Comparative Study of Various Double-stranded RNAs as Inducers of Human Interferon

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SUMMARY

Various double-stranded RNAs of either synthetic or natural origin have been compared for their interferon-inducing potency in human skin fibroblasts ‘primed’ with interferon and ‘superinduced’ with cycloheximide and actinomycin D. While natural double-stranded RNAs (extracted from either *Penicillium chrysogenum* mycophage, *f₂* bacteriophage or reovirus) and alternating copolymers [(A-U)n. (A-U)n, (G-C)n. (G-C)n, (I-C)n. (I-C)n]* proved relatively less effective in inducing interferon than (I)n. (C)n, a variety of synthetic homopolymer pairs, including (I)n. (brSC)n, (I)n. (s2C)n, (A)n. (rT)n and (A)n. (U)n, showed an interferon-inducing activity comparable to that of (I)n. (C)n.

Although double-strandedness is a prerequisite for the interferon-inducing capacity of polynucleotides, there appears to be little restriction of the base composition or sequence required for a double-stranded (ds) RNA to act as an interferon inducer. In fact, both natural ds RNAs and synthetic ds RNAs, whether homopolymer pairs or alternating copolymers, have been shown to induce the formation of interferon (De Clercq, 1974; Torrence & De Clercq, 1977). Quantitative differences in the interferon-inducing activity of these different ds RNA classes have, however, never been determined. We have now compared a large variety of ds RNAs for their ability to stimulate interferon production in human diploid cells. This study was aimed at delineating the structural characteristics of the most potent (polynucleotide) inducer of human interferon.

To obtain a maximal interferon response (Havell & Vilšek, 1972; Billiau, Joniau & De Somer, 1973), human diploid cells [VGS strain, grown to confluency in Eagle’s minimal essential medium (EMEM) supplemented with 10% foetal calf serum (FCS)] were first treated (‘primed’) with human fibroblast interferon (100 units/ml) for 20 h, exposed to different ds RNA concentrations (in serum-free EMEM) for 1 h and then incubated with cycloheximide (10 µg/ml) for 6 h, with actinomycin D (1 µg/ml) present during the last 2 h of the cycloheximide incubation period. After removal of the metabolic inhibitors, the cells were replenished with EMEM + 3% FCS. After an additional 20 h incubation period, the cell culture fluids were harvested and assayed for interferon by a c.p.e. reduction assay in human skin fibroblasts challenged with vesicular stomatitis virus (in microtitre plates). The interferon titres are expressed as units in terms of the British Research Standard B (69–19) of human leukocyte interferon.

It should be emphasized that all ds RNAs which were included in our comparative study had previously been shown to induce interferon or resistance to virus infection in one or another assay system, namely *Penicillium chrysogenum* (PC) ds RNA in mice (Buck, Chain

* Abbreviations: (I)n, poly(inosinic acid); (C)n, poly(cytidylic acid); (A)n, poly(adenylic acid); (U)n, poly(uridylic acid); (rT)n, poly(ribothymidylic acid); (brC)n, poly(5-bromocytidylic acid); (s2C)n, poly(2-thiocytidylic acid); (A-U)n, alternating copolymer of adenylc acid and uridylic acid; (I-C)n, alternating copolymer of inosinic acid and cytidylic acid; (G-C)n, alternating copolymer of guanylic acid and cytidylic acid.
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& Himmelweit, 1971); \( f_2 \) bacteriophage ds RNA in mice and mouse leukocyte cultures (Doskočil et al. 1971); reovirus ds RNA in rabbits (Tytell et al. 1967); the alternating copolymers \((A-U)_n\), \((A-U)_n\), \((I-C)_n\), \((I-C)_n\) and \((G-C)_n\) \((G-C)_n\) in human skin fibroblasts (De Clercq, Wells & Merigan, 1970); \((G)_n\), \((C)_n\) in chick embryo cells in the presence of DEAE-dextran (Colby & Chamberlin, 1969; Novokhatsky et al. 1975); \((A)_n\), \((U)_n\) and \((A)_n\), \((rT)_n\) in rabbit kidney cells superinduced with cycloheximide and actinomycin D (De Clercq, Torrence & Witkop, 1974); \((I)_n\), \((br\beta C)_n\) in ‘superinduced’ rabbit kidney cells and human skin fibroblasts (Torrence et al. 1974; De Clercq et al. 1976); \((I)_n\), \((s\beta C)_n\) in rabbit kidney cells, rabbits, rats and dogs (Reuss, Scheit & Saiko, 1976) and \((I)_n\), \((C)_n\) preparations of varying molecular size in mice and rabbits as well as mouse and rabbit cell cultures (Tytell et al. 1970; Black et al. 1973; Stewart & De Clercq, 1974). Polynucleotides which were deemed inactive as interferon inducers (e.g. single- and triple-stranded polynucleotides, or double-stranded polynucleotides in which the hydroxyl group at C-2' of the ribose moiety was replaced by another substituent, or polynucleotide complexes in which the purine ring was altered; see De Clercq, 1974; Torrence & De Clercq, 1977) were not included in our study.

