Invited Discussions
Double-Stranded Polynucleotides as Interferon Inducers

A. K. FIELD, A. A. TYTELL, G. P. LAMPSON, M. M. NEMES, and M. R. HILLEMAN

From the Division of Virus and Cell Biology Research, Merck Institute for Therapeutic Research, West Point, Pennsylvania 19486

ABSTRACT A discussion of factors considered influential in making a polynucleotide an efficient inducer of interferon was presented. These factors were double-strandedness of the polynucleotides, the sugar moiety of the polynucleotides, thermal stability, resistance to enzymatic degradation, and molecular size of the polynucleotides. Recent developments concerning interferon induction during virus infection were also discussed.

This discussion will be directed to the induction of interferon by polynucleotides and by viruses as well. Attention will be given to the factors which have been proposed as influential in making a polynucleotide an efficient inducer of interferon. Additionally, an attempt will be made to relate the present knowledge of interferon induction by polynucleotides to the fragmentary, but emerging picture seen for interferon induction by infection of cells with RNA and DNA viruses.

Factors considered influential in altering the capacity of polynucleotides to induce interferon are: (a) the degree of double- or multi-strandedness of the polynucleotides in question; (b) the nature of the sugar moiety; (c) the stability of the double-stranded polynucleotide, as measured by thermal dissociation; (d) the stability of the polynucleotide to enzymatic digestion; and (e) the molecular size of the polynucleotide complex. These factors are not independent of each other and, in fact, one may be influenced by alterations in another. Also, each factor may exert different degrees of influence depending upon whether one observes in vitro or in vivo induction of interferon or resistance against virus infection.

(a) Double-strandedness. In the fall of 1967, four successive papers by our group appeared in the Proceedings of the National Academy of Sciences (1–4). Each of these papers dealt with the induction of interferon and interferon-associated interference to virus infection. Each described induction with RNA from different sources. In each, the same conclusions could be drawn. The poly-
Invited Discussion

nucleotides which induced interferon were double-stranded (as defined by data on thermal transition, resistance to RNase digestion, and lack of availability of amino groups to formaldehyde binding). Double-stranded RNA was isolated from Penicillium funiculosum preparations. Complexes of certain synthetic homopolynucleotides (poly rI:poly rC, poly rA:poly rU) induced interferon. Double-stranded RNA prepared from reovirus virions and from Escherichia coli cells infected with MS2 bacteriophage induced interferon. For each of these, destruction of the double-strandedness resulted in destruction of the inducing capacity. Since that time, numerous double-stranded RNA's have been tested and found active as interferon inducers. Several mold preparations have also been shown to contain viruses which contain double-stranded RNA and induce interferon (5). Also, an interferon inducer extracted from mengovirus-infected cells was identified as double-stranded RNA (6). However, the majority of attention has been focused on the synthetic polynucleotides. The requirement of double-strandedness for interferon induction has been confirmed by Vilcek et al. (7). Falcoff and Perez-Bercoff (8), Colby and Chamberlin (9), and, most recently, by De Clercq and Merigan (10). The latter authors have suggested why, under some conditions, low level induction has been observed with certain homopolynucleotides. This is believed to be due to the formation of multi-stranded secondary structures of the homopolymers. Thus, for reasons still not clear, double- or multi-strandedness does appear to be a factor necessary for interferon induction by polynucleotides.

(b) Nature of the sugar moiety. With rare exceptions, only double-stranded ribose nucleic acids have been effective inducers. Very low level activity has been associated with the synthetic DNA–RNA hybrid molecules, and none with double-stranded DNA's so far investigated. In Table I, there are listed several examples to illustrate these differences in activity. The double-stranded RNA's (poly rI:poly rC, poly rA:poly rU, Rice Dwarf virus dsRNA, cytoplasmic polyhedrosis virus dsRNA) were all active inducers of interferon and of in vitro resistance against virus infection. The hybrid nucleic acids (poly rI:poly dC, F1 bacteriophage DNA–RNA) showed variable activity. The DNA's (φx174-RFDNA, calf thymus DNA, poly d [A-T]) were inactive. In addition to this list, Colby and Chamberlin (9) have found no activity associated with deoxyribonucleotide complexes—poly dI:poly dC, poly dG:poly dC, and lambda bacteriophage DNA. Thus, overwhelmingly, induction appears to be associated with double-stranded polyribonucleotide complexes.

