Prostaglandins Enhance Spread of Herpes Simplex Virus in Cell Cultures

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SUMMARY

Stimuli such as u.v. light or trauma which induce recurrence of herpes simplex may act by affecting virus replication in the skin. Such stimuli release pharmacologically active agents in the skin, including prostaglandins (PGs) such as PGE2. These agents, and other compounds which alter levels of adenosine cyclic monophosphate (cyclic AMP), were tested for their effect on the replication of herpes simplex virus (HSV) in Vero cells.

Prostaglandin E2 (PGE2) and prostaglandin F2α both increase the size of HSV plaques; PGE2 also increases the yield of virus inoculated at low m.o.i. Moreover, inhibitors of prostaglandin synthesis decrease plaque size and inhibit the growth of virus inoculated at low m.o.i.; such inhibition can be partially overcome by adding PGE2. Analysis of the results suggest that prostaglandins can enhance cell-to-cell spread of HSV, but that cyclic AMP is probably not involved in this effect.

INTRODUCTION

Little is known about the mechanisms controlling the latency and reactivation of herpes simplex virus (HSV). The virus has been detected in the sensory nerve ganglia of man and animals following primary infection (Baringer, 1975; Stevens, 1975). It has been proposed that virus latency is controlled in the ganglion, perhaps by antibody (Stevens & Cook, 1974), and that recurrent disease results from activation of virus in the ganglion following a temporary suppression of the control mechanisms (Klein, 1976).

More recently, Hill & Blyth (1976) put forward a ‘skin-trigger’ theory; this proposes that virus is frequently released from the ganglion, but development of recurrent disease is controlled in the skin. It is suggested that after a local stimulus, physiological changes in the skin allow enhanced virus growth, leading to recurrence of clinical disease. Such recurrences of herpes simplex can be produced by applying various stimuli to the skin; these include u.v. light (Wheeler, 1975; Blyth et al. 1976), mild trauma (Hurd & Robinson, 1977; Hill et al. 1978), and the injection of prostaglandin E2 (PGE2) or even phosphate buffered saline (Blyth et al. 1976).

After injury, several physiologically active agents, including PGE2, are present in the skin (Goldyne, 1975), any or all of which may play a crucial role in the production of recurrent herpes simplex. In an attempt to predict which agents might be involved in inducing reactivation of disease, their effect on herpes simplex virus and on its growth in vitro was studied.
METHODS

Cell cultures. The Vero cells used throughout these studies were grown as described previously (Hill et al. 1975).

Viruses. The same SC.16 strain of HSV type 1 (Hill et al. 1975) was used as in our animal experiments (Hill et al. 1975, 1978; Blyth et al. 1976; Harbour et al. 1977).

Stock suspensions were prepared from infected Vero cells, and assayed on Vero cell monolayers (Hill et al. 1975).

Chemicals. The following were purchased from Sigma Chemical Company: 5-hydroxytryptamine (5-HT); L-epinephrine bitartrate (adrenalin); histamine diphosphate; spermine diphosphate; spermidine phosphate; adenosine cyclic 3'-5' monophosphate (cyclic AMP); N6O2'-dibutyryl adenosine cyclic 3'-5' monophosphate (dbcAMP); indomethacin; colchicine.

Lithium carbonate and theophylline (1,3-dimethyl-xanthine) were purchased from British Drug Houses Ltd. 8-3H-adenosine 3'5' cyclic phosphate, ammonium salt, sp. act. 26 Ci/mmol, and 1-5-3H-arginine monohydrochloride, sp. act. 11 Ci/mmol were purchased from the Radiochemical Centre, Amersham.

Prostaglandins E2 and F2α were gifts from Dr J. Pike of Upjohn Ltd., and Dr D. P. Evans of Parke, Davis & Co. supplied the mefenamic acid.

Drugs were dissolved directly in medium 199 with two exceptions; mefenamic acid and indomethacin were dissolved initially in dimethylsulphoxide and further diluted in 0.1 N-NaOH before final dilution in medium 199 (Inglot & Woyton, 1971). Serum was not included in the medium for any experiments unless otherwise noted.

