Cyclic AMP-mediated inhibition of vesicular stomatitis virus and herpes simplex virus replication in mouse macrophage-like cells

Paola Borghi, Paola Di Marzio, Barbara Varano, Lucia Conti, Filippo Belardelli and Sandra Gessani*

Laboratory of Virology, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy

In this study, we have analysed the effects of cAMP inducers on the multiplication of vesicular stomatitis virus (VSV) and herpes simplex virus type 1 (HSV-1) in mouse macrophage-like cells. The addition of dibutryl cAMP (dB-cAMP) or cholera toxin to resting peritoneal macrophages aged in vitro or P388D1, cells resulted in a 10- to 100-fold reduction of VSV yield compared to control cultures. In contrast, no cAMP-dependent inhibition was found in VSV-infected L929 cells. In macrophage-like cells, the dB-cAMP-induced antiviral state was not inhibited by antibodies to interferon (IFN)-α/β and did not correlate with any increase in the intracellular levels of 2-5 oligo(A) synthetase. Di-butryl cAMP did not inhibit virus yields in mouse macrophages infected with encephalomyocarditis virus. In P388D1 cells, the addition of dB-cAMP resulted in an approximately 10-fold inhibition of HSV-1 replication with respect to control cultures, as evaluated both by TCID₅₀ and plaque assays on Vero cells. Dibutyryl cAMP did not affect VSV binding or entry into mouse macrophages and the cAMP-mediated anti-VSV state was significantly reduced by inhibitors of protein kinase C (i.e. staurosporine and H7). These data suggest that macrophages may acquire resistance to infection by VSV and HSV-1 after treatment with cAMP inducers. This cAMP-mediated antiviral activity does not depend on the modulation of the endogenous IFN system, suggesting that macrophages exhibit multiple resistance mechanisms (i.e. IFN-dependent and IFN-independent) to maintain their intrinsic antiviral activity.

Introduction

Macrophages play an important role in the natural defence against virus infections (for reviews, see Mogensen, 1979; Morahan et al., 1985). In particular, macrophages exhibit an intrinsic resistance to virus infections (Mogensen, 1979; Morahan et al., 1985). We have previously demonstrated that the intrinsic resistance of mouse peritoneal macrophages (PMs) to vesicular stomatitis virus (VSV) or to encephalomyocarditis virus (EMCV) was due largely to the spontaneous expression of mouse interferon (IFN)-α/β (Belardelli et al., 1984). In fact, injection of mice with antibodies to IFN-α/β rendered PMs permissive for VSV or EMCV (Belardelli et al., 1984). Likewise, in vitro cultivation of PMs resulted in a time-dependent decay of the anti-VSV state, which was accelerated markedly by the in vitro addition of antibodies to mouse IFN-α/β (Belardelli et al., 1987). Treatment of in vitro aged, virus-permissive PMs with different stimuli [such as lipopolysaccharide (LPS) from Gram-negative bacteria, IFN-γ, monoclonal antibodies to Mac-1 antigen, macrophage colony-stimulating factor] resulted in the establishment of an antiviral state to VSV, which was mediated by the induction of endogenous IFN-β (Di Marzio et al., 1990). In the course of these experiments, we observed that treatment of in vitro aged PMs with agents capable of increasing the intracellular levels of cAMP resulted in a significant inhibition of VSV yield. Previous studies have shown that an increased accumulation of intracellular cAMP levels resulted in an inhibition of virus replication in some cell systems (Robbins & Rapp, 1980; Beushausen et al., 1987), although some stimulatory effects have been reported also in other virus-cell models (Kuno et al., 1986). Therefore it was of interest to investigate in some detail the effect of cAMP inducers on the replication of different viruses in macrophages.

In this paper, we provide evidence indicating that inducers of intracellular cAMP production, such as N6,O2-dibutyryl-cAMP (dB-cAMP) or cholera toxin, inhibit the multiplication of VSV and herpes simplex virus type 1 (HSV-1) in mouse PMs and/or macrophage-like cells and that this inhibition is not mediated by endogenous IFN. These results, together with our
previous data, show that macrophages can exhibit multiple resistance mechanisms (IFN-dependent and IFN-independent) to maintain their intrinsic antiviral activity.

