Molecular Species of Interferon Induced in Mouse L Cells by Newcastle Disease Virus and Polyriboinosinic-polyribocytidylic Acid

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SUMMARY

Interferons were stimulated in mouse L cells by Newcastle disease virus (NDV) or by polyriboinosinic-polyribocytidylic acid poly(rI).poly(rC). These were fractionated by sequential affinity chromatography on bovine plasma albumin (BPA)-Sepharose and on ω-carboxypentyl (CH)-Sepharose. Based on their interaction with CH-Sepharose, interferon induced by NDV was resolved into three major bands of activity (L/NDV-1,2,3) and poly(rI).poly(rC)-interferon into two (L/rI:rC-1,2). These interferon components were purified to a specific activity of $3 \times 10^7$ to $4 \times 10^7$ units/mg protein by antibody affinity chromatography and examined by electrophoresis in SDS-polyacrylamide gels. A total of five molecular species was thus identified for NDV-induced interferon and three for poly(rI).poly(rC) induced interferon, as summarized in Table I. We conclude from our observations that mouse interferons can be produced by L cells in multiple forms with specific physiochemical properties and in proportions determined by the type of agent employed for induction.

INTRODUCTION

The heterogeneous nature of virus-induced mouse interferon has been documented by a variety of techniques including isoelectric focusing (Stanček et al. 1970), electrophoresis in polyacrylamide gels (Stanček & Paucker, 1971; Borecký et al. 1972; Paucker & Stanček, 1972; Kawade, 1973; Yamamoto et al. 1974), and ion exchange chromatography (Golgher & Paucker, 1973). More recently, two discrete molecular populations of mouse interferon were demonstrated by electrophoresis in SDS-polyacrylamide gels (Stewart II, 1974; Yamamoto & Kawade, 1976; De Maeyer-Guignard et al. 1978) and by chromatography on hydrophobic ligands (Davey et al. 1976). These were further resolved into a series of 10 to 11 active glycopeptides on the basis of electrophoretic mobilities in SDS-polyacrylamide gradient gels (Knight, 1975).

Except for a recent report (Maehara et al. 1977), the impact of non-viral inducers on the molecular composition of mouse interferon has not been systematically studied. Mouse interferons stimulated by viral and non-viral agents appear to be antigenically identical (Boxaca & Paucker, 1967; Fauconnier, 1967a, b; Ogburn et al. 1973). However, it has been suggested that interferons stimulated by NDV and poly(rI).poly(rC) in human foreskin fibroblasts may exhibit small but distinct differences in affinity for hydrophobic ligands.
(Jankowski et al. 1975). Therefore, in the present study we compared the distribution of the dominant molecular components of NDV- and poly(rI).poly(rC)-induced mouse L cell interferons by means of chromatographic procedures that have previously been used to purify and fractionate various interferon species.

**METHODS**

**Production of interferons.** Mouse interferon was obtained from NDV-treated L cells as previously described (Ogburn et al. 1973). Induction by poly(rI).poly(rC) was as detailed in an earlier report (Golgher & Paucker, 1973), except that optimal concentrations of the reagents were 10 µg/ml of the nucleotide and 500 µg/ml of DEAE-dextran (Pharmacia Fine Chemicals, Piscataway, New Jersey, U.S.A.).

**Interferon assay.** Mouse interferon was assayed in microtitre plates essentially as described for human interferon (Paucker et al. 1975) but with 50000 L cells seeded per well and encephalomyocarditis virus at an input multiplicity of 0·2 used for challenge. Titres are expressed in terms of the reference standard G002-904-511 for mouse interferon (assigned potency 12000 units) which titred 24000 units in our system.

