The effects of poly(I).poly(C12U) and interferon on the multiplication of a mammalian type C retrovirus in human cells

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Poly(I).poly(C12U) or interferon treatment inhibited multiplication of the xenotropic baboon type C endogenous retrovirus M7 in chronically infected human AV3-M7 cells, as determined by a reverse transcriptase (RT) assay and electron microscopy. Furthermore, this polynucleotide induced 2'5' oligoadenylate (2'5'A) synthetase activity. In contrast to interferon (IFN), poly(I).poly(C12U) did not give rise to the appearance of a trapping phenomenon observable by electron microscopy. When AV3-M7 cells were treated simultaneously with poly(I).poly(C12U) and anti-IFN-β/α antibodies, the induction of 2'5'A synthetase was abolished without any alteration of the inhibitory effect of RT activity. Taken together, these results suggest that different mechanisms are used by poly(I).poly(C12U) and IFN in blocking type C retrovirus multiplication.

Introduction

Double-stranded RNAs are potent inducers of interferon (IFN) α and β (Stewart, 1979; Lengyel, 1982), and are also involved in the mechanism of IFN action (Torrence et al., 1982; Baglioni & Nilsen, 1983; Hovanessian, 1991). IFNs exert their action first by binding to receptors on the cell surface (Pestka et al., 1987), with the result that many proteins are induced, two of which, a protein kinase and 2'5' oligoadenylate (2'5'A) synthetase, have been characterized. 2'5'A synthetase, after being activated in vitro by dsRNA, catalyses from ATP the synthesis of 2'5'A with 2'5' phosphodiester bonds. 2'5'A activates an endoribonuclease to degrade RNA and thereby block translation and virus replication (Ball, 1982). IFNs are known to have antiviral, antiproliferative and immuno-modulatory activities (Pestka et al., 1987; Clemens & McNurlan, 1985). DsRNAs, in addition to their capacity to induce IFN, also activate certain IFN-associated mediators, possess immuno-modulatory activity and inhibit tumour cell proliferation (Carter & De Clercq, 1974; Hearl & Johnston, 1986; Carter et al., 1976; Haines et al., 1991; Thang & Guschlbauer, 1992). The therapeutic value of dsRNAs as an antiviral/anticancer drug has been difficult to assess owing to the toxicity of the prototype molecule poly(I).poly(C) at every thirteenth residue, is much less toxic by being rendered sensitive to RNase destruction while retaining the biological properties mentioned above (Carter et al., 1972; Ts'o et al., 1976; O'Malley et al., 1979). It has been shown recently that poly(I).poly(C12U) is a more potent anti-human immunodeficiency virus (HIV) agent than IFN-α, IFN-β or IFN-γ, or all three IFNs combined (Montefiori & Mitchell, 1987), and that it can inhibit cellular growth independently of IFN induction (Hubbel et al., 1991).

In the present study, we compared the effect of poly(I).poly(C12U) and IFN on virus multiplication and 2'5'A synthetase induction in human AV3 cells chronically infected by the M7 strain of the endogenous baboon type C retrovirus. This system was particularly examined, considering that it could be used as a representative in vitro model of retrovirus replication.

Methods

Materials. Adenosine 5'-[α-32P]triphosphate (410 Ci/mmol) was obtained from Amersham. Poly(I).poly(C) and poly(I).poly(C)-agarose were from Pharmacia, poly(I).poly(C12U) was supplied as part of a cooperative program, by the Johns Hopkins University School of Hygiene and Public Health, Baltimore, Md., U.S.A. Polyethyleneimine (PEI) was a gift from BASF, France; cellulose MN 300 was from Macherey Nagel. PEI-cellulose thin-layer plates were prepared as described by Randerath & Randerath (1967). Eagle's medium, Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum (FBS) were supplied by InterMed, France.
**Cell culture.** The cells used were spontaneously transformed cells from human placental tissue (WISH), human fibroblast GM02767B and mouse fibroblast L929 cells. Amniotic human cells AV3, from the ATCC, were chronically infected with endogenous baboon type C virus strain M7 (Saal et al., 1981). AV3-M7 cells were maintained in McCoy culture medium and WISH cells were grown in DME, both media were supplemented with 10% FBS. GM02767B and L929 cells were grown in MEM supplemented with FBS: 10% for GM02767B and 5% for L929 cells.

