 cells. Approximately 1×10^6 target cells were suspended in 6 ml of infectious supernatant diluted in RPMI-1640 without serum. The cell suspension was incubated at 37°C for 2 h, then transferred to a Nunclon 50-ml tissue culture bottle, where an additional 15 ml of RPMI-1640 and 10% FCS were added. The infected cell suspension was grown at 37°C in the presence of 5% CO_2. Cultures were kept in a state of continuous infection by the addition of 1×10^6 fresh cells every 5 days. Only cells from continuously infected cultures were used in the present study, and the percentage of infected cells on the first day was assessed by immunofluorescence (I.F.) for each experiment. Various concentrations of DMSO, DMF or an association of DMF and DMSO were added to HHV-6-infected cultures, and the lytic effect was assessed 3, 8 or 9 days later.

Reagents

N,N,dimethyl formamide (DMF) (99+% spectrophotometric grade) and dimethyl sulphoxide (DMSO) anhydrous (99+%) were obtained from Aldrich-Chimie, Strasbourg, France. Usually, DMF and DMSO were diluted in PBS and stored at 4°C until use, but for some experiments the compounds were diluted directly in the medium. The range of concentrations used, 62.5–250 mM for DMSO and 12.5–125 mM for DMF, was that at which the chemicals have

been shown to have an effect on HIV or EBV and are not toxic for HSB_2 cells.

Assay of HHV-6

The HHV-6-infected cells were monitored by light microscopy for the presence of HHV-6-induced cytopathic effects characterized by the appearance of large refractile cells, clustering and fusion of infected cells, cell lysis and death (Ablashi et al., 1988a; Salahuddin et al., 1986). The time course of infection was monitored by immunofluorescence using a monoclonal antibody reacting with HHV-6 p41 as well as by the appearance of typically infected large refractile cells (Balachandran et al., 1989).

Assay of supernatants' infectivity

Cell supernatants from HHV-6-infected controls and HHV-6-infected cells treated with DMSO or DMF were cleared by centrifugation and filtered through 0.45 μ Millipore filters. Suspensions of fresh HSB_2 cells were seeded at about 2×10^5 cells/ml in wells from COSTAR Cluster^{24} trays and allowed to reach confluence. The cells were subsequently overlaid with 0.5 ml of the appropriate filtered supernatant. After 2 h at 37°C, the viral inoculum was removed by careful aspiration and an extra 2 ml of RPMI-1640 medium containing 10% FCS and 1% carboxymethyl cellulose were added to each well. Development of infection was monitored by the appearance of foci consisting of clusters of fused infected cells.

Virus titration in supernatants from infected cultures

Equal amounts of supernatants from control infected HSB_2 cultures and infected cultures treated with DMSO and/or DMF were collected on day 9 post-treatment. The virus in these supernatants was precipitated with polyethylene glycol and concentrated 10-fold in Tris-saline buffer, pH 7.4. Serial 10-fold dilutions of concentrated virus were made in the same buffer.

Aliquots of each dilution (1 ml) were added to duplicate samples of fresh HSB_2 cells (about 5×10^5 cells in 1 ml RPMI medium without serum) and the virus was allowed to adsorb at room temperature. After 1 h, the unadsorbed virus was removed by washing the cells in PBS and resuspension in RPMI and 10% FCS. Each sample, containing about 5×10^4 cells/ml, was equally divided into three wells from a COSTAR Cluster^{24} tray (2 ml/well). The cell cultures were incubated at 37°C in 5% CO_2. On day 10 post-infection, the cultures were scored for the presence or absence of any virus-induced cytopathic effects.

Electron microscopy (EM)

The cells were prepared for thin section electron microscopy as described by Biberfeld et al. (1987). Briefly, the cells were fixed for one hour at room

temperature in 2.5% glutaraldehyde buffered to pH 7.3 with 0.1 M sodium cacodylate. They were then washed with 0.2 M sodium cacodylate buffer, postfixed for 1 h with Dalton's chrome osmium, rinsed with distilled water, stained with 1% aqueous uranyl acetate and dehydrated in ethanol. The cells were subsequently immersed in propyleneoxide and embedded in T-epon 812 (Tousimis Research Corp., Rockville, MD). Blocks were sectioned in a Reichert microtome, picked up on uncoated copper grids, stained with uranyl acetate and lead citrate and scanned in a Siemens Elmiskop electron microscope.