**Affinity chromatography.** Crystallized bovine plasma albumin (BPA, Reheis Chemical Company, Chicago, Illinois, U.S.A.) was coupled to CNBr-activated Sepharose 4B (Pharmacia). Chromatography on BPA-Sepharose was carried out according to Davey et al. (1976). Interferon samples were dialysed against 0·05 m-sodium acetate, pH 5·0, for 18 h at 4 °C before being applied to a BPA-Sepharose column (20 × 150 mm) equilibrated with the same buffer. The breakthrough fraction containing any interferon and protein not bound by the columns was collected as a single pool. Washing with the equilibrating buffer was continued until the absorbance at 280 nm fell below 0·1. The buffer system was then changed to 0·02 m-sodium phosphate, pH 7·2 (E1), and individual fractions of 6 to 7 ml each were collected (see legend of Fig. 1). The composition of the elution buffer was subsequently changed again to 0·02 m-sodium phosphate, pH 7·2, 0·5 m-NaCl (E2) in a single step, to improve resolution of the interferon eluate. A flow rate of 60 ml/h was used throughout.

Chromatography on CH-Sepharose 4B (ω-carboxypentyl-agarose, Pharmacia) was carried out as described for BPA-Sepharose, but with a 15 × 100 mm column.

Chromatography of individual interferon species on immune globulins from rabbits injected with L cell-NDV interferon was performed as stated in an earlier publication (Ogburn et al. 1973). The size of the immunoabsorbent column was 10 × 300 mm. The specific activities of the eluted interferons ranged from 3 to 4 × 10³ units/mg protein. Eluates were concentrated about tenfold with Aquacide (Calbiochem, San Diego, California, U.S.A.) and dialysed against 0·01 m-sodium phosphate containing 0·1 % SDS.

**SDS-polyacrylamide gel electrophoresis.** Methods modified from Weber & Osborn (1969) were used. Gels of 13 % acrylamide (12·65 % acrylamide, 0·35 % bisacrylamide), 0·1 % SDS and 0·375 m-tris-HCl, pH 8·2, were polymerized in tubes of 5·5 mm inside diam. with 0·013 % ammonium persulphate (w/v) and 0·033 % (v/v) tetramethyl-ethylenediamine (Eastman Kodak Company, Rochester, New York). After 2 to 3 days, the gels were extruded from the tubes and dialysed against 0·01 m-sodium phosphate, pH 7·2, 0·1 % SDS (E-buffer) for at least 4 days. Such pre-dialysis of gels has been previously described (Weiner et al. 1972). During this step, the gels swelled to the extent of about 10 % in both linear dimensions. Gels, 100 mm long and equilibrated with E-buffer, were then sucked into glass tubes of 6 mm inside diam. and the lower ends of the tubes were covered with dialysis membranes held in place with a 3 mm section of rubber tubing.

Interferon samples were dialysed against E-buffer for 18 h at 23 °C before the addition of sucrose (final concentration 20 %, w/v) and bromophenol blue (0·005 %, w/v). Volumes
Mouse interferon molecular species

of 0.1 ml were electrophoresed at 23 °C, using a constant current of 3.5 mA/gel, for about 3.5 h, until the tracking dye had reached the end of the gel. The gels were then extruded from the tubes, frozen at −20 °C and cut into 2 mm sections. Each section was extracted with 2 ml of sodium/potassium phosphate-buffered saline, pH 7.2, containing 0.5% BPA and antibiotics, for 48 h at 23 °C, with gentle rocking. In a companion gel, a mixture of markers consisting of 10 µg each of ovalbumin, chymotrypsinogen, myoglobin and cytochrome c in 0.1 ml of E-buffer was co-electrophoresed with each experiment. This gel was fixed, stained with Coomassie blue R and destained as described by Weber & Osborn (1969). To compensate for any shrinkage or swelling of the gels during staining, destaining or freezing, the following adjustment was made: the distance migrated by each mol. wt. marker was divided by the length of the destained gel and the resulting ratio applied to find the corresponding position of the marker in the interferon gel.

Renaturation of interferon species. Incubation in the presence of SDS, β-mercaptoethanol and urea was essentially carried out as described by Stewart et al. (1974), except that the samples were treated for 18 h at room temperature.