**Human IFNs and anti-human IFN antibodies.** Purified natural IFN-β containing 10^8 international units/mg of protein was from Cytotech. The anti-human IFN-α (G-026-502-568) and IFN-β (G-028-501-568) antibodies, having 7.5 x 10^5 and 1.2 x 10^4 neutralizing units/ml, respectively, were from the National Institutes of Health. The anti-human IFN-γ antibodies, having 6.4 x 10^5 neutralizing units/ml, were a generous gift from Dr. J. Wietzerbin (Institut Curie, Paris, France).

**Determination of IFN titres.** Poly(I).poly(C12U)-induced IFN was titrated on WISH cells challenged with vesicular stomatitis virus (VSV). IFN titres, determined in terms of the amount of IFN required to produce 50% inhibition of the c.p.e., were expressed in relation to the human IFN-β reference (G-023-902-527, National Institutes of Health).

**Assay of 2′5′A synthetase.** After different treatments, cells were washed with buffer A containing 140 mM-NaCl, 3 mM-magnesium chloride and 35 mM-HEPES pH 7.5, and then lysed for 5 min at 0°C with 0.5% NP40 in buffer B containing 5 mM-magnesium acetate, 1 mM-DTT, 25 mM-potassium chloride, 10% glycerol and 20 mM-Tris-HCl pH 8. Cell lysates were then centrifuged for 6 min in an Eppendorf centrifuge, pellets were discarded and the cell extracts were stored at −80°C until assayed. For the 2′5′A synthetase assay, 100 μl of each cell extract was mixed with 15 μl poly(I).poly(C)-agarose (10 to 15 mg/ml); after 15 min incubation at room temperature, the matrix was washed twice with buffer B and resuspended in 10 μl of the same buffer containing 7 mM-[α-32P]ATP (0.05 μCi per incubation), 25 mM-magnesium acetate, 12 μg/ml poly(I).poly(C), 0.25 mg/ml BSA, 0.25 mg/ml creatine kinase and 10 mM-creatine phosphate. After 2 h at 37°C, the reaction was stopped by the addition of 20 μl of 50 mM-EDTA. 6 μl of each incubation mixture was spotted onto a PEI-cellulose plate and 2′5′A was separated from ATP by ascending chromatography in 2-methyl-Tris-HCl pH 8.6 (Justesen et al., 1980). After autoradiography, the spots were cut out and their radioactivity was determined using a linear scanner. The specific activity was calculated in relation to the amount of protein in the cell extract.

**Reverse transcriptase (RT) assay.** Culture supernatants were centrifuged on a 10% glycerol cushion and the virion-associated activity of the pellet on top of the cushion was measured using synthetic template poly(A).oligo(dT) and divalent cation Mn^2+ as described (Périès et al., 1979).

**Electron microscopy.** Thin sectioning procedures were used to detect and count type C virions in both treated and untreated cells. After removal of supernatant fluids, cells were fixed and processed for electron microscopy as already described (Canivet et al., 1983). The ultrathin sections were mounted on carbonized Formvar membranes on 150-mesh copper grids. In these grids, the area of the squares is large enough to observe 20 to 30 entire cell sections. For each sample at least 10 grid squares were systematically scanned and types of particles (budding, extracellular, immature and extracellular mature particles) counted, taking care to avoid counting the same or two adjacent fields.

**Results**

**Optimal poly(I).poly(C12U) treatment required to produce a simultaneous effect on virus replication and 2′5′A synthetase induction**

In preliminary experiments, AV3 cells acutely infected with M7 type C endogenous baboon retrovirus were subjected to short-term treatment with mismatched RNA. Cultures were exposed to poly(I).poly(C12U) (50 μg/ml) for 48 h before challenging with the M7 isolate at an m.o.i. of about 0.5. After 7 days no morphological changes were observed in either untreated or treated cultures. Electron microscopy showed no significant differences in the number of budding or free virions present in both cases. RT activity levels were comparable in the supernatants of both groups (data not shown), indicating that the mismatched dsRNA provides no or little protection against virus multiplication in AV3 cells acutely infected with M7 retrovirus.