Results

Table 1 illustrates the effect of DMSO or DMF on the viability of HHV-6-infected cells as estimated by trypan blue exclusion: the addition of DMSO or DMF in the cultures resulted in a significant decrease in cell mortality. On the third day after the addition of the compounds, the viability was 60–70% in the DMSO-treated cultures and 45–55% in the DMF cultures, whereas it was only 18% in the controls. On the eighth day, cell viability was nil for the control cultures, whereas 55% viable cells were found in the cultures treated with 250 mM of DMSO. In a different series of experiments (Table 2), the effect of various concentrations of DMSO and/or DMF were assayed on HHV-6-infected cells. A marked protective effect was noticed, viability being at least 45% in all treated cultures, whereas it was nil in the control.

In electron microscopy, intact cells with cytoplasmic coated nucleocapsids and nucleocapsids in the nucleus could be seen in cultures treated with 250 mM of DMSO. Extracellular virions were also observed (Fig. 1). In contrast, all cells of the control cultures were lysed and no extracellular virus was evident (Fig. 2).

TABLE 1

Effect of DMSO or DMF on the viability of HHV-6 (GS)-infected HSB_2 cells[a]

Treatment	Post-treatment day 3		Post-treatment day 8	
	Viable cells[b] (%)	Cell lysis[c]	Viable cells[b] (%)	Cell lysis[c]
Infection control	18 ± 2	+ +	None	+ + + +
250 mM of DMSO	70 ± 3	−	55 ± 5	+/−
125 mM of DMSO	60 ± 3	−	35 ± 5	+
125 mM of DMF	45 ± 5	+	None	+ +
62.5 mM of DMF	45 ± 4	+	None	+ +
12.5 mM of DMF	55 ± 4	−	7 ± 2	+

[a] HSB_2 cultures infected with HHV-6 (GS) were treated with various concentrations of DMSO and DMF. 77% of cells were reactive in immunofluorescence to an anti-p41 monoclonal antibody on the first day of treatment. The effect was assessed on post-treatment days 3 and 8.

[b] Cell viability was estimated by trypan blue exclusion counting 300 cells.

[c] + + + +, total cell lysis; −, absence of cell lysis.

TABLE 2

Cell viability on day 8 of DMSO and/or DMF treatment of HHV-6-infected HSB_2 cells[a]

Treatment	Viable cells[b] (%)	Cell lysis[c]
Infection control	None	+ + + +
250 mM of DMSO	70 ± 3	–
125 mM of DMSO	70 ± 2	+/–
62.5 mM of DMSO	45 ± 5	+
125 mM of DMF	45 ± 6	+
62.5 mM of DMF	50 ± 3	+
12.5 mM of DMF	55 ± 5	+/–
62.5 mM of DMSO + 62.5 mM of DMF	45 ± 6	+
62.5 mM of DMSO + 12.5 mM of DMF	55 ± 6	+/–

[a] HSB_2 cultures infected with HHV-6 (GS) were treated with various concentrations of DMSO and/or DMF. 48% of the cells were found reactive to an anti-p41 monoclonal antibody on the first day of treatment.

[b]Cell viability was estimated by trypan blue exclusion counting 300 cells.

[c]+ + + +, total cell lysis; –, absence of cell lysis.