RESULTS

Fractionation of mouse interferons on BPA-Sepharose

Chromatography of L cell interferon induced either by NDV or poly(rI).poly(rC) on BPA-Sepharose always led to the separation of two components (Fig. 1). In the presence of low molar phosphate (E1), a variable portion of the interferon was desorbed from the column. In 27 experiments with NDV-induced interferon (Fig. 1a), this fraction (designated as BPA-E1) contained between 17% and 44% of the initial titre. In three separate runs with poly(rI).poly(rC)-induced interferon (Fig. 1b), that percentage ranged from 4% to 29%. BPA-E1 preparations of both types tended to become turbid shortly after elution, unless NaCl was added to a final concentration of 0.15 M. The remainder of the interferon (designated as BPA-E2) was displaced from the column by the E2-buffer. This fraction containing 0.5 M-NaCl was entirely stable on prolonged storage in the cold, in contrast to observations by others (Kawakita et al. 1978).

In order to determine whether the interferon in the BPA-E1 fraction resulted from overloading of the column, representative materials were re-chromatographed on BPA-Sepharose. In each instance, the activity was quantitatively recovered in the same fraction as before (data not shown), thus confirming an affinity behaviour distinct from that of BPA-E2 interferon.

Fractionation of mouse interferons on CH-Sepharose

BPA-E1 fractions of interferons induced either by NDV or poly(rI).poly(rC) were excluded from CH-Sepharose under our prevailing conditions and thus could not be further separated by this ligand. On the other hand, BPA-E2 fractions of both types of interferon adsorbed to CH-Sepharose. No further fractionation was achieved with poly(rI).poly(rC)-interferon, all of the activity being recovered by elution with E1 buffer alone (Fig. 2b). In contrast, the BPA-E2 fraction of NDV-interferon was segregated into two components approximately equal in size by elution with E1 and E2 buffers, respectively (Fig. 2a). These findings were reproduced for each of the interferons on at least three separate occasions.

Since NDV-stimulated interferon was routinely treated at pH 2.0 for several days to inactivate the inducer virus, whereas no such treatment was applied to poly(rI).poly(rC)-interferon, it seemed possible that the activity peak of NDV-interferon, eluted from CH-Sepharose by E2 might be an artifact resulting from exposure to low pH. To investigate this possibility, an NDV-induced interferon preparation was divided into two portions. One was
**Fig. 1.** Chromatography of mouse L cell interferons on BPA-Sepharose. (a) Interferon induced with NDV, input $1.5 \times 10^6$ units in 100 ml; breakthrough fraction (not shown), $< 6.4 \times 10^4$ units ($< 1\%$); E1 eluate, $3.7 \times 10^3$ units (33%); E2 eluate, $8 \times 10^5$ units (70%); fraction size was 6 ml. (b) Interferon induced with poly(rI), poly(rC), input $1.5 \times 10^6$ units in 75 ml; breakthrough fraction (not shown), $< 4.2 \times 10^4$ units ($< 1\%$); E1 eluate, $3.9 \times 10^5$ units (29%); E2 eluate, $7.2 \times 10^6$ units (53%); fraction size was 7 ml. Arrows indicate positions where elution buffers were applied.

**Fig. 2.** Chromatography of mouse L cell interferons on CH-Sepharose after desorption from BPA-Sepharose by E2 elution buffer (see Fig. 1). BPA (0.5%, w/v) was added as a carrier protein. (a) Interferon induced with NDV, input $4.7 \times 10^6$ units in 39 ml; breakthrough fraction (not shown), $1.8 \times 10^5$ units (4%); E1 eluate, $3.3 \times 10^4$ units (25%); E2 eluate, $1.1 \times 10^6$ units (23%); fraction size was 5 ml. (b) Interferon induced with poly(rI), poly(rC), input $6 \times 10^6$ units in 30 ml; breakthrough fraction (not shown), $9.7 \times 10^5$ units (16%); E1 eluate, $3.2 \times 10^6$ units (54%); fraction size was 5 ml. Arrows indicate positions where elution buffers were applied.
Mouse interferon molecular species

Fig. 3. SDS-PAGE of NDV-induced mouse L cell interferon species separated on CH-Sepharose (see text for details of fractions tested). (a) L/NDV-1, excluded from CH-Sepharose, input 8 x 10^4 units; recovery 2.8 x 10^4 units (35%). (b) L/NDV-2, eluted from CH-Sepharose by E1 elution buffer, input 8 x 10^3 units; recovery, 2.1 x 10^3 units (26%). (c) L/NDV-3, eluted from CH-Sepharose by E2 elution buffer, input 6.4 x 10^3 units; recovery 1.1 x 10^3 units (17%). ○--○, Interferon (units/ml); ■--■, mol.wt. markers.