These results led us to adopt another dsRNA treatment. We examined the effect of poly(I).poly(C12U) on chronically infected AV3-M7 cultures. AV3-M7 cells were treated once (time 0) or every 48 h for 5 days with 50 μg/ml of poly(I).poly(C12U). Table 1 shows the RT and 2′5′A synthetase activities under these two conditions of double-stranded polynucleotide treatment. 2′5′A synthetase activity increased slightly in control infected cells compared to non-infected cells (Table 1). Poly(I).poly(C12U) added once (time 0) did not alter RT and 2′5′A synthetase activities in AV3-M7 cultures; however, three successive additions (at 0 h, 48 h and 96 h) inhibited extracellular production of M7 virus as measured by the RT assay. In parallel, the same treatment caused the induction of 2′5′A synthetase activity (Table 1). These results are comparable to those obtained when cultures of AV3-M7 cells were treated with 100 international units/ml of human IFN-β (Table 2).

**Table 1. Effect of mismatched dsRNA on virus replication and induction of 2′5′A synthetase after 7 days of treatment**

<table>
<thead>
<tr>
<th></th>
<th>RT activity (c.p.m. x 10^-3)</th>
<th>Reduction in RT activity (%)</th>
<th>2′5′A synthetase (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-infected cells</td>
<td>2</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td>Infected cells</td>
<td>389.7</td>
<td>–</td>
<td>10</td>
</tr>
<tr>
<td>Series 1</td>
<td>372.3</td>
<td>4-4</td>
<td>10</td>
</tr>
<tr>
<td>Series 2</td>
<td>89.2</td>
<td>77</td>
<td>70</td>
</tr>
</tbody>
</table>

* Chronically infected AV3-M7 cells were incubated for 7 days in the absence or the presence of 50 μg/ml poly(I).poly(C12U). One series of cells (Series 1) was treated once (time 0) and a second series (Series 2) every 48 h (0 h, 48 h and 96 h). At the end of the incubation, supernatants were taken for the test of RT activity and the cells were lysed for the determination of 2′5′A synthetase activity as described in Methods. RT activity is expressed as [3H]TMP incorporated into poly(dT) over 60 min.
Table 2. Effect of mismatched dsRNA and IFN on virus replication and induction of 2′5′A synthetase activity

<table>
<thead>
<tr>
<th>Addition</th>
<th>RT activity (c.p.m. × 10⁻³)</th>
<th>Reduction in RT activity (%)</th>
<th>2′5′A synthetase (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>355.6</td>
<td>-</td>
<td>15</td>
</tr>
<tr>
<td>IFN-β (100 units/ml)</td>
<td>98.9</td>
<td>72.2</td>
<td>122</td>
</tr>
<tr>
<td>Poly(I).poly(C₁₂U) (50 μg/ml)</td>
<td>117.8</td>
<td>66.8</td>
<td>68</td>
</tr>
</tbody>
</table>

* Triplicate confluent monolayers of AV3-M7 cell cultures in 25 cm² Falcon flasks were exposed to the treatment indicated every 48 h. On day 7, RT activity in the supernatant and 2′5′A synthetase activity in the cell lysates were assayed as described in Methods. RT activity is expressed as [³H]TMP incorporated into poly(dT) over 60 min.

Poly(I).poly(C₁₂U)-induced IFN

Since it is known that dsRNA is an IFN inducer (Stewart, 1979; Johnston & Torrence, 1984), we determined whether IFN is induced and secreted in double-stranded polynucleotide-treated AV3-M7 cells. For this the supernatant, taken after 7 days from AV3-M7 cells treated every 48 h with poly(I).poly(C₁₂U), was exposed at 70 °C for 30 min in order to inactivate residual poly(I).poly(C₁₂U) (Aurelian et al., 1987). The presence of IFN in fibroblast GM02767 B cells was determined by the capacity of the supernatant obtained as above to induce 2′5′A synthetase activity. Supernatant from untreated AV3-M7 did not induce any increase in 2′5′A synthetase activity in GM02767B cells (17 units/mg compared to 13 units/mg with no addition). This demonstrates that untreated cells did not secrete detectable IFN activity. In contrast, supernatant from AV3-M7 cells treated with poly(I).poly(C₁₂U) induces enzyme activity (71 units/mg) in GM02767B cells. This inducing capacity was attributable to induced and secreted IFN, which was of type β/α as demonstrated by antibody cross-reactions; its 2′5′A synthetase-inducing capacity in GM02767B cells was totally abolished by anti-IFN-β/α antibodies (15 units/mg). This IFN was titrated on human WISH cells challenged with VSV, and expressed in relation to the human IFN-β reference. The result showed that poly(I).poly(C₁₂U) caused the induction and the secretion by AV3-M7 cells of an IFN with an antiviral titre of 60 international units/ml.