PC ds RNA samples derived either from the mycelial macerate (Lemke & Ness, 1970) or purified mycophages (Nash et al. 1973) were generously provided by R. J. Douthart (Lilly Research Laboratories, Indianapolis, Indiana). Their physicochemical properties have been described extensively (Lemke & Ness, 1970; Nash et al. 1973; Burnett, Frank & Douthart, 1975). \( f_2 \) ds RNA was extracted from \( E. coli \) infected with \( f_2 \) phage (Doskočil et al. 1971). It was purified as described by Tábořský, Doskočil & Zajicek (1974) and, kindly provided by L. Borecký (Institute of Virology, Slovak Academy of Sciences, Bratislava, Czechoslovakia). Reovirus ds RNA was a gift from J. Content (Pasteur Institute, Brussels, Belgium). It had been extracted from purified reovirus type 3 (Dearing strain) virions. The alternating copolymers \((A-U)_n\), \((A-U)_n\), \((I-C)_n\), \((I-C)_n\) and \((G-C)_n\), \((G-C)_n\) were purchased from Biogenics Research Corporation (Chagrin Falls, Ohio; lot numbers 377 247 and 391, respectively). The ‘standard’ complexes \((I)_n\), \((C)_n\) and \((A)_n\), \((U)_n\) were constituted with homopolymers obtained from P-L Biochemicals (Milwaukee, Wisconsin). The sedimentation values \((s_{20}, w)\) of \((I)_n\), \((C)_n\), \((A)_n\) and \((U)_n\) were 9.4, 10.6, 9.8 and 7.0S, respectively. The sources of \((I)_n\), \((br\beta C)_n\) and \((A)_n\), \((rT)_n\) have been described previously (De Clercq et al. 1974, 1976; Torrence et al. 1974). \((I)_n\), \((s\beta C)_n\) was kindly supplied by K. Reuss (E. Merck, Darmstadt, Germany). To constitute \((G)_n\), \((C)_n\), \((G)_n\) and \((C)_n\) were first deproteinized with a chloroform-isoamyl alcohol mixture, chromatographed on Sephadex G-100, dialysed against 0.1 M-NaCl, 1 mM-EDTA (pH 7), then 1 M-NaCl and then against distilled water, and finally annealed in different conditions (25°C in 0.1 M-NaCl, 0.05 M-HEPES, pH 7.0; at 90°C in distilled water; or at 90°C in 8 M-urea). \((I)_n\), \((C)_n\) polymers of different size \(s_{20}, w\): 12.5 or 2.5S for \((I)_n\); \(s_{20}, w\): 13.2 or 3.1S for \((C)_n\) were purchased from P-L Biochemicals (Milwaukee, Wisconsin). To constitute the homopolymer duplexes, \((I)_{12.5}\) or \((C)_{12.5}\) \((C)_{13.2}\) or \((C)_{3.1}\) were annealed at 1:1 mole P stoichiometry.

