Polyinosinic-Polycytidylic Acid in Association with Cyclic Nucleotides Activates the Antiviral Factor (AVF) in Plant Tissues

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SUMMARY

Leaves and callus cultures from *Nicotiana glutinosa* L. were treated with a mixture of polyinosinic-polycytidylic acid [poly(I)-poly(C)], cyclic AMP and cyclic GMP. A new antiviral activity appearing in the treated tissues co-purified with the antiviral factor AVF. Poly(I)-poly(C) can replace tobacco mosaic virus infection in the induction of AVF activity, the latter being also mediated to a certain extent by cyclic nucleotides. The kinetics of the stimulation of AVF in these *in vivo* studies suggests that AVF is released by a rapid activation of a pre-existing precursor, thus supporting previous results.

AVF is an antiviral factor appearing in virus-infected plants (Sela & Applebaum, 1962). The AVF from tobacco mosaic virus (TMV)-infected *Nicotiana glutinosa* L. leaves has been purified to various degrees (Sela et al., 1964; Antignus et al., 1977) and characterized as a glycoprotein (Mozes et al., 1978). It has recently been more fully purified, and at picogram levels totally inhibits TMV multiplication (Jacobi et al., unpublished results). The *N. glutinosa* AVF is associated with the N gene of some *Nicotiana* species (Antignus et al., 1975, 1977; Sela et al., 1978), a gene which is responsible for the localization of TMV infection in these plants. AVF induces another activity in treated leaves, a deacylation factor (DF) which removes histidine from histidinyl-TMV-RNA (Sela et al., 1976; Devash et al., 1981).

In a previous study (Sela et al., 1978), it was demonstrated that AVF could be activated *in vitro* by cyclic 3':5'-adenosine monophosphate (cAMP) and cyclic 3':5'-guanosine monophosphate (cGMP). Polyinosinic-polycytidylic acid [poly(I)-poly(C)] also seemed to play some role in this activation. The flow of events was tentatively concluded to be the following. The N gene determines an enzyme activity which processes a pre-existing precursor of AVF to produce active AVF. This enzyme is dormant or slow-working, and the release, or acceleration of its activity is coupled with cyclic nucleotide-dependent protein phosphorylation. It was assumed that TMV infection releases this phosphorylation and hence brings about the stimulation of AVF activity in N gene-carrying plants.

Various substances, among them poly(I)-poly(C), polyacrylic acid and transfer RNA were reported to induce resistance to virus infection in N gene-carrying plants (Stahman & Gothoskar, 1958; Gicherman & Loebenstein, 1968; Stein & Loebenstein, 1970, 1972; Gianinazzi & Kassanis, 1974). It has been suggested that the above, and other agents, induce resistance by substituting for TMV in the stimulation of phosphorylation leading to AVF activation (Sela, 1981a).

The present study was initiated in an attempt to find out whether poly(I)-poly(C) can indeed release AVF activity and whether cyclic nucleotides are involved in the *in vivo* induction of AVF, as indicated by the *in vitro* studies. The general experimental approach in this study was to find out whether the various treatments induce a new antiviral activity, and whether the induced antiviral activity could be attributed to AVF. The required criteria were that it could be purified, like AVF and exhibited antiviral activity at less than 1 ng protein per ml.

Leaves of *N. glutinosa*, or callus cultures grown on Murashige and Skoog (1962) agar medium, were immersed in the ‘inducing mixture’ [0.1 μM-N6,6'-dibutyryl cAMP, 0.1 μM-N2,2'-dibutyryl cAMP, 10 μg/ml poly(I)-poly(C) in 0-01 M-sodium phosphate buffer pH 7.6 and control material was tested similarly but with buffer only. After an incubation period of 30 min at room temperature, the inducing mixture was removed, the plant material was washed with the standard buffer and further incubated on wet filter paper in Petri dishes for various ‘waiting
Table 1. *Antiviral activity induced in leaves and callus cultures of N. glutinosa by the inducing mixture*

<table>
<thead>
<tr>
<th>Fraction period (h)</th>
<th>Leaves</th>
<th>Callus</th>
<th>Buffer-treated</th>
<th>Inducing mixture-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 0.25 1 24</td>
<td>0.25 1 24</td>
<td>0 0.25 1 24</td>
<td>0 0.25 1 24</td>
</tr>
<tr>
<td>Clarified extract</td>
<td>ND ND 41 53</td>
<td>20 35 31</td>
<td>ND ND 17 ND</td>
<td>ND ND 17 ND</td>
</tr>
<tr>
<td>Crude preparation</td>
<td>0 0 43 ND</td>
<td>ND ND 17 ND</td>
<td>422 496 384 405</td>
<td>364 516 322 420</td>
</tr>
<tr>
<td>DE-0.3</td>
<td>0 0 0 0</td>
<td>0 0 0</td>
<td>355 505 466 424</td>
<td>518 440 440 492</td>
</tr>
<tr>
<td>DE-0.65</td>
<td>0 25 32 51</td>
<td>0 45 45</td>
<td>439 405 388 414</td>
<td>382 190 45 52</td>
</tr>
</tbody>
</table>

* Tested on half-leaves of *D. stramonium* and expressed as percentage protection. Each number is an average of three to five different experiments, and is statistically significant (*t*-test for related samples; *p* = 0.05).