Methods

Mice. Male C3H/HeN mice (5 to 8 weeks old) were obtained from Charles River.

Reagents. RPMI 1640 medium (Microbiological Associates Bioproducts) was supplemented with penicillin [100 units (U)/ml], streptomycin (100 μg/ml), 2 mM-glutamine and foetal calf serum (FCS) (Biological Industries, Kibbutz Beth Haemek, Israel), at a final concentration of 10% (v/v). All tissue culture reagents were purchased as endotoxin-free, as assessed by the Limulus amoebocyte assay.

Cells. Peritoneal cells were harvested by washing the peritoneal cavity with 10% FCS-RPMI 1640 medium and seeded in 24-well plates (1 x 10^6/well). After 3 h at 37 °C, non-adherent cells were removed by three washes with medium. PMs were then 'aged' for 4 days, to render them permissive for viral replication.

P388D1 cells, a mouse macrophage cell line (Koren et al., 1975), were cultivated in 10% FCS-RPMI 1640 medium.

Virus titration. The origin, methods of preparation, and assay of VSV (Indiana strain) cells and EMCV in mouse L929 cells have been described previously (Belardelli et al., 1984). HSV-1 was prepared in Vero cells and titrated by plaque assay as described elsewhere (Leary et al., 1985). HSV-1-infected cells were frozen and thawed three times and then centrifuged at 1500 g for 5 min at 4 °C. The resulting virus-rich supernatant was titrated on Vero cell monolayers.

VSV, EMCV and HSV-1 yield assays. A sample (0.2 ml) of a viral dilution was added to PMs or P388D1 cells. The m.o.i.s used were 0.1 p.f.u./cell for VSV and EMCV and 0.3 p.f.u./cell for HSV-1. After a 1 h incubation at 37 °C, the cell monolayers were washed extensively and 1 ml of medium containing 10% FCS was added. After 18 h at 37 °C, supernatants were harvested and virus yields were titrated on L929 cells, as described in Methods. There were three experimental points for each cell line.

Determination of intracellular 2-5A synthetase activity was measured as previously described (Gresser et al., 1985).

S1 mapping of IFN-β and IFN-α mRNAs. Total cellular RNA was extracted as described (Chirgwin et al., 1979). The synthesis of complementary RNA probes and solution hybridization were carried out as previously described (Gessani et al., 1989).

Determination of intracellular cAMP levels in PMs. Cellular extracts were prepared as described elsewhere (Belardelli et al., 1980). The cAMP concentration was measured by the protein-binding competition method (cyclic AMP [3H] assay system, Amersham).

Preparation of 35S-labelled VSV. Confluent monolayers of L929 cells were maintained for 3 h in methionine-free medium containing 0.5% dialysed FCS. The cells were then infected with VSV at 0.3 p.f.u./cell. After 2 h at 37 °C, the virus was removed and medium containing [35S]methionine (Amersham, 370 MBq or 10 mCi) was added. After 18 h, the total virus was collected and purified by sucrose gradient centrifugation, as previously described (Belardelli et al., 1984).

Statistical analysis. Data were analysed by Student's t-test.

Results

Inhibition of VSV yield by treatment of PMs or P388D1 cells with db-cAMP or cholera toxin

As shown in Fig. 1, pretreatment of mouse PMs or P388D1 macrophage-like cells with db-cAMP resulted in an approximately 10- to 100-fold inhibition of VSV yield, as compared to control cultures. Cholera toxin, a known inducer of adenylate cyclase activity, also induced resistance to VSV infection in PMs (Fig. 1) and in P388D1 cells (data not shown). In contrast, no reduction of VSV yield was observed in L929 cells after pretreatment with db-cAMP (Fig. 1) or cholera toxin (data not shown).

These results indicated that agents capable of increasing the intracellular level of cAMP induced a definite inhibition of virus yield after infection with VSV of macrophage-like cells (i.e. PMs and P388D1 cells), but not of other cell types (i.e. L929 cells).

