Interferon Stabilization and Enhancement by Rare Earth Salts

(Accepted 18 August 1980)

SUMMARY

The addition of salts of the rare earth elements (lanthanides) enhances by a factor of two or more the initial activity of human fibroblast, leukocyte and immune interferons as well as mouse L-cell interferon. Furthermore, the salts of the lanthanides (0.01 to 0.002 M) preserve the activity of human fibroblast interferon not only after 4 days of heating at 37 °C but also after exposure to severe shearing forces; human leukocyte interferon was stabilized to a lesser degree, albeit significantly.

Interferons can become especially unstable during purification or the preparation of large quantities for clinical use. Approaches that have been reported to stabilize interferons against thermal and/or mechanical inactivation include the use of (i) low pH (Edy et al., 1974; Jariwalla et al., 1975; Marshall et al., 1972); (ii) detergents, such as sodium dodecyl sulphate (Stewart et al., 1975; Knight, 1976) or Tween 80 (De Somer et al., 1974; Sedmak et al., 1978); (iii) thioctic acid and other chemicals which inhibit disulphide bond formation (Cartwright et al., 1977b); (iv) ethylene glycol (Heine et al., 1978); (v) addition of proteins, such as bovine serum albumin (Davey et al., 1976) or cytochrome c (Anfinsen et al., 1974); and (vi) chaotropic salts, such as guanidine thiocyanate (Jariwalla et al., 1977).

We report here that relatively non-toxic salts (Haley, 1965) of rare earth elements (atomic numbers 57 to 71) at concentrations as low as 0.002 M not only effectively protect human fibroblast interferon from thermal and shearing forces but, to a lesser degree, also protect human leukocyte and mouse L-cell interferons against thermal inactivation. All the lanthanides (99.9% or greater purity) were obtained from Apache Chemicals, Seward, Ill., U.S.A. Antiviral activity of the human interferons was determined by inhibition of encephalomyocarditis (EMC) virus haemagglutinin yield in the BUD-8 strain of human skin fibroblasts (Jameson et al., 1977). This assay measured a geometric mean of 4.54 (s.d. 0.141) log_{10} units for the international human fibroblast interferon standard G023-902-527, or about 3.5 times the 4.0 log_{10} units assigned to it, and 5.26 (S.D. 0.32) log_{10} units for the NIH human leukocyte standard G023-901-527, or about nine times the 4.3 log_{10} units assigned to it. Mouse interferon was assayed by inhibition of the yield of GDVII virus haemagglutinin in mouse L-cells (Oie et al., 1972), a method measuring 4.13 (s.d. 0.28) log_{10} units, or about the same value (4.08) assigned to the international mouse L-cell interferon standard G002-904-511.

Fibroblast interferon supplied by NIH had been prepared by superinduction by Dr Vilcek (Havell & Vilcek, 1972) in human foreskin cell with poly(I).poly(C) and purified by C. Anfinsen by immuno-affinity chromatography (Anfinsen et al., 1974). This preparation had a specific activity of 1.6 × 10^6 units/mg protein after the addition of 500 µg/ml cytochrome c. It was diluted to 5 µg/ml protein for stability studies. Human leukocyte interferon was obtained as partially purified, Sendai virus-induced interferon from Dr K. Cantell (Helsinki, Finland), with a specific activity of 4.8 × 10^6 units/mg protein. Immune interferon, a crude preparation (sp. act. 54 units/mg protein) from cultures of lymphocytes and macrophages treated with phytohaemagglutinin, was provided by Dr L. Epstein, San Francisco, Calif., U.S.A. Mouse L-cell interferon was induced with Newcastle disease virus (NDV) and after
**Short communications**

Table 1. *Stability of human fibroblast interferon at 37 °C and in a vortical agitation test in the presence of lanthanide salts*

<table>
<thead>
<tr>
<th>Lanthanide salt*</th>
<th>Molarity</th>
<th>Activity (units/ml) after heating† at 37 °C for</th>
<th>After agitation‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 day</td>
<td>1 day</td>
</tr>
<tr>
<td>Control</td>
<td>0.01</td>
<td>5000</td>
<td>800(16)§</td>
</tr>
<tr>
<td>CeCl₃</td>
<td>0.01</td>
<td>12000</td>
<td>9000(75)</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>12000</td>
<td>12000(100)</td>
</tr>
<tr>
<td>DyCl₃</td>
<td>0.01</td>
<td>17000</td>
<td>12000(71)</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>17000</td>
<td>17000(100)</td>
</tr>
<tr>
<td>ErCl₃</td>
<td>0.01</td>
<td>7000</td>
<td>7000(100)</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>7000</td>
<td>7500(107)</td>
</tr>
<tr>
<td>SmCl₃</td>
<td>0.01</td>
<td>17000</td>
<td>14000(78)</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>17000</td>
<td>20000(118)</td>
</tr>
<tr>
<td>GdCl₃</td>
<td>0.01</td>
<td>17000</td>
<td>20000(118)</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>17000</td>
<td>24000(90)</td>
</tr>
<tr>
<td>DyCl₃</td>
<td>0.01</td>
<td>20000</td>
<td>17000(85)</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>20000</td>
<td>24000(90)</td>
</tr>
<tr>
<td>YbCl₃</td>
<td>0.01</td>
<td>17000</td>
<td>17000(100)</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>17000</td>
<td>20000(100)</td>
</tr>
</tbody>
</table>

* Stock solutions (1 M) of the lanthanide salts were prepared in 0.5 M-HCl. For stability tests the salts were then diluted into 0.05 M-tris-HCl and the pH adjusted to 7.2. The buffer alone was used for the control sample.