The results obtained with all polynucleotides are listed in Table 1. Of these polynucleotides only \((I)_n\), \((br\beta C)_n\) has previously been compared to \((I)_n\), \((C)_n\) for its interferon-inducing ability in ‘superinduced’ human skin fibroblasts (De Clercq et al. 1976). As noted before (De Clercq et al. 1976), \((I)_n\), \((br\beta C)_n\) is a more effective interferon inducer than \((I)_n\), \((C)_n\) when assayed at relatively low doses (0.01 to 0.1 μg/ml). Other ds RNAs which exhibited an interferon-inducing activity comparable to that of \((I)_n\), \((C)_n\) were \((I)_n\), \((s\beta C)_n\), \((A)_n\), \((rT)_n\) and \((A)_n\), \((U)_n\). Irrespective of the annealing procedure employed, \((G)_n\), \((C)_n\) proved completely ineffective in inducing interferon, even at 10 μg/ml, the highest concentration tested.
### Table 1. Interferon induction by various ds RNAs in human skin fibroblasts ‘primed’ with interferon and ‘superinduced’ with cycloheximide and actinomycin D

<table>
<thead>
<tr>
<th>Polynucleotide</th>
<th>Interferon titre (log₁₀ units/ml) obtained with the following concentrations of polynucleotide (µg/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>Natural ds RNAs</td>
<td></td>
</tr>
<tr>
<td>PC ds RNA (mycelium-extracted)</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>PC ds RNA (mycophage-extracted)</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>F₂ ds RNA</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Reovirus ds RNA</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Alternating copolymers</td>
<td></td>
</tr>
<tr>
<td>(A-U)ₙ . (A-U)ₙ</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>(I-C)ₙ . (I-C)ₙ</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>(G-C)ₙ . (G-C)ₙ</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Homopolymer pairs</td>
<td></td>
</tr>
<tr>
<td>(I)ₙ . (C)ₙ</td>
<td>1.5</td>
</tr>
<tr>
<td>(G)ₙ . (C)ₙ</td>
<td>—</td>
</tr>
<tr>
<td>(A)ₙ . (U)ₙ</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>(Aₙ . (rT)ₙ</td>
<td>1.7</td>
</tr>
<tr>
<td>(Iₙ . (s²C)ₙ</td>
<td>3.1</td>
</tr>
<tr>
<td>(Iₙ . (s²C)ₙ)</td>
<td>1.0</td>
</tr>
<tr>
<td>(Iₙ . (C)₁₂.₂)</td>
<td>3.0</td>
</tr>
<tr>
<td>(Iₙ . (C)₁₂.₃)</td>
<td>3.1</td>
</tr>
<tr>
<td>(Iₙ . (C)₁₂.₃)</td>
<td>2.6</td>
</tr>
<tr>
<td>(Iₙ . (C)₁₂.₃)</td>
<td>2.5</td>
</tr>
</tbody>
</table>

* All data represent average values for at least three determinations. The range of the individual values was ±0.2, ±0.3, ±0.5, ±0.7 and ±0.5 log₁₀ units/ml for the interferon titres obtained with 100, 10, 1, 0.1 and 0.01 µg/ml of polynucleotide, respectively.

At all doses, the natural (mycophage, bacteriophage or reovirus) ds RNAs elicited an interferon response which was 0.5 to 1 log₁₀ lower than the interferon response to the homopolymer pairs (I)ₙ . (C)ₙ, (I)ₙ . (s²C)ₙ, (Aₙ . (U)ₙ and (Aₙ . (rT)ₙ. The most effective interferon inducer of the three natural ds RNAs was the PC mycophage ds RNA. The ds RNA extracted from the purified PC virions did not markedly differ in activity from the ds RNA extracted from the whole mycelium.

