STIMULATION OF INTERFERON YIELDS FROM CULTURED HUMAN CELLS BY CALCIUM SALTS

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1. Introduction

Interferon is a glycoprotein with antiviral effects produced by many cell types in response to viral infections [1] or by treatment with natural and synthetic double-stranded ribopolynucleotides such as polyinosinic : polycytidylic acid (poly(IC)) [2]. In view of the current considerations of possible uses of interferon in treating diseases, methods of increasing interferon yields in cell cultures are of interest. The yields of interferon produced by cultured cells in response to inducing agents vary considerably. High yields are often not reached by simple induction procedures such as the single application of poly(IC) and it has been found that use of inhibitors of protein and RNA synthesis, e.g., the combination of cycloheximide and actinomycin D, are required for maximum amounts of interferon to be produced after poly(IC) induction [3–5]. This has been called superinduction [4]. Pre-treating cells with homologous interferon prior to poly(IC) induction (priming) is another method often used to increase interferon yield [6,7]. We report here that the addition of soluble calcium salts to the medium of cultured human cells, leads to enhanced production of human interferon when the cells are induced with poly(IC).

2. Materials and methods

The human cell lines used were:

(i) Human embryo fibroblasts (HEF).
(ii) An SV40-transformed human cell line, WI26 VA4 FU3 obtained from Dr L. Diamond, Wistar Institute, PA [8].
(iii) An osteosarcoma cell line, MG63, obtained from Dr A. Billiau, Rega Institute, Leuven [9].

All cells were cultured in Glasgow modified minimal essential medium supplemented with non-essential amino acids, 10%, v/v, foetal bovine serum (FBS), (Flow laboratories, Irvine, Ayrshire) and penicillin (200 units/ml) and streptomycin (100 μg/ml). Induction with poly(IC) was carried out in serum-free medium after extensive washing of confluent cell monolayers on 50 mm plastic dishes. Cultures were incubated with poly(IC) (PL Biochemicals, Milwaukee, WI) at 50 μg/ml in 2 ml serum-free medium for 1 h at 37°C, washed several times with serum-free medium, and then incubated with maintenance medium (2% FBS) at 37°C until interferon fluids were collected. Interferon samples were dialysed for 5 days at 4°C against phosphate-buffered saline (PBS, pH 7) to eliminate excess calcium and inhibitors when used, and treated with ribonuclease (pancreatic ribonuclease, 50 μg/ml + T_1 ribonuclease, 300 units/ml) to remove any residual poly(IC) for 1 h at 37°C before assaying on HEF cells or a line of human foreskin fibroblasts (HFF). Interferon assays were carried out as in [10]. Interferon titres are expressed in reference research units and all assays contained an internal standard.

The preparation of poly([5-^{203}Hg]C) was carried out by a modification of the method in [11]. Poly(C) (1 mg) in 100 mM sodium acetate was treated with a 5-fold excess of mercuric acetate, containing ^{203}Hg, at 50°C for 24 h. The reaction mixture was dialysed against a solution of 200 mM sodium chloride and 10 mM Tris–HCl (pH 7.4), with several changes of buffer, for 5–6 days. The labelled poly(C) was precipitated at −20°C with 5 vol. ethanol, dissolved in PBS and hybridized with an equivalent amount of poly(I).

The specific activity was 1.1×10^4 cpm/μg.

Pancreatic ribonuclease, T_1 ribonuclease and cycloheximide were purchased from Sigma Chemical Co., London. Actinomycin D was a gift from Merck, Sharp and Dohme Ltd.

3. Results and discussion

When the three human cell lines were induced with poly(IC) at 50 μg/ml, MG63 cells gave the highest amounts of interferon (table 1) in agreement [9]. All cell lines produced increased interferon yields in a superinduction schedule of a 0–6.5 h treatment with cycloheximide (50 μg/ml) followed by a 6.5–7.0 h treatment with actinomycin D (5 μg/ml) (table 1). HEF (and other human diploid cell lines [6,7]) and WI26 VA4 FU3 produced even higher amounts of interferon when priming and superinduction schemes were combined (table 1).