† Interferon (0.5 ml) in the indicated salt solution was incubated in a 37 °C water bath for the indicated time and then frozen at −75 °C until assayed for antiviral activity.

‡ Samples (1 ml) of interferon in the indicated solution were subjected to agitation at fast-speed setting with a Lab-line Instruments vortex action mixer no. 1290. The agitation was done in 30-s bursts at 4 °C to obviate thermal inactivation due to friction.

§ Values in parentheses are the % residual activity after heating or agitation relative to the unheated or unagitated preparation containing the same concentration of lanthanide salt. The heating and agitation tests were run independently.

purification by Sephadex G-200 column chromatography had a specific activity of 9 × 10⁵ units/mg protein.

Table 1 shows the effect of the chloride salts of various elements in the lanthanide series on the preservation of activity of human fibroblast interferon heated at 37 °C. Tests conducted at this temperature were more predictive of the long-term stability of fibroblast interferon held at commonly used storage temperatures than were screening tests at higher temperatures (Sedmak *et al.*, 1978). Fibroblast interferon retained 40 to 100% of its activity when heated for 4 days in the presence of 0.01 M- or 0.002 M-lanthanide salts compared to <2.5 % activity in the control. The chloride salts of other metallic ions such as Mg²⁺ and Mn²⁺ at even higher concentrations (Sedmak *et al.*, 1978) did not enhance stability. Lanthanum acetate was as effective as LaCl₃ in preventing thermal inactivation, whereas magnesium acetate was ineffective. All fibroblast interferon activity was recovered even after 18 days of heating at 37 °C in the presence of 0.002 M-YbCl₃ and 10 % of the activity remained after 42 days of heating. LaCl₃ at 0.002 M enhanced the stability of fibroblast interferon over a range of pH values from 3 to 9 in different buffers; however, phosphate buffers caused the formation of insoluble lanthanum phosphates.

A problem encountered during the processing of human fibroblast interferon has been its inactivation under conditions of mechanical stress that may occur with agitation of liquid preparations (De Somer *et al.*, 1974; Cartwright *et al.*, 1977a). Low pH or Tween 80 (De Somer *et al.*, 1974) and reagents which inhibit disulphide bond formation (Cartwright *et al.*, 1977b) have been reported to protect fibroblast interferon in mechanical stress tests. We
have found that the lanthanide salts at 0.002 M and higher concentrations also prevent such inactivation (Table 1) in a vortical agitational test (Sedmak et al., 1978). After 2 min of vigorous vortex agitation the control preparation retained only 14% of its potency, whereas > 70% of the activity was recovered in the presence of all the lanthanide salts, except cerium.

Also apparent from Table 1 is the 1.4- to 6-fold enhancement of interferon activity by the lanthanides, even in unheated preparations. In some instances greater than 6-fold enhancement of fibroblast interferon activity has been observed. This enhanced activity was also noted for human leukocyte and immune interferons, as well as mouse L-cell interferon (Table 2); however, the rare earth salts were less effective in stabilizing these interferons against thermal inactivation. Since leukocyte interferon is more stable than the fibroblast type, the former was heated to 50 instead of 37 °C. Various lanthanides stabilized leukocyte interferon such that 15 to 40% activity remained at 24 h, compared to 10% for the control; and after 4 days of heating, the residual activity was 5 to 10% for the samples containing lanthanides, compared to 0.1 to 1% for the control.

The mechanism of lanthanide stabilization of human fibroblast interferon is presently not understood. It has been reported that fibroblast interferon has an affinity for chelates of Cu\(^{2+}\) (Chadha et al., 1979) and Zn\(^{2+}\) (Edy et al., 1977), indicating that fibroblast interferon may undergo coordination with transition metal cations. If human fibroblast interferon also complexes with lanthanide cations, the binding of these cations may impart rigidity to the interferon such that it does not unfold under denaturing conditions. Such a mechanism has been postulated in the case of the thermal stability of a bacterial \(\alpha\)-amylase bound to calcium (Hasegawa & Imahori, 1976). Indeed, if the lanthanide cations bind to interferon (for which we have preliminary evidence), the higher interferon titres that result from the simple addition of the cations may result from a greater affinity of the interferon–cation complex for interferon receptors on cell membranes, an hypothesis we are now testing experimentally.

Lanthanide chlorides have only slight toxicity (Haley, 1965). The LD\(_{50}\) mg/kg for LaCl\(_3\) given intravenously to a rabbit is 200 to 250, compared to an LD\(_{50}\) of 2910 for NaCl. Based on this low toxicity in rabbits it seems likely that lanthanide ions at a concentration of 0.002 M, or even at higher concentrations, would be acceptable in preparations for human injection and thus suitable for enhancing or stabilizing interferons during preparation, processing, purification, shipment and storage.

This investigation was supported by an award from the National Institute of Health, N01 AI42520. We express our appreciation to M. Dixon and C. Schoenherr for their excellent technical assistance.
REFERENCES


(Received 4 March 1980)