The inhibition of VSV yield by db-cAMP in macrophage-like cells is not mediated by the induction of endogenous IFN and does not correlate with any increase in the intracellular levels of 2-5A synthetase

We had previously demonstrated that endogenous IFN-β was responsible for the resistance to VSV infection induced in virus-permissive PMs by different stimuli (Di Marzio et al., 1990). It was of interest, therefore, to investigate whether the db-cAMP-induced inhibition of
VSV yield was neutralized by the simultaneous addition of antibodies to IFN-α/β. No inhibition of the dB-cAMP-induced antiviral state was observed in PMs treated with a potent preparation of antibodies to IFN-α/β (data not shown), which effectively neutralized the antiviral state induced in PMs by LPS or IFN-γ (Gessani et al., 1989). As shown in Fig. 2, dB-cAMP induced a 10- to 100-fold inhibition of VSV yield in PMs without resulting in any increase in the intracellular levels of 2-

Table 1. Effects of dB-cAMP on the multiplication of VSV and EMCV in PMs and of VSV and HSV-1 in P388D1 cells*

<table>
<thead>
<tr>
<th>Cell type</th>
<th>dB-cAMP</th>
<th>Virus challenge</th>
<th>TCID_{50} (log_{10})</th>
<th>P.f.u./ml (log_{10})</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM - VSV</td>
<td>+</td>
<td>VSV</td>
<td>4.7 ± 0.3</td>
<td>5.1 ± 0.2</td>
</tr>
<tr>
<td>PM + VSV</td>
<td>-</td>
<td>VSV</td>
<td>3.8 ± 0.2</td>
<td>4.0 ± 0.3</td>
</tr>
<tr>
<td>PM - EMCV</td>
<td>+</td>
<td>EMCV</td>
<td>6.6 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td>PM + EMCV</td>
<td>-</td>
<td>EMCV</td>
<td>6.8 ± 0.1</td>
<td>ND</td>
</tr>
<tr>
<td>P388D1 - VSV</td>
<td>+</td>
<td>VSV</td>
<td>6.5 ± 0.1</td>
<td>ND</td>
</tr>
<tr>
<td>P388D1 + VSV</td>
<td>-</td>
<td>HSV</td>
<td>5.6 ± 0.2</td>
<td>7.2 ± 0.1</td>
</tr>
<tr>
<td>P388D1 + HSV</td>
<td>+</td>
<td>HSV</td>
<td>4.5 ± 0.4</td>
<td>6.5 ± 0.3</td>
</tr>
</tbody>
</table>

* Evaluation of virus yields by TCID_{50} and plaque assays. Mouse PM and P388D1 cells were treated for 24 h with dB-cAMP (5 mM), before infection with VSV, EMCV or HSV-1 as described in Methods. Virus yields were titrated on L929 or Vero cells (see Methods). There were three different cultures for each experimental point.

† ND, Not determined.

5A synthetase. This enzyme activity was greatly increased after IFN-α/β treatment of PMs.

To investigate directly the possible effect of dB-cAMP on the expression of IFN genes in PMs, we performed S1 mapping analysis of RNAs extracted from PMs. No accumulation of IFN-α2 or IFN-β mRNAs was detected in dB-cAMP-treated mouse PMs, whereas high levels of these mRNAs were found in Newcastle disease virus (NDV)-infected cultures (Fig. 3).

Effects of dB-cAMP on the multiplication of VSV, EMCV and HSV-1 in macrophage-like cells, as evaluated by TCID_{50} or plaque assays

It was of interest to investigate whether dB-cAMP was effective in inhibiting virus multiplication in macrophage-like cells infected with other RNA viruses (for instance with non-enveloped picornaviruses, such as EMCV) or with DNA viruses (i.e. HSV-1). As shown in Table 1, dB-cAMP did not inhibit the multiplication of EMCV in PMs, whereas in the same experiment, it was markedly effective in inhibiting VSV yields, as measured in either way (Table 1).

dB-cAMP does not affect VSV binding or entry into mouse PMs

As shown in Table 2, pretreatment of mouse PMs with dB-cAMP did not inhibit the binding or uptake of ^{35}S-labelled VSV. In replicate samples infected with the
Table 2. Lack of correlation between the binding and uptake of 35S-labelled VSV and virus yield in PMs treated with dB-cAMP*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell-associated VSV† (c.p.m.)</th>
<th>Internalized VSV‡ (c.p.m.)</th>
<th>VSV yield§ (log_{10})</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>963 ± 6</td>
<td>315 ± 21</td>
<td>6.0 ± 0.2</td>
</tr>
<tr>
<td>dB-cAMP</td>
<td>1025 ± 71</td>
<td>309 ± 11</td>
<td>4.7 ± 0.2</td>
</tr>
</tbody>
</table>

