Effect of Fragmentation on Interferon Induction by Double-stranded Virus RNA

(Accepted 14 January 1974)

SUMMARY

Fragmentation by several techniques of the double-stranded RNA of Penicillium chrysogenum viruses reduces its in vivo interferon-inducing capacity in mice.

The capacity of double-stranded RNA (ds-RNA) to induce interferon and to give protection against virus infection was discovered some years ago (Field et al. 1967; Lampson et al. 1967; Banks et al. 1968) and a great number of different polynucleotides have since been tested in different laboratories as to their activity in inducing the production of interferon in vivo and in vitro, their antiviral and antitumour activities, and toxicity (see reviews by Colby, 1971; Gresser, 1972; Kleinschmidt, 1972).

Numerous studies have failed, however, to establish exactly the structural requirements of a potent inducer, except for the necessity of a rather stable double-stranded structure and a free 2'-OH in the ribose molecules. A quantitative comparison of the results obtained with different natural ds-RNAs or synthetic double-stranded polynucleotides of different composition is rather difficult, partly because of the different techniques and assay systems used by different authors and partly because of the uncertainty of several structural features (mol. wt., secondary structure, stability) of the synthetic polymers used. Detailed investigations have been carried out on poly(I), poly(C) (Jameson & Grossberg, 1970; Lampson et al. 1970; Tytell et al. 1970; Niblack & McCreary 1971; Morahan et al. 1972) which stress the importance of the high mol. wt. of the inducer. Variation of the biological activities of this polymer with the size of one or both of its strands have been studied by different authors with not quite consistent results.

The differences may be caused by the polydispersity of the starting material and by some uncontrolled factors inherent in the techniques used for producing smaller fragments. Annealing separately synthesized or separately degraded single-stranded polynucleotides in all probability yields a heterogeneous mixture of mainly double-stranded molecules. It may also give rise, however, to imperfect double-stranded structures containing single-stranded tails, nicks or longer single-stranded regions inside the double-stranded polynucleotide chain. The differences in biological activity obtained with such preparations may reflect therefore not only the effect of the reduced mol. wt. but also that of changes in the secondary structure.

It was partly for this reason that the studies reported here were undertaken in which we attempted to avoid the above mentioned uncertainties by using a well defined starting material [ds-RNA from Penicillium chrysogenum virus (Banks et al. 1969; Wood & Bozarth, 1972) which contains 3 double-stranded RNA species of mol. wt. 2.18, 1.99 and 1.89 x 10^6, respectively] and applying degradation techniques which yield smaller molecular fragments as perfectly double-stranded as the starting material. A special interest of these studies is that the relationship between mol. wt. and biological activity has been tested on natural double-stranded RNA. In spite of many similarities in their action, it is not self-evident that any structural alteration should have the same influence on the biological effects of natural ds-RNAs and of synthetic double-stranded polynucleotides.
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Fig. 1. Diagram of polyacrylamide gel electrophoretic patterns of double-stranded RNA digested by pancreatic RNase, RNase III or ultrasonic irradiation. (a) digested by RNase III, for conditions see in Table 1. (b) digested with pancreatic RNase A, for conditions see in Table 1. The gels contained 5% acrylamide, 0.17% bis-acrylamide in 0.04 M-tris-acetate pH 8.2 and 0.001 M-EDTA. In each gel the first sample on the left shows the position of the intact RNA. The length of the runs were different, the arrows show the position of the bromophenol blue marker. (c) RNA, concentration 1 mg/ml, was irradiated as described in Table 2, for 0, 3, 5, 10 and 20 min.

The RNA preparations used were the gift of Beecham Research Laboratories, for which the authors wish to express their gratitude. They were unfractionated mixtures of all three double-stranded RNA components of Penicillium chrysogenum virus. Fragmentation of RNA was accomplished by (i) digestion with RNase III, an endonuclease specific for double-stranded RNA (Robertson, Webster & Zinder, 1968); (ii) digestion with pancreatic RNase A under conditions when the extent of degradation could be controlled by both salt concentration and enzyme: substrate ratio (V. G. Edy, M. Szekely, C. Dreyer, unpublished results); (iii) by ultrasonic disintegration. The sites attacked in the RNA molecule by these treatments are expected to be different, corresponding to the different specificities of the two enzymes and the basically different mode of action of ultrasonication. Although the degradation products were always heterodisperse, conditions could be controlled so as to yield breakdown products within a given size range. This was checked in each experiment by polyacrylamide gel electrophoresis (Fig. 1) or sucrose density gradient sedimentation of the digests. The secondary
Table 1. Effect of nuclease treatment on interferon induction by double-stranded RNA

<table>
<thead>
<tr>
<th>Treatment of RNA</th>
<th>Interferon titre</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>1341 U</td>
</tr>
<tr>
<td>Digested with RNase III</td>
<td>797 U</td>
</tr>
<tr>
<td>None</td>
<td>1708 U</td>
</tr>
<tr>
<td>Digested with pancreatic RNase A</td>
<td>27 U</td>
</tr>
</tbody>
</table>

Digestion with RNase III was carried out in 0.02 M-tris-HCl, pH 7.6, 0.01 M-Mg-acetate, 0.1 M-NH₄Cl at 36 °C for 1 h, using 700 U of enzyme for 1 mg RNA. After incubation 1% SDS was added and the fragmented RNA isolated by phenol extraction, ethanol precipitation, followed by dialysis against PBS. The extent of digestion is shown in Fig. 1(a). Digestion with pancreatic RNase A (125 μg for 1 mg RNA) was in SSC for 1 h at 36 °C. Digestion was terminated by the addition of 20 μl/ml diethyl pyrocarbonate, and fragmented RNA isolated as above. The extent of digestion is shown in Fig. 1(b). Assays of interferon-inducing capacity were done using groups of 15 mice for each sample.