Estimation of incorporation of tritiated arginine into cell proteins. The method of Knowles et al. (1975) was used, except that the final precipitate was dissolved in 250 µl of Soluene 350 and then mixed with 5 ml Dimilume (both from Packard Inc.) before counting in a Tri-Carb 3330 scintillation counter.

Assay of cyclic AMP. Cyclic AMP was assayed as described by Brown et al. (1972). To each cell culture tube, 1 ml of 0.05 M-tris-HCl buffer containing 8 mM-theophylline and 6 mM-2-mercaptoethanol was added. The cells were homogenized by shaking with glass beads on a 'Whirlimixer' (Fisons Scientific Apparatus Ltd.) and the suspensions were placed in a boiling water-bath for 3 min. They were then centrifuged at 3000 g for 5 min and the supernatants were stored at −35 °C before assay. The efficiency of the extraction method was 36 to 79 %, judged by the recovery of added tritiated cyclic AMP from cultures.

The assay mixture was filtered through a 0.45 µm Millipore membrane filter (Walton & Garren, 1970) after the 2 h incubation period. Membranes were dissolved in PCS solubilizer (Amersham-Searle) before counting in a scintillation counter. The binding protein used in the assay was kindly given by Dr J. D. M. Albano (Department of Medicine, Bristol).

For the determination of intracellular levels of cyclic AMP, cells were washed free of medium with the buffer before proceeding as indicated above.

Protein was estimated by the method of Lowry et al. (1951).

Estimation of cytotoxicity. A range of concentrations of each drug was tested; a solution of the drug was added (1 ml to each of five wells) to monolayers of Vero cells grown in ‘Multidishes’ (Sterilin Ltd.). The cultures were incubated at 35 °C in an atmosphere of 5 % CO2 and observed at 40× magnification daily for one week for morphological signs of cytotoxicity. Drugs were then tested at the highest concentrations that produced no such changes in three further ways, namely by measuring their effects on cell density, mitotic activity and the incorporation of 3H-arginine into cell proteins.
To estimate cell density and mitotic activity, confluent cultures on glass coverslips were treated for 24 h at 35 °C with the drug under test. The cells were then fixed with ethanol and stained with methylene blue. For the estimation of mitotic activity, colchicine to a final concentration of 10⁻⁶ M was added to the cultures 4 h before fixing. The cells, or the mitotic figures in 20 microscope fields, were counted at a magnification of 400 x.

For assay of incorporation of tritiated arginine into cell protein, culture medium was replaced by 1 ml of medium 199 containing the drug to be tested. At various times thereafter, 50 µl of medium 199 containing 1 µCi of ³H-arginine was added to each of four control cultures and four with the drug; 3 h later these were taken for the assay.

Estimation of direct virucidal activity. A solution of drug was mixed with an equal volume of medium 199 containing 1·2 × 10⁶ p.f.u. HSV. The mixture was incubated at 35 °C for 30 min and then diluted and assayed by plaque count on Vero cell monolayers.

Effect of drugs on efficiency of plaquing and plaque size. Cells were treated with the compound under test for 24 h before infection with virus at about 80 p.f.u./well for estimates of efficiency of plaquing, or 20 p.f.u./well for measurement of plaque size. After a period of adsorption in drug-free medium, medium with drug was added and the number of plaques produced was counted after 2 days incubation (Hill et al. 1975). For measurement of plaque size the cells were fixed with ethanol after 2 days incubation and lightly stained with 0·05% (w/v) malachite green. The areas of at least 26 plaques were measured at a magnification of 40 x using an eyepiece graticule divided into 0·25 mm² squares, and the mean areas were calculated. In some experiments on plaque size cultures were treated with drugs for 72 h before infection.

Results of measuring cell density, efficiency of plaquing and plaque size were analysed by Student's t-test.

RESULTS

Cytotoxic activity of drugs

At the concentrations used in subsequent experiments none of the drugs affected the cell density of confluent cultures or caused morphological signs of cytotoxicity, or altered the mitotic activity. Mefenamic acid, indomethacin or PGE₂ had little effect on the incorporation of ³H-arginine into cell proteins but PGF₂α (10 µg/ml) increased this by about 50% from 20 to 70 h after addition of the drug. With adrenalin (10 µg/ml) incorporation was depressed by about 20% at 20 to 30 h after addition of the drug, and by 40% after 48 h. With dbcAMP (500 µg/ml) or theophylline (100 µg/ml) incorporation was depressed by about 50% at 20 to 30 h after addition of the drug but thereafter returned to the levels in the control cultures.