* PMs were aged in vitro for 4 days and subsequently treated for 24 h with dB-cAMP (5 mM) or left untreated. PMs were then infected with 35S-labelled VSV (0.1 p.f.u./cell; 14000 c.p.m./well).
† After 1 h at 37 °C, cells were washed three times with PBS, solubilized in 150 μl of 1% SDS, and added to 10 ml of scintillation liquid (Lumagel from Lumac). There were two wells for each experimental point.
‡ After 1 h at 37 °C, cells were washed twice with PBS, treated with trypsin (1 mg/ml) and EDTA (1 mM), transferred to plastic tubes, and centrifuged. Cells were washed further with PBS and pellets were solubilized in 150 μl of 1% SDS and added to 10 ml of scintillation liquid.
§ Virus yields were harvested 18 h after infection of PMs and titrated for TCID_{50}, as described elsewhere (Belardelli et al., 1984).

Effects of different inhibitors of protein kinases (PKs) on the dB-cAMP-induced resistance to VSV infection in mouse PMs

We then examined the effect of different inhibitors of PK on the induction of an antiviral state by dB-cAMP in PMs. PMs were treated with staurosporine, a PK C inhibitor (Tamaoki et al., 1986), or with other PK inhibitors (i.e. H7, H8 and HA1004). H7 is the most potent PK C inhibitor among the isoquinolinesulphonamide group of inhibitors, whereas H8 inhibits cAMP-dependent PK more efficiently than does H7 (Hidaka et al., 1984). HA1004 is the weakest PK C inhibitor among these compounds, and it is commonly used as a control for H7 (Hidaka et al., 1984). In cells treated with H7 or staurosporine, the induction of the antiviral state by dB-cAMP was significantly reduced (Table 3). The treatment with H8 resulted in a slight inhibition of the dB-cAMP antiviral effect, which was not statistically significant. HA1004 had no inhibitory effect.

Decay of the antiviral state to VSV of freshly explanted PMs during in vitro cultivation does not correlate with any change in the intracellular levels of cAMP

Previously we demonstrated that the in vitro decay of the antiviral state to VSV of freshly harvested mouse PMs was markedly enhanced by the addition of antibodies to IFN-α/β (Belardelli et al., 1987). This decay in the antiviral state paralleled a time-dependent decrease in the levels of IFN-β mRNA (our unpublished observations). In the light of the results reported above, it was of interest to investigate whether freshly harvested PMs exhibited higher levels of intracellular cAMP than in vitro aged virus-permissive PMs. Similar intracellular levels of cAMP (i.e. 1 to 4 pmol of cAMP/10⁶ cells) were found in both non-permissive freshly harvested PMs and in 4 day-cultured permissive macrophages.

Discussion

The results reported in this article indicate that agents capable of increasing the intracellular levels of cAMP (i.e. dB-cAMP and cholera toxin) are capable of inhibiting the multiplication of VSV in mouse PMs and in macrophage-like cell lines (i.e. P388D1 cells). However, cAMP-dependent inhibition of VSV yield was not observed in L929 cells (Fig. 1). In P388D1 cells, dB-cAMP also induced a marked reduction of HSV-1 yield as compared with control cultures (Table 1).

Previously we demonstrated that PMs exhibit a specific ability to respond to different stimuli (such as LPS, IFN-γ and other biological response modifiers) for the production of IFN-β (Gessani et al., 1989; Di Marzio et al., 1990). It was possible to envisage therefore that the cAMP-mediated antiviral activity observed in PMs could be due to the induction of endogenous IFN. However the data presented in this paper clearly indicate that IFN is not involved in this phenomenon. In fact, antibodies to IFN-α/β did not neutralize the cAMP-mediated inhibition of VSV yield. Likewise, the addition of dB-cAMP to PMs did not result in any accumulation of specific mRNAs for IFN-α and IFN-β (Fig. 3) and was not associated with any increase in the intracellular...
levels of 2-5A synthetase activity, an enzyme positively regulated by IFNs in PMs (Gresser et al., 1985).