Poly(I).poly(C₁₂U) does not lead to the trapping of extracellular virions on the cell surface

As shown in Tables 1 and 2, poly(I).poly(C₁₂U) or IFN produced inhibition of extracellular virus production as measured by virus-associated RT activity. In both cases, the direct in vitro mixing of poly(I).poly(C₁₂U) or an IFN preparation with supernatant fluids containing M7 virus did not alter the RT activity (data not shown). This clearly indicates that the two products have no direct effect on virus particles. Thus, poly(I).poly(C₁₂U), like IFN, seems to act by an intracellular mechanism.

To gain further insight into this mechanism, poly(I).poly(C₁₂U)-treated and untreated AV3-M7 cultures were checked by electron microscopy. No morphological differences were observed between untreated and treated cells. Nevertheless, the total number of budding and extracellular immature or mature virions was clearly lower in cultures treated with mismatched RNA preparations (Fig. 1).

Fig. 1. Number of virus particles observed by electron microscopy on day 7 in (a) untreated cultures and (b) poly(I).poly(C₁₂U)-treated AV3-M7 cell cultures (50 μg/ml every 48 h).

It is relevant to mention here that, in contrast to IFN, poly(I).poly(C₁₂U) did not cause trapping of extracellular virions at the cell surface (Fig. 2). This suggests that the characteristics of poly(I).poly(C₁₂U) inhibition of M7 virus multiplication were different from those described for IFN, which caused trapping of type C and type D retrovirus particles produced by infected cells (Billiau et al., 1976; Periès et al., 1979; Canivet et al., 1983).

Effect of anti-IFN antibodies on RT and 2′5′A synthetase activity

From the results of electron microscopy, it seems reasonable to assume that the high anti-retroviral effect we observed may not depend on the induction of endogenous IFN by mismatched dsRNA. To evaluate this hypothesis, we studied the effect of anti-IFN antibodies on poly(I).poly(C₁₂U)-induced 2′5′A synthetase and on the inhibition of extracellular virus production. Since the results above show that poly(I).poly(C₁₂U) caused in AV3-M7 cells the induction and
secretion of an IFN with a titre of 60 international units/ml, each of the anti-IFN antibodies was used at a concentration capable of neutralizing 400 international units/ml of IFN. Table 3 shows that basal level of 2′5′A synthetase in AV3-M7 cells was not altered by the presence of anti-IFN-α, -β, or -γ antibodies, whereas poly(I).poly(C12U)-induced 2′5′A synthetase was abolished strongly by anti-IFN-β, slightly by anti-IFN-α and not at all by anti-IFN-γ antibodies. In the same context, the anti-IFN (-α, -β or -γ) antibodies had no effect on RT activity in chronically infected AV3-M7 cells and did not alter the inhibitory effect of poly(I).poly(C12U), as determined by an RT assay (Table 3).

These results suggest that 2′5′A synthetase was induced by IFN-β/α synthesis and that the inhibition of virus replication was totally independent of IFN production, because the anti-IFN antibodies blocked the induction of the enzyme but did not alter the inhibition of virus multiplication due to poly(I).poly(C12U) treatment. This was confirmed by electron microscopy: the number of virus particles in cultures treated with the double-stranded polynucleotide in the absence or presence of specific anti-IFN-α, -β or -γ antisera (data not shown) was comparable. Thus the effects of poly(I).poly(C12U) on virus multiplication are not likely to be mediated through the production of IFN and its action via the endonuclease-2′5′A synthetase pathway. Other mechanisms of action should be explored to explain this retrovirus inhibition effect.

**Discussion**

In this report, we have shown the ability of poly(I).poly(C12U) to inhibit the multiplication of the baboon type C endogenous retrovirus M7 in chronically infected human AV3 cells, as assayed by RT activity and electron microscopy. We have also demonstrated that the mismatched dsRNA induces 2′5′A synthetase activity in AV3-M7 cells. Our model can be considered as an example of oncornavirinae infection in human cells. In fact, this taxonomic retrovirus subfamily determines a characteristic type of chronic steady-state infection and can produce proliferative malignancies and demyelinating neurological diseases in animals and humans (Coffin, 1990).