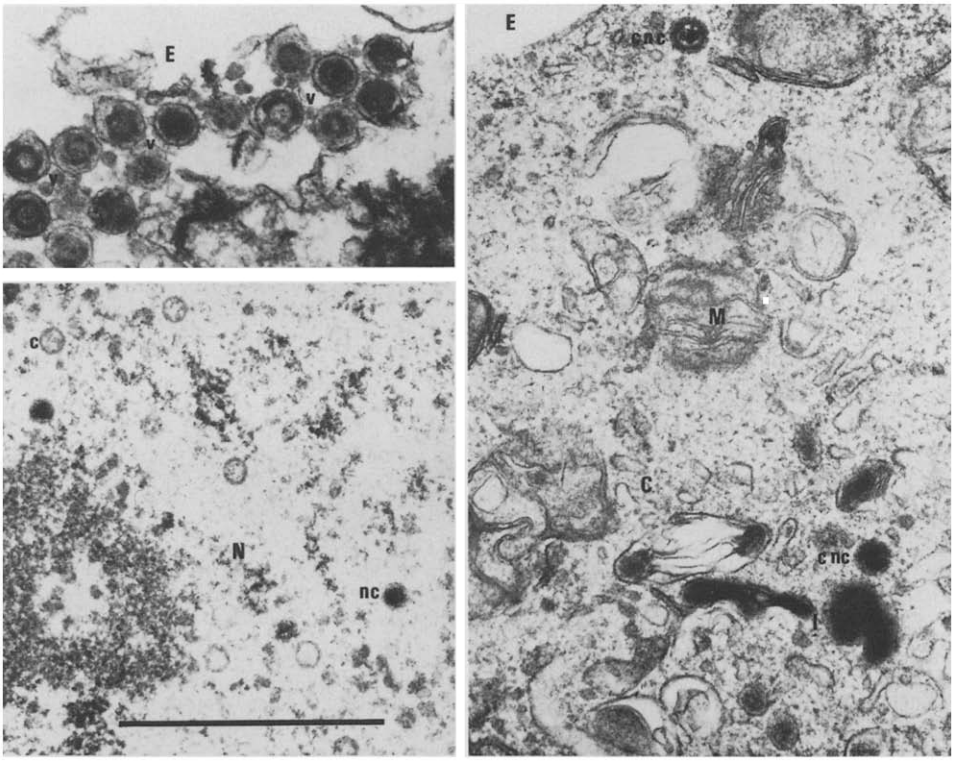

Fig. 1. HHV-6-infected cells cultured in presence of 250 mM of DMSO on post-treatment day 9. Many lysed cells, but also intact cells, are present. The figure shows capsids (c) and nucleocapsids (nc) in the nucleus (N). Coated nucleocapsids (cnc) are seen in the cytoplasm (C). M, mitochondrion. A cluster of virions (v) is seen in the extracellular space (E). The bar represents one micrometer.