treated in the usual manner for five days at pH 2.0 in the cold and the other was irradiated for 10 min with u.v. light (Paucker et al. 1970). Each portion was then chromatographed on BPA-Sepharose, and the BPA-E2 eluates were chromatographed on CH-Sepharose. The acid-treated and irradiated preparations were similarly resolved into two components under the elution conditions described. Hence, the NDV-interferon peak eluting from CH-Sepharose in the presence of E2 buffer appears to be an authentic component which is not elicited in L cells by poly(rI), poly(rC).

Therefore, based on the interferon inducer and elution conditions from CH-Sepharose, five different interferon fractions can be defined. To facilitate their discussion, those fractions excluded from CH-Sepharose have been designated as L/NDV-1 and L/rI:rC-1, those fractions eluted by E1 as L/NDV-2 and L/rI:rC-2 and the fraction eluted by E2 as L/NDV-3.

Characterization of mouse interferon fractions by SDS-polyacrylamide gel electrophoresis (SDS–PAGE)

We next sought to determine whether the NDV- and poly(rI), poly(rC)-interferon species recovered from CH-Sepharose under the same conditions were of comparable molecular size as defined by SDS–PAGE. To that end, portions of the five interferon fractions obtained by CH-Sepharose chromatography were first purified on anti-interferon globulins (see Methods) and samples concentrated approximately 10-fold were electrophoresed on at least
Fig. 4. SDS–PAGE of poly(rI), poly(rC)-induced mouse L cell interferon species separated on CH-Sepharose (see text for details of fractions tested). (a) L/rI:rC-1, excluded from CH-Sepharose, input 4 × 10⁸ units; recovery 1.1 × 10⁸ units (28%). (b) L/rI:rC-2, eluted from CH-Sepharose by EI elution buffer, input 2.8 × 10⁶ units; recovery 5.8 × 10⁸ units (21%). ○—○, Interferon (units/SDS–PAGE)

Table 1. Classification of mouse L cell interferon species according to the inducer, interaction with CH-Sepharose 4B and electrophoretic mobility on SDS–PAGE

<table>
<thead>
<tr>
<th>Designation</th>
<th>Inducer</th>
<th>CH-Sepharose 4B*</th>
<th>SDS–PAGE</th>
<th>Apparent mol.wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>L/NDV-1S</td>
<td>NDV</td>
<td>Excluded</td>
<td>Slow</td>
<td>42000</td>
</tr>
<tr>
<td>L/NDV-1F</td>
<td></td>
<td>Excluded</td>
<td>Fast</td>
<td>28000</td>
</tr>
<tr>
<td>L/NDV-2S</td>
<td></td>
<td>Low salt elution</td>
<td>Slow</td>
<td>40000</td>
</tr>
<tr>
<td>L/NDV-2F</td>
<td></td>
<td>Low salt elution</td>
<td>Fast</td>
<td>30000</td>
</tr>
<tr>
<td>L/NDV-3S</td>
<td></td>
<td>High salt elution</td>
<td>Slow</td>
<td>38000</td>
</tr>
<tr>
<td>L/rI:rC-1S</td>
<td>Poly(rI), poly(rC)</td>
<td>Excluded</td>
<td>Slow</td>
<td>38000</td>
</tr>
<tr>
<td>L/rI:rC-1F</td>
<td></td>
<td>Excluded</td>
<td>Fast</td>
<td>23000</td>
</tr>
<tr>
<td>L/rI:rC-2S</td>
<td></td>
<td>Low salt elution</td>
<td>Slow</td>
<td>37000</td>
</tr>
</tbody>
</table>

* Low salt denotes 0.02 M-sodium phosphate, pH 7.2 (EI). High salt denotes EI plus 0.5 M-NaCl.

two separate occasions in SDS–polyacrylamide gels. The results illustrated in Fig. 3 show that L/NDV-1 (panel a) and L/NDV-2 (panel b) were each resolved into two subspecies with different electrophoretic mobilities. Apparent mol.wt. of the more slowly migrating components were in the range of 40000 to 42000 and those of the faster component 28000 to 30000. L/NDV-3 (Fig. 3c) migrated as a single homogeneous fraction with an apparent mol.wt. of 38000.