Virucidal activity of drugs and their effect on plaquing efficiency

When used at the highest concentrations listed in Tables 1 and 2 none of the drugs was directly virucidal nor caused a decrease in the number of plaques in infected cultures. Similarly there was no effect with histamine (100 µg/ml), 5HT (100 µg/ml), dbcAMP (500 µg/ml), spermine (50 µg/ml), spermidine (100 µg/ml) or lithium carbonate (37 µg/ml).

Effect of drugs on plaque size

Treatment of cultures for 24 h with PGE₂ (0·1 to 10 µg/ml) or PGF₂α (10 µg/ml) before infection with HSV produced a significant increase in the size of plaques (Table 1). Similar treatment with 5HT (10 or 100 µg/ml) produced a smaller increase (about 20%, P < 0·05). By contrast, dbcAMP (500 µg/ml) or mefenamic acid (50 µg/ml) decreased plaque size.
Table 1. Effect on the size of HSV plaques of treating Vero cells with various chemicals for 24 h before infection

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (μg/ml)</th>
<th>Plaque size in mm² (mean ± s.d.)</th>
<th>% difference of mean from control mean</th>
<th>P (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>1.35 ± 0.77</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PGE₂</td>
<td>0.1</td>
<td>2.00 ± 0.83</td>
<td>+48 %</td>
<td>&lt;0.001</td>
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<tr>
<td></td>
<td>1.0</td>
<td>2.79 ± 1.53</td>
<td>+107 %</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.31 ± 1.07</td>
<td>+71 %</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PGF₁₂</td>
<td>0.1</td>
<td>1.76 ± 1.15</td>
<td>+30 %</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.56 ± 0.73</td>
<td>+16 %</td>
<td>&lt;0.05</td>
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<tr>
<td></td>
<td>10</td>
<td>2.12 ± 1.23</td>
<td>+57 %</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>None</td>
<td>—</td>
<td>1.40 ± 0.57</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Mefenamic acid</td>
<td>50</td>
<td>1.03 ± 0.51</td>
<td>-26 %</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>dbcAMP</td>
<td>50</td>
<td>1.07 ± 0.80</td>
<td>-24 %</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0.89 ± 0.56</td>
<td>-36 %</td>
<td>&lt;0.01</td>
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</tbody>
</table>

Table 2. Effect on the size of HSV plaques of treating Vero cells with various chemicals for 72 h before infection

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (μg/ml)</th>
<th>Plaque size in mm² (mean ± s.d.)</th>
<th>% difference of mean from control mean</th>
<th>P (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>2.36 ± 1.01</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PGE₂</td>
<td>1.0</td>
<td>2.16 ± 1.09</td>
<td>-8 %</td>
<td>&gt;0.05</td>
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<tr>
<td></td>
<td>10</td>
<td>3.17 ± 1.28</td>
<td>+34 %</td>
<td>&lt;0.01</td>
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<tr>
<td>PGF₁₂</td>
<td>0.1</td>
<td>2.49 ± 1.29</td>
<td>+6 %</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>2.70 ± 1.35</td>
<td>+14 %</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.03 ± 1.09</td>
<td>+28 %</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Theophylline</td>
<td>1.0</td>
<td>2.27 ± 1.05</td>
<td>-4 %</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.91 ± 0.87</td>
<td>-19 %</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.46 ± 0.47</td>
<td>-38 %</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Adrenalin</td>
<td>0.2</td>
<td>1.87 ± 0.85</td>
<td>-21 %</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>1.71 ± 0.84</td>
<td>-28 %</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.54 ± 0.99</td>
<td>-35 %</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>None</td>
<td>—</td>
<td>1.77 ± 1.01</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Mefenamic acid</td>
<td>50</td>
<td>1.28 ± 0.58</td>
<td>-28 %</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

(Table 1) as did 100 μg/ml spermidine or 50 μg/ml spermine (about 20 % decrease, P < 0.05, for each drug). Histamine (100 μg/ml) and lithium carbonate (37 μg/ml) were without effect.