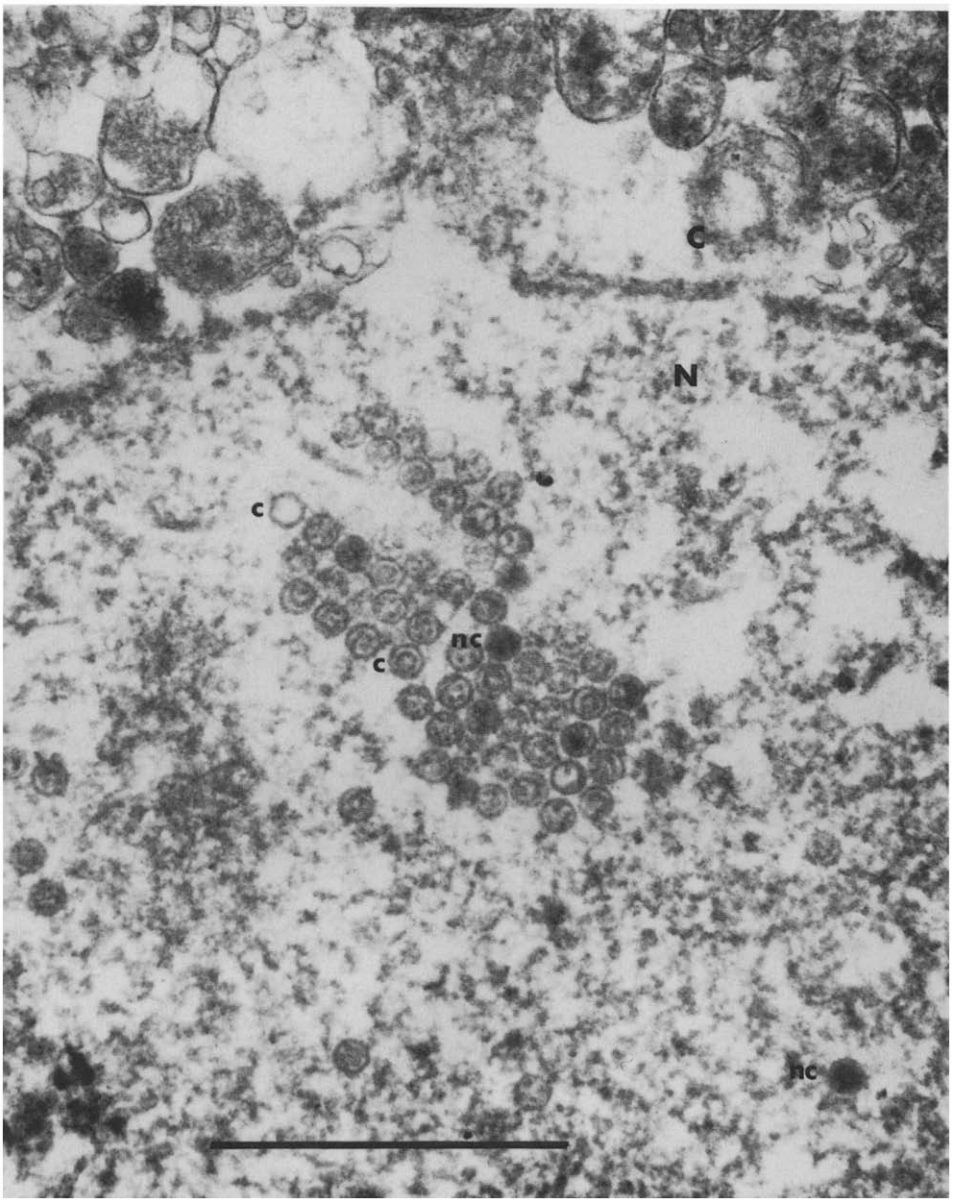

Fig. 2. The same experiment as in Fig. 1 but without DMSO. All cells are lysed. Neither extracellular virus nor cytoplasmic (coated) nucleocapsids are seen. Nuclear capsids, in more or less intact nuclei, are fairly common; some contain nucleic acid and are therefore nucleocapsids. The figure shows such a nucleus (N) containing capsids (c) and nucleocapsids (nc). C, remnants of cytoplasm. The bar represents one micrometer.

In another experiment (Table 3), six days after the addition of the chemicals, the number of p41-positive cells in the cultures containing the polar compounds

TABLE 3

Immunofluorescence of HHV-6-infected HSB_2 cells in presence of DMSO or DMF[a]

Treatment	Post-treatment day 6			Post-treatment day 9		
	IF+ cells[b] (%)	Giant cells[c] (%)	Viable cells[d] (%)	IF+ cells[b] (%)	Giant cells[c] (%)	Viable cells[d] (%)
Infection control	77.0 ± 3	36.0 ± 3	43 ± 4	39.0 ± 4	15.0 ± 2	18 ± 5
250 mM of DMSO	36.5 ± 3	14.0 ± 2	75 ± 2	36.0 ± 3	29.5 ± 3	67 ± 2
125 mM of DMSO	36.0 ± 2	15.0 ± 4	74 ± 5	21.0 ± 2	26.0 ± 3	71 ± 2
62.5 mM of DMSO	44.0 ± 2	18.0 ± 3	69 ± 1	57.0 ± 3	31.0 ± 2	56 ± 3
125 mM of DMF	50.0 ± 3	29.5 ± 2	60 ± 2	38.0 ± 2	49.0 ± 5	56 ± 5
62.5 mM of DMF	34.5 ± 4	18.5 ± 3	73 ± 4	44.0 ± 3	23.0 ± 2	66 ± 5
12.5 mM of DMF	30.5 ± 3	5.0 ± 2	67 ± 5	43.0 ± 5	23.0 ± 3	60 ± 4

[a] HHV-6-infected HSB_2 cells were treated with various concentrations of DMSO and/or DMF. 35% of cells were reactive to anti-p41 antibody on first day of treatment.
[b] Using a monoclonal anti-p41 antibody, the number of HHV-6-infected HSB_2 cells was evaluated by I.F. on days 6 and 9 after the addition of the polar compounds. 300 cells were counted per sample. [c] The percentage of giant cells, typical of HHV-6 infection, is also shown.
[d] Cell viability was estimated by trypan blue exclusion.