With poly(rI), poly(rC)-interferon (Fig. 4), L/rI:rC-1 was also resolved into two molecular species with distinct electrophoretic mobilities (Fig. 4a), corresponding to apparent mol.wt. of 38000 and 23000 respectively. However, L/rI:rC-2 migrated as a single peak (apparent mol.wt. 37000), as shown in Fig. 4(b).

Thus, a total of five molecular species has been identified for NDV-interferon and three for poly(rI), poly(rC)-interferon, on the basis of their interaction with CH-Sepharose 4B and electrophoresis in SDS–polyacrylamide gels. They are designated as shown in Table 1.
**Table 2. Differences in renaturation of inactivated L/NDV-2 and L/rI:rC-2 mouse interferon species**

<table>
<thead>
<tr>
<th>Treatment†</th>
<th>L/NDV-2</th>
<th>L/rI:rC-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before freezing</td>
<td>15,000 (100%)</td>
<td>20,000 (100%)</td>
</tr>
<tr>
<td>After thawing</td>
<td>&lt;1,000 (&lt;7%)</td>
<td>&lt;1,000 (&lt;5%)</td>
</tr>
<tr>
<td>SDS</td>
<td>&lt;1,000 (&lt;7%)</td>
<td>1,500 (7.5%)</td>
</tr>
<tr>
<td>ME</td>
<td>&lt;1,000 (&lt;7%)</td>
<td>&lt;1,000 (&lt;5%)</td>
</tr>
<tr>
<td>U</td>
<td>&lt;1,000 (&lt;7%)</td>
<td>&lt;1,000 (&lt;5%)</td>
</tr>
<tr>
<td>SDS/ME</td>
<td>&lt;1,000 (&lt;7%)</td>
<td>8,000 (40%)</td>
</tr>
<tr>
<td>SDS/U</td>
<td>&lt;1,000 (&lt;7%)</td>
<td>&lt;1,000 (&lt;5%)</td>
</tr>
<tr>
<td>SDS/ME/U</td>
<td>&lt;1,000 (&lt;7%)</td>
<td>16,000 (80%)</td>
</tr>
</tbody>
</table>

* See Methods.
† Abbreviations denote sodium dodecyl sulphate (SDS), β-mercaptoethanol (ME) and urea (U).

**Stability of L/NDV-2 and L/rI:rC-2 interferon species**

It has been shown that despite their comparable elution behaviour from CH-Sepharose, L/NDV-2 and L/rI:rC-2 differed in the number of subspecies they contained. Further differences became apparent when it was discovered that freezing and thawing of these preparations in the presence of the low salt containing E1 buffer resulted essentially in complete loss of activity. However, the antiviral activity of L/rI:rC-2 could be restored, whereas that of L/NDV-2 was irretrievably lost. In the experiment presented in Table 2, a sample of each interferon species in E1 buffer was frozen at -20 °C, thawed at room temperature and incubated for 18 h at 23 °C in the presence of 1% SDS, 1% β-mercaptoethanol, 4 M-urea, either with each ingredient alone, or in the combinations shown in the table. The results indicate that the interferon activity of L/rI:rC-2 was partially recovered by incubation with a mixture of SDS, mercaptoethanol and urea, or of SDS and mercaptoethanol, but not that of L/NDV-2.

In other experiments not detailed here, it was found that in its inactive form after freezing and thawing, L/rI:rC-2 retained full antigenicity, as shown by the ability of Sepharose-bound anti-mouse interferon globulins to remove from such preparations the potential for renaturation.