(c) Thermal stability of the double-stranded RNA. Aside from a minimal thermal stability sufficient to allow and maintain double-strandedness under physiological conditions, no striking correlation between thermal stability of a double-stranded polyribonucleotide and its capacity to induce interferon has been observed. I have attempted to illustrate this in Table II. Thus poly rI:poly rC has about a 29°C lower Tm than does the complex of poly rI with
pol y rBC. Both complexes were active interferon and host resistance inducers. Poly rA:poly rU and poly r(A-U), the alternating copolymer, have a 10°C difference in Tm, and yet they are similarly poor inducers of rabbit interferon and good inducers of resistance in cell culture. The “natural” double-stranded RNA's are all considerably more stable than poly rI:poly rC and yet are very

### Table 1

**INTERFERON INDUCTION BY POLYNUCLEOTIDE COMPLEXES**

<table>
<thead>
<tr>
<th>Polynucleotide complex</th>
<th>Minimum dose to induce</th>
<th>Interference†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μg/animal</td>
<td>μg/ml</td>
</tr>
<tr>
<td>Poly rI:poly rC</td>
<td>0.5</td>
<td>0.001</td>
</tr>
<tr>
<td>Poly rA:poly rU</td>
<td>25</td>
<td>0.001</td>
</tr>
<tr>
<td>Rice Dwarf virus dsRNA§</td>
<td>10</td>
<td>0.01</td>
</tr>
<tr>
<td>Cytoplasmic polyhedrosis virus dsRNA§</td>
<td>11</td>
<td>0.03</td>
</tr>
<tr>
<td>Poly rI:poly dC¶</td>
<td>Not active at 10</td>
<td>Not active at 5</td>
</tr>
<tr>
<td>F1 bacteriophage DNA-RNA¶</td>
<td>5.0</td>
<td>0.6</td>
</tr>
<tr>
<td>φX174-RFDNA**</td>
<td>Not active at 10</td>
<td>Not active at 1</td>
</tr>
<tr>
<td>Calf thymus DNA</td>
<td>Not active at 100</td>
<td>Not active at 100</td>
</tr>
<tr>
<td>Poly d(A-T)</td>
<td>Not active at 10</td>
<td>Not active at 100</td>
</tr>
</tbody>
</table>

* Interferon assayed in serum taken 2 hr after i.v. injection of inducer.
† Minimum concentration (μg/ml) required to protect primary rabbit kidney cell cultures against infection with vesicular stomatitis virus.
§ Received from Dr. K. Miura, Nagoya University, Nagoya, Japan.
¶ Minimum dose not determined.
** Received from Doctors M. Rush and R. Warner, New York University, New York.

### Table II

**THERMAL STABILITY AND INTERFERON INDUCTION BY DOUBLE-STRANDED RNA**

<table>
<thead>
<tr>
<th>Interferon inducer</th>
<th>Thermal transition midpoint (Tm)</th>
<th>Ionic strength</th>
<th>Minimum dose to induce</th>
<th>Interference‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>°C</td>
<td>Ionic strength</td>
<td>μg/animal</td>
<td>μg/ml</td>
</tr>
<tr>
<td>Poly rI:poly rC</td>
<td>60-62</td>
<td>0.1</td>
<td>0.5</td>
<td>0.001</td>
</tr>
<tr>
<td>Poly rI:poly rBC</td>
<td>89</td>
<td>0.1</td>
<td>10.5 or less§</td>
<td>0.08</td>
</tr>
<tr>
<td>Poly rA:poly rU</td>
<td>56</td>
<td>0.1</td>
<td>25</td>
<td>0.001</td>
</tr>
<tr>
<td>Poly r(A-U)</td>
<td>66</td>
<td>0.1</td>
<td>Not active @ 15</td>
<td>0.04</td>
</tr>
<tr>
<td>Reovirus type 3 dsRNA</td>
<td>92</td>
<td>0.02</td>
<td>0.5</td>
<td>0.04</td>
</tr>
<tr>
<td>MS2-RF-dsRNA</td>
<td>90-92</td>
<td>0.02</td>
<td>0.5</td>
<td>0.04</td>
</tr>
</tbody>
</table>

* Presence of interferon determined for sera taken 2 hr after intravenous injection of inducer.
† Minimum concentration (μg/ml) to protect primary rabbit kidney cell cultures against infection with vesicular stomatitis virus.
§ Minimum not determined.
similar in their inducing capacities. Recently, De Clercq and Merigan (10) did suggest that although differences in Tm above 60°C may not be important, below 60°C inducing capacity may be lost. As I shall point out, our data are not inconsistent with this possibility.