was significantly lower than in the control, as assessed by immunofluorescence. Similarly, the percentage of giant cells, a marker of HHV-6 infection, was significantly higher in the controls. However, by day 9 the picture changed: the number of I.F.-positive cells and giant cells in the control cultures decreased due to the reduction of cell viability caused by the progression of the infection, whereas the number of giant cells and/or I.F.-positive cells increased in the cultures containing the polar compounds. It should be noted that the level of infection in these cultures was 35% at the beginning of the experiment and thus, some viable cells were present in the control cultures on day 9. In

TABLE 4

Infectivity of HHV-6-infected HSB_2 culture supernatants on day 9 of DMSO and/or DMF treatment[a]

Treatment	Post-infection day 10
	Foci of fused cells/field[b]
Infection control	9.00 ± 0.63
250 mM of DMSO	0.83 ± 0.40
125 mM of DMSO	2.66 ± 0.52
62.5 mM of DMSO	7.66 ± 0.27
125 mM of DMF	3.50 ± 0.30
62.5 mM of DMF	0.16 ± 0.40
12.5 mM of DMF	0.50 ± 0.54
62.5 mM of DMSO + 62.5 mM of DMF	5.83 ± 0.2
62.5 mM of DMSO + 12.5 mM of DMF	3.83 ± 0.17

[a] Supernatants from HHV-6-infected HSB_2 cells, with or without DMSO and/or DMF, were collected on post-treatment day 9 and used to infect fresh HSB_2 cells.
[b] The percentage of foci of fused cells per field (6 fields/sample) in these cultures was estimated on post-infection day 10.

TABLE 5

Virus titration of supernatants from HHV-6-infected HSB_2 cell cultures on day 9 of DMSO or DMF treatment[a]

Treatment	$TCID_{50}$/ml[b]
Infection control	$10^{7.48}$
250 mM of DMSO	$10^{4.24}$
125 mM of DMSO	$10^{4.50}$
62.5 mM of DMSO	$10^{7.59}$
Infection control	$10^{7.74}$
125 mM of DMF	$10^{3.50}$
62.5 mM of DMF	$10^{3.74}$
12.5 mM of DMF	$10^{4.59}$

[a] Supernatants from HHV-6-infected HSB_2 cell cultures treated with DMSO or DMF were collected on day 9 and concentrated 10-fold by PEG precipitation. The infection controls were treated in the same way. Serial 10-fold dilutions were used to infect fresh HSB_2 cell cultures. The presence of virus-induced cytopathic effects was scored on day 10 post-infection (two sets of triplicates for each viral dilution sample). The appearance of clear cytopathic effects in the infection controls preceded the appearance of similar effects in the equivalent DMSO- or DMF-treated samples by at least 48 h. Absence of infection was confirmed by I.F. using the anti-p41 monoclonal antibody.

[b] $TCID_{50}$/ml was estimated by the method of Reed and Muench (1938).

contrast, in the experiments shown on Tables 1 and 2, the level of infection was higher on the first day, and the viability of the control cultures was nil by day 8.

It should be mentioned that neither DMSO nor DMF affected cell viability of HSB_2 cells at the concentrations used, and no virucidal effect was noticed when the virus was incubated alone with the compounds for 1 or 2 h.

The infectivity of supernatants from infected cultures in the presence and in the absence of DMSO and/or DMF on day 9 post-treatment is shown on Table 4. On post-infection day 10, a significant difference was observed between the number of foci of fused infected cells in the control and that in the cultures infected with supernatants from the polar compound-treated samples.

Titration of virus from DMSO-treated cultures showed that concentrations of 250 and 125 mM DMSO could reduce the titer of infectious virus by at least 3 $\log_{10}$ when compared to infected controls. Similarly, 125 mM and 62.5 mM DMF treatment reduced the virus titer by at least 4 $\log_{10}$, while 12.5 mM of DMF reduced such titers by at least 3 $\log_{10}$ when compared to the infected control (Table 5).

Discussion

DMSO and DMF are polar compounds known for their effect on cell differentiation (Collins et al., 1978; Francis et al., 1985; Friend et al., 1971; Preisler and Lyman, 1975; Preisler et al., 1973; Scher et al., 1973; Tanaka et al., 1975). DMSO has also been reported to have an effect on certain viruses. For

instance, it stabilizes enveloped viruses, like herpesviruses, thus protecting them from inactivation caused by freezing and thawing. This stabilization is achieved with concentrations of DMSO $\geqslant 5\%$ (Wallis and Melnick, 1968). West et al. (1989) observed that incubation of cells in the presence of DMSO and/or dexamethasone enhances the detection of herpes simplex viruses, this phenomenon being followed by an earlier and more extensive appearance of cytopathic effects in the treated cultures. In contrast, Chan and Gadebuch (1968) showed that high concentrations of DMSO ($\geqslant 70\%$) inactivate both DNA and RNA viruses.