In our experiments, poly(I).poly(C12U) caused the induction and secretion of IFN-β/α, with an antiviral
titre of 60 international units/ml. However, the inhibition of M7 retrovirus multiplication by poly(I).poly(C12U) does not seem to be mediated by the IFN induced because anti-IFN-α, -β or -γ antibodies did not alter the effect of poly(I).poly(C12U) on virus multiplication, as determined by an RT assay. These results were confirmed by electron microscopy, which indicated that the quantity of virus particles was comparable in cell cultures treated with mismatched dsRNA in the absence or the presence of anti-IFN-α, -β or -γ antibodies. However, anti-IFN-β/α antibodies were efficient in abolishing the 2′5′A synthetase activity induced by poly(I).poly(C12U) in AV3-M7 cells. Thus, in contrast to the induction of enzyme activity, the anti-retrovirus effect of poly(I).poly(C12U) did not seem to be dependent on IFN production.

It is known that dsRNA is a potent inducer of IFN and that it is also involved in the mechanism of IFN action (Stewart, 1979; Lengyel, 1982). There has been debate as to whether induction of different biological events by dsRNA is mediated by IFN. Some observations are in favour of a direct effect of dsRNA. It has been shown recently that dsRNA directly induces the expression of many IFN-inducible genes (Whathelet et al., 1987, 1988; Pince et al., 1990; Mémet et al., 1991). Evidence also indicates that dsRNA can inhibit cellular growth independently of IFN induction: tumour cells show differential sensitivity to IFN and dsRNA (Lin et al., 1984), which can synergistically inhibit tumour cell growth, and anti-IFN antibodies do not interfere with the antiproliferative effects of dsRNA (Hubbell et al., 1984; Chapekar et al., 1988; Chapekar & Glazer, 1983, 1985, 1986). Moreover, Hubbell et al. (1991) have reported that a rapid increase in the intracellular concentration of cAMP is sufficient for poly(I).poly(C12U)-induced growth inhibition in human glioma cell line A1235, which is incapable of producing detectable levels of IFN in response to this dsRNA. However, it should be recalled that poly(I).poly(C12U) is unable to increase cAMP concentration in another cellular system (Chelbi-Alix et al., 1991).

Poly(I).poly(C12U) seems also to have the ability to restore immunological function, and to control both HIV replication (Carter et al., 1987; Laurence et al., 1987; Montefiori et al., 1989a) and herpes simplex virus type 2 infection ( Aurelian et al., 1987). Furthermore, poly(I).poly(C12U) is a more potent anti-HIV agent than IFN-α, IFN-β or IFN-γ, or all three IFNs combined (Montefiori & Mitchell, 1987), and acts synergistically with IFN or azidothymidine in inhibiting HIV infection (Montefiori et al., 1989b; Mitchell et al., 1987). From this, it is clear that the effect of the mismatched dsRNA cannot be attributed only to IFN production. Thus, two different mechanisms seem to be manifested by poly(I).poly(C12U) and IFN. It has also been shown (Montefiori & Mitchell, 1987) that mismatched dsRNA has no effect on HIV replication in infected cultures of CEM or H9 cells. However, in these experiments infected cultures were treated once with poly(I).poly(C12U) at 50 μg/ml. Under the same conditions (Table 1) we also found that poly(I).poly(C12U) does not alter RT activity in AV3-M7 cultures. To obtain an antiviral effect, chronically infected AV3-M7 cells have to be treated with mismatched dsRNA every 48 h for 7 days.

We have also shown that, in contrast to IFN, poly(I).poly(C12U) does not cause the trapping of extracellular virions at the cell surface. This fact suggests that the mechanism of action of poly(I).poly(C12U) is different from that described for IFN on oncornavirinae multiplication (Billiau et al., 1976; Périès et al., 1979; Canivet et al., 1983). Our results are in favour of the hypothesis that poly(I).poly(C12U) inhibits this category of retrovirus in an IFN-independent manner. Thus, it seems that its effect is unlikely to be mediated by the production of IFN and its actions via the endonuclease-2′5′A synthetase pathway.

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