It has been reported that cAMP inducers can potentiate the antiviral activity of IFN (Friedman & Pastan, 1969; Allen et al., 1974; Weber & Stewart, 1975). In the light of these data, it was possible to assume therefore that the expression of low levels of endogenous IFNs in mouse PMs may synergize with cAMP in inducing an increased antiviral state. However, the finding that dB-cAMP also induced inhibition of VSV and HSV-1 multiplication in P388D1, cells, which apparently do not express any spontaneous IFN, supports the conclusion that the cAMP-mediated antiviral activity observed in macrophage-like cells does not require any basal expression of IFN.

It has been previously reported that modifications of the intracellular levels of cyclic nucleotides can markedly affect virus replication in various cell types (reviewed by Ongradi & Telekes, 1990). This influence may be on either the activation of infectious progeny formation from a virus in a latent state or the suppression of replication. This type of control over virus expression may be somewhat specific for each virus group and the particular host cell (Ongradi & Telekes, 1990). The mechanisms by which an increased intracellular accumulation of cAMP may result in the inhibition of virus multiplication in macrophages are not yet defined. The finding that treatment of PMs with dB-cAMP does not alter the binding and uptake of labelled VSV (Table 2) indicates that the early entry steps of the virus cycle are not affected by cAMP. Inducers of cAMP have been shown to induce a variety of alterations in macrophage-like cells, including an increased Fc receptor-mediated phagocytosis (Muschel et al., 1977), a decreased production of LPS-induced cytokines (Katakami et al., 1988; Taffet et al., 1989; Tannenbaum & Hamilton, 1989; Ohmori et al., 1990) and changed cell morphology and decreased cell proliferation (Rosen et al., 1979). cAMP regulates the expression of a number of genes through a conserved promoter element, the CRE (Roesler et al., 1988). Moreover, cAMP induced stable transcription of specific genes such as the c-fos gene in macrophages, and this induction is qualitatively different from the response to cAMP of other cell types (Bravo et al., 1987). These data underline the specific responses of macrophages to cAMP. It is possible to assume, therefore, that an increased accumulation of intracellular cAMP in macrophages may result in specific molecular and morphological changes capable of interfering with some intracellular steps of the virus cycle. The finding that dB-cAMP did not inhibit the multiplication of EMCV, neither in PMs (Table 1) nor in P388D1 cells (data not shown), might suggest that the cAMP-dependent virus inhibition in macrophage-like cells is related to specific events interfering with the cycle of enveloped viruses. It is of interest to note that the cAMP-mediated antiviral state to VSV was almost completely abolished by inhibitors of PK C such as staurosporine or H7. These findings suggest that PK C may be involved in the cAMP-induced suppression of VSV and HSV-1 in macrophages.

Lastly, it is possible to speculate that, in macrophages, cAMP may alter the autocrine production of putative factors such as prostaglandins (PGs) or cytokines, capable of affecting VSV or HSV-1 production in infected cultures. In particular, some PGs (i.e. PGAs and PGJs) have been shown to exhibit a potent antiviral activity against VSV and HSV and this antiviral effect is not mediated by the IFN system (Ongradi & Telekes, 1990; Santoro et al., 1991). It is possible to speculate therefore that an increased production of such factors in dB-cAMP-treated macrophages may result in some inhibition of virus replication.

The intrinsic antiviral activity of macrophages is considered to play important roles in natural restriction of virus infection (Mogensen, 1979; Morahan et al., 1985). We had previously demonstrated that the spontaneous expression of IFN in PMs was mostly responsible for the non-permissiveness of these cells to VSV and EMCV (Belardelli et al., 1984; Gresser et al., 1985). In addition to endogenous IFN, other factors have been shown to play a role in the resistance of PMs to infection with HSV-1 (Sit et al., 1988). The data reported in this article provide the first evidence, to the best of our knowledge, indicating that the intrinsic antiviral activity of macrophages against some viruses can partially depend on the intracellular levels of cAMP. We can envisage that several factors such as PGs and other biological response modifiers might stimulate the intracellular levels of cAMP in PMs in vivo, thus resulting in the enhancement of the natural resistance to some virus infections. Our results further underline the antiviral role of macrophages and their specific ability to respond to a variety of stimuli by increasing their intrinsic antiviral activity.

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References


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