† Callus cultures were treated for 30 min with the standard buffer or with inducing mixture. The various tissue extracts made at the indicated times were applied to TMV-infected leaf discs. Virus content was determined by ELISA 72 h later; results are expressed as ng TMV per disc.

‡ ND, Not done.
Table 2. The requirements for inducing antiviral activity in callus cultures of N. glutinosa as measured by the ELISA test

<table>
<thead>
<tr>
<th>Composition of inducing mixture</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (buffer only)</td>
<td>315</td>
<td>406</td>
<td>292</td>
</tr>
<tr>
<td>Complete mixture</td>
<td>36</td>
<td>31</td>
<td>46</td>
</tr>
<tr>
<td>Poly(I)-poly(C) only</td>
<td>42</td>
<td>28</td>
<td>38</td>
</tr>
<tr>
<td>Cyclic nucleotides only</td>
<td>206</td>
<td>74</td>
<td>98</td>
</tr>
</tbody>
</table>

* Callus was treated with the indicated mixtures and, after 1 h, the DE-0.65 fraction was isolated and applied to TMV-inoculated tobacco leaf discs. The virus content of the discs was determined by ELISA 72 h later.

periods' to allow the build-up of the anticipated antiviral activity. At the ends of these times, the plant material was homogenized and centrifuged at low speed to give the clarified extract. Extracts from treated and control samples were purified by mixing with hydrated calcium phosphate ('crude preparations') and further by chromatography of the unadsorbed material on DEAE-cellulose, collecting the various fractions described by Devash et al. (1981). The samples at the various levels of purification were diluted 1:1000 in the phosphate buffer and brought to 5 μg/ml with purified TMV. The infectivity in the control samples was assayed on half-leaves of Datura stramonium L. for comparison with that of the fractions derived from treated tissues. Alternatively, diluted fractions, eluted from DEAE-cellulose and containing only 0.1 ng protein per ml (Sedmak & Grossberg, 1977) were applied to TMV-infected leaf discs (1 h post-inoculation) and their TMV content was determined 72 h later by ELISA as described by Orchansky et al. (1982).

Antiviral activity did indeed appear in plant tissues treated with the inducing mixture, and the active agent could be separated in a manner similar to AVF. The new activity appeared quite rapidly after the tissue had been exposed to the inducing mixture, but a short latent period was observed (Table 1).

To assess which of the constituents were essential for the release of the antiviral activity, tissue culture samples were treated with the complete inducing mixture or with mixtures from which certain components had been omitted. To ensure that any observed inhibition in the release of antiviral activity was indeed due to the lack of stimulation of AVF, these tests were carried out with the DE-0.65 fraction. As shown in Table 2, the antiviral activity was induced by the complete mixture. Omission of the cyclic nucleotides had only negligible effects on the level of the induced antiviral activity. However, in some cases, omission of poly(I)-poly(C) resulted in a considerable reduction of stimulation of the activity. It is quite evident that in these in vivo experiments the cyclic nucleotides are auxiliary to poly(I)-poly(C) in the induction of antiviral activity, i.e. they can sometimes replace poly(I)-poly(C) but are not indispensable.

Poly(I)-poly(C) has been shown to induce resistance to virus infection in plants (Stein & Loebenstein, 1970), and the above data show that it is a good inducer of AVF. This supports the suggestion that poly(I)-poly(C) induces resistance via the activation of AVF.

The build-up of AVF activity following treatment with the inducing mixture is very rapid, usually detected within 15 min and reaches a more or less steady state at 60 min. This supports the suggestion that the release of AVF involves a process of activation of a pre-existing precursor, rather than, or in addition to, the induction of new activities on part of the host genome (Sela et al., 1978).

Poly(I)-poly(C) possibly mimics the virus double-stranded RNA. Activation of AVF in TMV-infected, N gene-carrying plants is a late event detectable only after a lag period of 24 to 48 h (Antignus et al., 1977). The poly(I)-poly(C)-directed AVF activation, however, is almost instantaneous, presumably because in the case of virus infection a certain time should elapse before a sufficient amount of double-stranded TMV RNA is synthesized.

When tested in vitro, cyclic nucleotides were necessary for AVF activation, while the role of poly(I)-poly(C) seemed to be of auxiliary nature only (Sela et al., 1978). However, in the present in vivo studies, polynucleotide appears to be an essential factor for AVF activation. It is
suggested that poly(I)-poly(C), or possibly the replicative form of TMV RNA, triggers the release of cyclic nucleotides and thus starts the chain of events leading to AVF activation. Hence, poly(I)-poly(C), by itself, may release enough cyclic nucleotides inside the cells, and the addition of exogenous nucleotides would become merely auxiliary.

Recently, the presence of cAMP in N. glutinosa was verified. It was also demonstrated that a pulse of cAMP was induced between 20 and 40 h after TMV inoculation (Rosenberg et al., 1982), which is correlated with the time of AVF induction (Antignus et al., 1977) and of the beginning of local lesion production (Otsuki et al., 1972).

In another recent study (Orchansky et al., 1982), human interferons (especially the γ3 sub-species of human leukocyte interferon) have been reported to protect plant tissue from TMV infection. Some other features of AVF resemble those of interferon (Sela, 1981b) and the induction of AVF activity by poly(I)-poly(C) adds to this resemblance.

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REFERENCES


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