Table 2. Effect of progressive sonic degradation on interferon induction by double-stranded RNA

<table>
<thead>
<tr>
<th>Time of irradiation (min)</th>
<th>Interferon titre</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>1078 U</td>
</tr>
<tr>
<td>3</td>
<td>656 U</td>
</tr>
<tr>
<td>5</td>
<td>255 U</td>
</tr>
<tr>
<td>10</td>
<td>67 U</td>
</tr>
<tr>
<td>20</td>
<td>48 U</td>
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</table>

1.5 mg samples of RNA in 1.5 ml were irradiated for the periods indicated in an MSE 100 W ultrasonic disintegrator, using the 9 mm trip diam. probe. The frequency of irradiation was 20 kHz, transducer amplitude 7 to 8 μm peak to peak. Sample temperature was kept below 20 °C by ice cooling and restricting the irradiation periods to 5 min followed by a 10 min cooling periods in the samples where longer irradiation was needed. The irradiated samples were centrifuged to remove metal particles thrown off the probe, the degraded RNA was precipitated by ethanol and dialysed against PBS. Interferon-inducing capacity was assayed using groups of 15 mice per sample. The approximate extent of degradation in the different samples can be seen in Fig. 1(c).

Structure of the fragments was shown to be fully double-stranded by determination of their melting profiles and their failure to react with formaldehyde. Interferon induction was assayed by i.v. injection of RNA into groups of 15 mice (18 g ± 1 g), strain BS4S at a dose rate of 4 mg/kg. After 8 h the mice were bled and the pooled sera assayed for interferon by the dye uptake method (Finter, 1969).

Fragmentation by any of the three techniques led to a significant fall in the capacity of RNA to induce the formation of circulatory interferon in the mice (Tables 1 and 2). As shown in Table 2, progressive fragmentation by increasing the time of ultrasonic treatment resulted in a progressive fall in interferon-inducing capacity. The gel electrophoresis patterns of these preparations (Fig. 1c) showed a parallel decrease in the size range of the fragments, reaching approx. the range of 100 000 to 1 000 000 in the last sample. This relationship between mol. wt. and interferon-inducing capacity is in agreement with that found for poly(I), poly(C) by several authors (Jameson & Grossberg, 1970; Lampson et al. 1970; Morahan et al. 1972). A different behaviour was found, however, with regard to the toxicity of fragmented RNA. Mice given intact RNA survived the experimental period without any visible ill-effect. Mice receiving fragmented RNA produced by any of the above techniques were frequently
ill in appearance (ruffled fur, hunched posture) and several mice out of each group (between 3 and 7 out of each group of 15) died in the 8 h period before sacrifice.

No increased toxicity of fragmented poly(I), poly(C) has been reported; on the contrary, some authors claim to have found a slight decrease in toxicity with decreasing size of the polymer (Levy et al. 1970; Hilleman et al. 1971; Niblack & McCready, 1971; Lampson et al. 1972; Morahan et al. 1972). The increased toxicity of fragmented *Penicillium chrysogenum* RNA did not appear related to the interferon-inducing capacity of the fragments, and its cause is unknown.

The decrease of interferon induction upon fragmentation can be interpreted in two ways: the ability to induce interferon may decrease gradually with the size of the fragments, or there may be a threshold value for the mol. wt. of the inducer, under which no induction is taking place. The changes caused by fragmenting RNA might even be of a more complex nature, as according to Jameson & Grossberg (1970) the kinetics of induction and thus the time of finding the highest level of interferon in the serum may also vary with the size of the inducer.

The effect of mol. wt. on interferon induction is of special interest because of the presence of a double-stranded RNase in serum (Stern, 1970). It depends on the speed by which double stranded RNA is taken up by the cells whether the inducer reaches the receptor site intact or at least in the form of a molecule of a sufficiently high mol. wt.

Our results can be summarized by stating that double-stranded fragments of virus RNA produced by different degradation techniques show a decrease in interferon-inducing activity, which seems to fall parallel with the size of range of the RNA fragments.

The work reported here forms part of study on fungal viruses and non-specific immunity in collaboration with Beecham Research Laboratories, Brockham Park, Betchworth, Surrey. We thank H. Jacobs for technical assistance. V. G. E. was in receipt of a Medical Research Council Scholarship during the period of this work.

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REFERENCES


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(Received 19 November 1973)