**DISCUSSION**

In the present report, we have identified several mouse interferon species by means of sequential affinity chromatography on BPA- and CH-Sepharose columns. These findings differ in some respects from those contained in two earlier publications (Davey et al. 1976; Maehara et al. 1977). Our data indicate that both NDV- and poly(rI).poly(rC)-induced mouse interferons can be fractionated on BPA-Sepharose into two components, one requiring NaCl for elution (BPA-E2), the other not (BPA-E1). During subsequent chromatography on CH-Sepharose, BPA-E1 was completely excluded from the ligand whereas NDV-induced BPA-E2 was further resolved into two subspecies one of which again needed NaCl for elution (L/NDV-3), but the second did not (L/NDV-2). On the other hand, poly(rI).poly(rC)-induced BPA-E2 interferon eluted as a single fraction in the absence of NaCl (L/rI:rC-2).

According to Davey et al. (1976) who pioneered the use of BPA-Sepharose chromatography for purification of mouse interferon, only one fraction of activity was eluted from this column in an NaCl gradient of rising molarity, at neutral pH, and no interferon activity was detected in the breakthrough fraction from CH-Sepharose. Machara et al. (1977)
demonstrated the existence of three zones of interferon activity comparable to those described here, based on the interaction of mouse interferons from several sources with CH-Sepharose, but found that interferons induced by NDV and poly(rI).poly(rC) in the same cell population were similar. Others concluded also that the cell source rather than the inducer determined the composition of human interferons (Cesario et al. 1977). The reasons for these discrepancies are not clear, but they may reflect the variable distribution of interferon sub-species in individual preparations. Furthermore, the resolution of minor components may be difficult when small amounts of interferon are used for fractionation.

It is of interest that each of the interferon species identified by SDS-PAGE had an electrophoretic mobility corresponding to one of the two general mol.wt. regions previously observed with unfractionated mouse interferons (Stewart II, 1974; Knight, 1975; Yamamoto & Kawade, 1976; De Maeyer-Guignard et al. 1978). Therefore, our data indicate that within each of the ‘fast’ and ‘slow’ components of unfractionated virus- or poly(rI).poly(rC)-induced mouse interferon, subspecies exist that can be separated on the basis of their affinity for CH-Sepharose. The nature of this affinity could be electrostatic, hydrophobic, or a combination of the two forces, possibly compounded by any conformational shifts that may occur in individual interferon components under elution conditions that involve substantial changes in pH and ionic strength. The existence of differently charged components of comparable molecular size has also been described for human leukocyte interferon (Lin et al. 1978).

Although mouse interferons stimulated by NDV (Stewart II, 1974) and MM virus (Knight, 1975) in L cells, and by NDV in C-243 cells (Stewart II et al. 1976), present comparable electrophoretic profiles in SDS-polyacrylamide gels, the recent report by Maehara et al. (1977) clearly indicates that the cell source may profoundly affect the molecular composition of mouse interferons. It has also been shown previously that the type of inducer can determine the antigenic and other properties of interferons elicited in the mouse (Youngner & Salvin, 1973; Salvin et al. 1975). We have now shown that even the same population of mouse cells may respond in different ways to a viral and synthetic inducer. Similar findings were recently reported with virus- and poly(rI).poly(rC)-induced interferons in cultures of human fibroblasts (Havell et al. 1978).

All the subspecies of L cell interferon, stimulated by NDV and poly(rI).poly(rC) were partially purified on the same antibody column, which confirms earlier findings from this laboratory that these two mouse interferons are antigenically similar (Ogburn et al. 1973). However, this does not preclude the existence of minor antigenic differences among individual subspecies which would not be detected by a serum containing mixtures of antibodies with specificities for several distinct interferon antigens.

At least two of the subspecies, L/NDV-2 and L/rI: rC-2, which eluted from CH-Sepharose under identical conditions had further configurational features which were not resolved by this hydrophobic ligand. This points to the likelihood that additional molecular species with distinctive physicochemical properties may yet be identified in these mouse interferons. The remarkable multiplicity of mouse interferon components may be due to minor structural adornments which have no physiological significance, as has also been postulated by others (Knight, 1975; Stewart II et al. 1977). Alternatively, mouse interferon molecular populations may differ considerably in a number of antiviral and other attributes that have been ascribed to them. Experiments to resolve these interpretations are in progress.
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REFERENCES


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