In cultures treated for 72 h before infection with HSV a somewhat different picture was consistently seen (Table 2). The increases in plaque size induced by PGE₂ and PGF₁₂ were smaller than before. However adrenalin and theophylline produced significant decreases in plaque size whereas, with pre-treatment for 24 h, this was not seen. Mefenamic acid (50 μg/ml) caused a decrease similar to that seen with 24 h pre-treatment. Spermine, spermidine, dbcAMP, histamine, 5HT or lithium carbonate (concentrations as above) produced no effects.
Yield of virus at low multiplicity of infection

The medium was removed from each of a number of 10 × 1 cm glass tubes each containing confluent monolayers of Vero cells, and a 50 μl inoculum containing 0.01 p.f.u./cell (low m.o.i.) was added. After 1 h adsorption at 35 °C, 1 ml of medium 199, with or without the drug under test, was added and the tubes were incubated at 35 °C. For each treatment four tubes were taken at various times after infection and the cells were disrupted by freezing and thawing three times. Infectious virus was then assayed in monolayers of Vero cells. In other experiments, the cells were treated with the compound under test for 24 h before infection with virus, the drug being replenished after adsorption.

Prostaglandin E₂ (10 μg/ml) and PGF₂α (1 μg/ml) both increased virus yield (Fig. 1, 2). Other experiments showed that when the concentration of PGF₂α was increased from 1 μg/ml to 10 μg/ml, yields of virus were depressed by three- to ten-fold, which may represent a bell-shaped dose response curve to PG concentration (Horrobin, 1977). In contrast, theophylline (Fig. 1), mefenamic acid (50 μg/ml) or indomethacin (Fig. 2) all decreased virus yields. Mefenamic acid (10 μg/ml) also decreased yields, in this case by about seven-fold throughout the period 20 to 70 h after infection. Similar results were obtained whether the cultures were treated before infection for 24 h, or when the drugs were added immediately after adsorption of virus. To check the specificity of the decrease in virus yield by prostaglandin synthetase inhibitors, monolayers of cells were treated simultaneously with PGE₂ and either mefenamic acid or indomethacin (Fig. 2). With the concentrations of drugs shown in Fig. 2 the yield of virus was raised above that obtained with inhibitors alone, but rarely reached control levels. However, when a lower dose (10 μg/ml) of mefenamic acid was mixed with PGE₂ (10 μg/ml) there was no decrease in the yield of virus.

The results with theophylline suggested that cyclic AMP might be involved in altering virus yields, since this compound can increase cyclic AMP levels (Chlapowski et al. 1975). Similar experiments were therefore done to test the effect of cyclic AMP (500 μg/ml) on virus yield, but even within one experiment the yields of virus varied in a seemingly random manner. However, exogenous cyclic AMP does not penetrate cells readily and is rapidly
Fig. 2. Effect of inhibitors of prostaglandin synthesis and PGE$_2$ on yield of HSV at low multiplicity of infection: ▲, 10 μg/ml PGE$_2$; ○, 50 μg/ml mefenamic acid; ●, 50 μg/ml mefenamic acid + 10 μg/ml PGE$_2$; □, 18 μg/ml indomethacin; ■, 18 μg/ml indomethacin + 10 μg/ml PGE$_2$.

Each point represents the mean yield from 2 to 4 experiments. Bars represent ± s.e. between experiments. * log geometric mean titre with drug minus log geometric mean titre without drug.

broken down by phosphodiesterase. The experiments were repeated using dbcAMP which may enter cells more easily (Robison et al. 1971) and is more resistant to degradation by phosphodiesterase (Kaukel & Hilz, 1972). The results obtained with this compound were similar to those with theophylline in that virus yields were consistently decreased (Fig. 1). When adrenalin (20 μg/ml) was added to medium in similar experiments it had little effect on virus yields. In all experiments only the maximum yield of virus was altered in the presence of drugs; there was no effect on the time at which infectious virus was first seen, or on the rate of its production.
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Yield of virus at high multiplicity of infection

With a multiplicity of infection of 10 p.f.u./cell, the effect of theophylline (100 μg/ml), dbcAMP (500 μg/ml), mefenamic acid (50 μg/ml) or PGE₂ (10 μg/ml) on the yield of HSV was tested in experiments similar to those using a low m.o.i., but carried on only until 22 h after infection. None of the drugs affected the time at which infectious virus was first produced, its rate of production or the maximum yield.