(d) Nuclease sensitivity of the double-stranded RNA. The role of ribonuclease resistance of a double-stranded RNA and its capacity to induce interferon is more open to debate. The suggestion has been made that single-stranded RNA's have not been efficient interferon inducers because of their marked sensitivity to nucleases. Yet several polynucleotides such as poly rA and poly rI alone, or even the complex poly rA:poly rI are relatively resistant and do not induce interferon. Further, the two complexed polynucleotides poly rI:poly rC and poly r(I-C), the alternating copolymer, are both efficient inducers of interferon and of resistance to viral infection in vitro and in vivo. Yet the alternating copolymer is about 4.5 times more sensitive to pancreatic RNase digestion than is poly rI:poly rC. Colby and Chamberlin (9) have shown that in chick embryo cell cultures poly r(I-C), an efficient inducer of host resistance, and poly rI:poly dC, a noninducer, were broken down at about the same rates. Recently, De Clercq, Eckstein, and Merigan (11) have noted that substitution of sulfur for a nonester oxygen in the phosphate linkages of the alternating copolymer of adenylic and uridylic acids resulted in enhancement of capacity of the copolymer to induce resistance to virus infection in vitro and increased capacity to induce interferon in vivo. Such a substitution also resulted in greater resistance of the copolymers to digestion by ribonucleases. Thus, their data suggest one instance of a correlation between ribonuclease resistance and capacity of the polynucleotide complex to induce interferon.

All that can be concluded with certainty from these various reports is that the dependence of interferon-inducing capacity by a complexed polynucleotide on resistance to enzymatic digestion is still an open question.

(e) Molecular size. Little or no attention has been paid the relationship of chain length of the polynucleotide complexes and their inducing capacity. It is known that preparations of poly rI and poly rC can greatly vary in average chain length and distribution of lengths. It has also been shown previously by our group that poly rI and poly rC obtained from different sources can vary widely in degree of complex formation and capacity to induce interferon. In studies to be published more extensively elsewhere, poly rI:poly rC has been prepared in smaller molecular weight complexes. These complexes have been characterized according to molecular weight, Tm, RNase sensitivity, and inducing capacities.

In Table III we have chosen to compare two extremes of average molecular size for poly rI:poly rC. A 20-fold reduction in average molecular weight was accompanied by a 5°C decrease in Tm and more than a twofold increase in the rate of digestion by pancreatic RNase. No significant difference could
be observed in capacity to induce interferon in rabbits or interference in cell culture.

Fractionation of the polydisperse poly rI:poly rC preparations permitted us to compare even smaller molecular size of poly rI:poly rC fragments. As seen in Table IV, a reduction of molecular size below approximately $1.5 \times 10^6$ resulted in reduction of both interferon and host resistance induction. Thus, chain length of the complex within the range tested did influence capacity to induce interferon and stability of the complex. We do not know whether the decrease in this inducing capacity was related to the increased susceptibility to enzymatic digestion or to the slightly reduced Tm, but we do know that any critical comparisons of chemically different polynucleotide

<table>
<thead>
<tr>
<th>Mw, w</th>
<th>Approximate average molecular weight*</th>
<th>Tm</th>
<th>Relative R.Nase sensitivity</th>
<th>Rabbit serum interferon induction</th>
<th>Minimum protective dose in PRK§</th>
</tr>
</thead>
<tbody>
<tr>
<td>21.1</td>
<td>$6.4 \times 10^6$</td>
<td>63.5</td>
<td>1.0</td>
<td>5</td>
<td>&gt;640, 160</td>
</tr>
<tr>
<td>6.7</td>
<td>$2.7 \times 10^4$</td>
<td>58.5</td>
<td>2.2</td>
<td>5</td>
<td>&gt;640, 160</td>
</tr>
</tbody>
</table>

* Mw = $1.7 \times 10^8$ (Mw, w)$^{2.7}$ (see reference 14).
‡ Tm in buffer at ionic strength = 0.15.
§ Primary rabbit kidney cell culture.

interferon inducers will be in question unless differences in molecular size are also taken into account.