An effect of DMSO on retroviruses was first suggested by the work of Sato et al. (1971): its presence presents release of Friend leukaemia virus from infected cells in culture. We have recently observed that DMSO and other polar compounds have an inhibitory effect on HIV production in vitro (Viza et al., 1989, 1991). Using similar concentrations, Yanase and Sugawara (1989) have shown inhibition of EBV induction in vitro in the presence of various polar compounds, including DMF and DMSO.

However, it is worth pointing out that despite the numerous publications describing the effect of these compounds, especially that of DMSO, the mechanism of action on cell differentiation and viral replication is not yet understood.

The results presented here suggest that both DMSO and DMF, used at concentrations similar to those previously described, also have an inhibitory effect on HHV-6 production. Indeed, the viability of HHV-6-infected cells incubated with the compounds was significantly higher than that of non-treated cells. Viral inhibition is partial: the cells eventually develop terminal infection but, in contrast to the non-treated cells, cell death occurs without widespread cell lysis (Figs. 1 and 2, Tables 1 and 2). Furthermore, in immunofluorescence, a few days after the beginning of the experiments, the number of infected cells is higher in the control cultures as compared with those containing DMSO and/or DMF, thus also implying a lower rate of infection in the treated cultures (Table 3). The effect is dose-dependent for DMSO, i.e., 250 mM of DMSO prevents cell lysis to a greater extent than 125 or 62.5 mM, whereas for DMF, the dose-effect relationship is less clear.

The association of DMF and DMSO does not produce any additive or synergistic effect, in some instances they appear even antagonistic, and no clear correlation between the protective effect and the concentration of the chemicals used could be established in these experiments. No plausible explanation can be offered at this stage for these observations. Albeit both DMF and DMSO are polar compounds, they might act through different mechanisms. Indeed, in an unrelated set of experiments, using the LDV/7 cell line (Viza et al., 1982), we observed that DMF's effect on cell morphology is strikingly different from that of DMSO. It might also be pertinent to mention here reports describing an antagonistic effect on cell differentiation between DMSO and butyric acid, which, although a non-polar compound, is also a cell differentiation inducer (Leder and Leder, 1975).

We have previously reported that treatment with DMSO of cell cultures infected by human immunodeficiency virus (HIV) enhances the accumulation of certain viral proteins on the surface of the infected cells, e.g. HIV p24, but at the same time, significantly reduces the amount of both viral p24 and HIV infectious particles released into the culture medium (Viza et al., 1989, 1991). It thus seems that the effect of incubating virus-infected cells in the presence of DMSO may vary according to the particular virus and/or cell line involved.

In the present experiments it was shown that the two chemicals have no direct virucidal effect, the pre-incubation of the virus with the compounds does not reduce its infectivity, nor do they enhance viral infection. Furthermore, they do not specifically inhibit certain viral proteins: immunoprecipitation of HHV-6-specific polypeptides showed the presence of the same range of viral proteins in both treated and non-treated cells, though there seems to be a reduction in the amount of viral proteins synthesized by the DMSO-treated cells (data not shown). One may thus hypothesize that these compounds inhibit viral production by acting on cellular control mechanisms. They seem to partially inhibit or to slow down early viral synthesis and subsequent release into the culture medium: supernatants from infected cultures treated with the polar compounds have reduced numbers of infectious virions in comparison to the controls (Table 5).

The data presented here suggest that polar compounds inhibit the production of infectious HHV-6, another member of the herpesvirus family. Since certain compounds, such as DMSO, have a low toxicity in vivo (Willson et al., 1965), their potential use for control of infections where HHV-6 is constantly reactivated, e.g. chronic fatigue syndrome, systemic lupus erythematosus, Kikuchi syndrome, AIDS, transplant patients (Ablashi et al., 1991), warrants further investigation.

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