Effect of drugs on levels of cyclic AMP

The medium was removed from confluent monolayers of cells in 10 x 1 cm tubes and replaced by 1 ml of a solution of the drug to be tested. In control cultures, 1 ml of medium 199 was added instead of the drug solution. At various intervals, four test and four control tubes were taken and assayed for cyclic AMP.

The level of cyclic AMP in untreated cells was very low, generally < 40 pmol/mg protein. Addition of the medium by itself usually raised the basal levels of total and intracellular cyclic AMP. After treatment with PGE₂, theophylline, or dbcAMP, both total and intracellular levels of cyclic AMP were further increased.

In cultures infected with 10 p.f.u./cell of HSV, no rise was seen in levels of cyclic AMP during the 50 h after infection.

DISCUSSION

In monolayers of Vero cells treated with PGE₂ the cell density, mitotic activity and incorporation of tritiated arginine into cell proteins are all unaffected. Nevertheless, in the presence of the drug, HSV produces larger plaques than in untreated cultures. Moreover, at low m.o.i. the virus yields are increased by treatment with PGE₂ whereas at high m.o.i. there is no such effect. This indicates that PGE₂ does not enhance the growth of the virus by increasing the amount produced per cell, but enables the virus to spread from cell to cell more efficiently. Since the rate of production of infectious virus is also unaffected by PGE₂, the drug must enhance virus spread in some way other than by accelerating virus replication.

The fact that two inhibitors of PG biosynthesis, mefenamic acid and indomethacin, each decreased HSV yields at low m.o.i. but not at high m.o.i. suggests that endogenous PGs also are involved in spread of virus from cell to cell. When either of the inhibitors was added together with PGE₂, the decrease in yield was smaller than with inhibitor alone. Thus exogenous PGE₂ is, in part at least, able to overcome the effect of the inhibitors of prostaglandin synthetase. The fact that PGE₂ only partially reversed the action of these drugs suggests that other PGs, whose synthesis they would also block, might play some role in the spread of HSV from cell to cell. PGF₂α also increases plaque size, and to some extent virus yield, although it is less active than PGE₂. PGF₂α also increases protein synthesis (as indicated by incorporation of tritiated arginine) which may be favourable for virus production.

We and others (Chlapowski et al. 1975) have shown that addition of PGE₂ increases levels of cyclic AMP in cell cultures. This seems unlikely to be related to its effect on the spread of HSV, since addition of dbcAMP or theophylline, each of which also increases cyclic AMP levels, decreases the size of plaques and the yield of virus. Stanwick et al. (1977) found a similar decrease in virus yield from human embryo fibroblasts treated with dbcAMP, theophylline or papaverine. PGs must therefore enhance virus spread in some other way; possibly by an effect on the cell membrane.
Prostaglandin $E_2$ or PG synthetase inhibitors had little or no effect on two RNA viruses, measles virus and Coxsackie virus B1 (D. A. Harbour, W. A. Blyth & T. J. Hill, unpublished results). The result with measles virus is perhaps surprising, as it is enveloped and also spreads directly from cell to cell.

The results obtained here may help to shed some light on the pathogenesis of recurrent herpes simplex. If PGs enhance virus spread \textit{in vivo} as well as \textit{in vitro} they would be good candidates for a 'skin-trigger', since they are released in the skin following many types of damage (Goldyne, 1975). Injection of PGE$_2$ into the skin of mice induces recurrent disease (Blyth \textit{et al} 1976) but several other mediators of inflammation are also produced by the trauma of injection. Further work to clarify the relative importance of the various mediators in the induction of recurrent disease \textit{in vivo} is now in progress.

The finding that mefenamic acid and indomethacin decrease virus spread \textit{in vitro} raises the possibility of their use as anti-herpetic agents. Indeed, Inglot & Woyton (1971) have already reported on the efficacy of these drugs for treating HSV infections.

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