The interferon-inducing behaviour of the alternating (A-U) copolymer was quite comparable to that of the mycophage ds RNA. The two other alternating copolymers, (I-C)ₙ and (G-C)ₙ . (G-C)ₙ, were less effective than (A-U)ₙ . (A-U)ₙ. Similar differences were observed previously (De Clercq et al. 1970) for the direct antiviral effects of alternating copolymers in human skin fibroblasts.

The ‘sized’ homopolymer pairs (I)₁₂.₅ . (C)₁₃.₂ and (I)₁₂.₅ . (C)₉.₁ proved superior in inducing interferon to all other ds RNAs, including the ‘standard’ (I)ₙ . (C)ₙ preparation. The differences in the interferon-inducing potency of the ‘sized’ and ‘standard’ (I)ₙ . (C)ₙ preparations may be related to the more heterogeneous character of the latter. In keeping with earlier findings (Tytell et al. 1970; Stewart & De Clercq, 1974), the interferon-inducing capacity of (I)ₙ . (C)ₙ depended more upon maintaining a high molecular size for (I)ₙ than for (C)ₙ. Yet the differences in activity between the complexes containing a (I)ₙ of high mol. wt. [(I)₁₂.₅ . (C)₁₃.₂ and (I)₁₂.₅ . (C)₉.₁] and the complexes containing a (I)ₙ of low mol. wt. [(I)₁₂.₅ . (C)₁₃.₂ and (I)₂₅ . (C)₉.₁] were relatively small (0.5 log₁₀ difference in interferon titre).

The main conclusions that emerge from our results, can be summarized as follows: (i)
Synthetic homopolynucleotide pairs \([\text{I}]_n\cdot\text{(C)}_n, \text{(I)}_n\cdot\text{(brS)}_n, \ldots\) are more efficient inducers of interferon than natural (mycophage, bacteriophage and reovirus) ds RNAs, as demonstrated in human diploid cells (Table 1) and other cell cultures (e.g. primary rabbit kidney cells; Thang et al. 1977). From data reported previously (Field et al. 1967), it also appears that to achieve direct resistance to virus infection in rabbit kidney cell cultures, significantly higher doses of natural ds RNAs are required than of \((\text{I})_n\cdot\text{(C)}_n\). The reasons for the differential behaviour of natural ds RNAs and synthetic homopolymer pairs are not clear at present. Long & Burke (1971) noted that interferon induction in chick cells by \((\text{I})_n\cdot\text{(C)}_n\) was considerably less sensitive to the effects of metabolic inhibitors than the interferon response to reovirus (ds RNA). Recently, we have found that natural ds RNAs inhibited protein synthesis in rabbit reticulocyte lysates at concentrations which were lower by several orders of magnitude than those at which the homopolymer pairs \((\text{I})_n\cdot\text{(C)}_n, \text{(I)}_n\cdot\text{(brS)}_n\) and \((\text{I})_n\cdot\text{(sS)}_n\) inhibited protein synthesis (J. Content, B. Lebleu & E. De Clercq, submitted for publication, 1977). Whether the potent inhibitory effect of natural ds RNAs on \textit{in vitro} protein synthesis accounts for their relatively poor interferon-inducing capacity in cultured cells, remains to be established. (2) Several homopolymer pairs, including \((\text{I})_n\cdot\text{(brS)}_n, \text{(I)}_n\cdot\text{(sS)}_n, \text{(A)}_n\cdot\text{(rT)}_n\) and \((\text{A})_n\cdot\text{(U)}_n\), proved nearly as active as \((\text{I})_n\cdot\text{(C)}_n\) in terms of interferon induction, if not more active. The most active were ‘sized’ \((\text{I})_n\cdot\text{(C)}_n\) complexes in which the \((\text{I})_n\) size was particularly high (12-55). It would appear, therefore, that for the induction of human interferon, \((\text{I})_n\cdot\text{(C)}_n\) could be advantageously replaced by some of its analogues. These analogues may prove suitable not only for the large-scale production of human interferon in fibroblast cultures, but, possibly, also for direct administration into man.

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\textbf{REFERENCES}  
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