After finding that double-stranded RNA's were efficient inducers of interferon, our group proposed that double-stranded RNA's were likely responsible for interferon induction during virus infection. Although this idea was compatible with what was known about replication of RNA viruses, no evidence existed suggesting that double-stranded RNA played any role in replication of DNA viruses. Another objection to our proposal was that viruses, inactivated to destroy their capacity to carry out a complete replicative cycle, had been used to induce interferon formation. After all, how could a replicative form be the inducer if no replication occurred?

Within the past 6 months two very pertinent papers have been published. Colby and Duesberg (12) investigated the nucleic acids produced during vaccinia replication in chick cells. Vaccinia virus-infected cells readily produce interferon. The authors observed that during vaccinia replication RNase-
resistant RNA labeled with tritiated uridine specifically capable of hybridizing with vaccinia virus DNA was produced. This was further identified as double-stranded RNA, rather than a DNA-RNA hybrid. The double-stranded, virus specific RNA was capable of inducing interferon, and this activity was lost upon destruction of the double helix by thermal denaturation. The Colby and Duesberg paper has introduced a new concept of the possible role of double-stranded RNA in replication of DNA viruses. The second paper, by Huppert, Hillova, and Gresland (13), described the synthesis of virus-specific RNA in chick embryo cells infected with ultraviolet-irradiated Newcastle disease virus, which was incapable of producing infectious virus. The synthesis of a new virus-specific RNA suggested that some transient double-stranded RNA was also produced. Thus, from what we have discovered concerning the efficiency of double-stranded RNA’s as interferon inducers, and from the data that have been published concerning replication of RNA and DNA viruses, we are further convinced that double-stranded RNA plays a major role in interferon induction during virus infection.

### Table IV

CAPACITY OF VARIOUS MOLECULAR WEIGHT FRACTIONS OF POLY rI:POLY rC TO INDUCE INTERFERON IN RABBITS AND INTERFERENCE IN PRK CELL CULTURES

<table>
<thead>
<tr>
<th>Estimated average molecular weight*</th>
<th>Dose per rabbit</th>
<th>Rabbit serum interferon titer</th>
<th>Interference§ in PRK cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;1,000,000</td>
<td>2</td>
<td>40-80, 80-160</td>
<td>100</td>
</tr>
<tr>
<td>700,000</td>
<td>2</td>
<td>320-640, 80-160</td>
<td>—</td>
</tr>
<tr>
<td>400,000</td>
<td>2</td>
<td>320, &lt;5</td>
<td>100-200</td>
</tr>
<tr>
<td>270,000</td>
<td>2</td>
<td>10-20, 40-80</td>
<td>50-100</td>
</tr>
<tr>
<td>230,000</td>
<td>2</td>
<td>10-20, 10-20</td>
<td>50-100</td>
</tr>
<tr>
<td>160,000</td>
<td>2</td>
<td>&lt;5, 80-160</td>
<td>—</td>
</tr>
<tr>
<td>150,000</td>
<td>—</td>
<td>—</td>
<td>25-50</td>
</tr>
<tr>
<td>125,000</td>
<td>2.8</td>
<td>&lt;5, &lt;5</td>
<td>—</td>
</tr>
<tr>
<td>100,000</td>
<td>—</td>
<td>&lt;5, &lt;5</td>
<td>6.25-12.5</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>&lt;5, &lt;5</td>
<td>—</td>
</tr>
</tbody>
</table>

* Calculated from $s_{20,w}$ using mol wt = $1.74 \times 10^4 (s_{20,w})^{2.7}$ (see reference 14).

§Per cent of protective activity of inducer relative to protective activity of highest molecular weight poly rI:poly rC. 100% protection was obtained using 0.06 µg/ml.

**References**