It was found that soluble calcium salts also enhanced the interferon yields from poly(IC)-induced human cells during a programme designed to test the effect of various nucleotides, drugs and salts on interferon production. Typical results are shown in table 2. The $CaCl_2$ was added to culture medium, which already contained 2.0 mM calcium chloride, prior to, during and after poly(IC) induction. The maximal amounts of interferon were produced when $CaCl_2$ at 12 mM was present continuously from 12 h before poly(IC) (50 μg/ml for 1 h) induction until 20 h after induction when the interferon was harvested. Increasing the calcium ion concentration above 12 mM led to heavy precipitation of calcium salts, probably calcium phosphate, cytotoxicity and reduced interferon yields. Addition of calcium salts (chloride or nitrate) prior to or after poly(IC) induction also enhanced interferon yields, but not to the levels produced when calcium chloride at 12 mM was maintained throughout the induction schedule. When the $CaCl_2$ was added to the cultures only during the period that poly(IC) was in contact with them, a small stimulation of interferon was still observed. Additions of equivalent amounts of sodium and magnesium salts did not produce the effect and virus (e.g., Newcastle disease virus)-induced interferon yields were not altered in the presence of excess calcium salts.

When the cells were superinduced in the presence of increasing concentrations of $CaCl_2$, yields of interferon were generally not as high as those produced by poly(IC) induction in the presence of $CaCl_2$ alone, i.e., there is no summative effect (table 2). A possible reason for this is the severe cytotoxicity caused by the combined use of $CaCl_2$, cycloheximide and actinomycin D. Priming cultures in the presence of 12 mM $CaCl_2$ also did not further increase interferon yields (A. M., unpublished results).

Table 1
Interferon inductions in human cells with poly(IC); comparison of superinduction and priming effects

Cell line	Interferon yields[a] affected by			
	Poly(IC) alone	Poly(IC) plus superinduction[b]	Poly(IC) plus priming[c]	Poly(IC) plus priming and superinduction
HEF	200–400	6000	2000	24 000
WI26 VA4 FU3	790	1050	1480	5000
MG63	6250	44 000	n.d.[d]	n.d.

[a] Yields expressed as reference research units/ml/10^6 cells

[b] Superinduction schedule: at time 0 cell monolayers were incubated at 37°C with poly(IC) (50 μg/ml) in 2 ml serum-free medium containing cycloheximide (50 μg/ml). One hour later poly(IC) was removed and replaced by 2 ml maintenance containing cycloheximide (50 μg/ml). Actinomycin D was added at 6.5 h to final conc. 5 μg/ml. At 7 h, the monolayers were washed extensively and re-incubated with 2 ml maintenance medium for a further 16 h [5]

[c] Priming schedule: cell monolayers were pretreated overnight with purified human leucocyte interferon at 200 reference research units/ml

[d] n.d., not done

Table 2
Effects of $CaCl_2$ additions on interferon yields induced by poly(IC) (A) and poly(IC) plus superinduction (B)

$CaCl_2$	Interferon yields[c]		
(mM)[b]	HEF	WI-26 VA4 FU3	MG63
A. Simple poly(IC) induction[a]			
2	250	700	6250
7	1780	3400	28 000
12	3550	4960	35 000
22	n.d.[e]	1580	n.d.
B. Poly(IC) plus superinduction[d]			
2	8000	1000	44 000
7	2820	3160	25 000
12	1260	4140	15 600
22	n.d.	1100	n.d.

[a] Induction schedule: cells pretreated with $CaCl_2$, 12–0 h, poly(IC) (50 μm/ml) with $CaCl_2$, 0–1 h; maintenance medium with $CaCl_2$, 1–20 h
[b] Normal growth medium contained 2 mM $CaCl_2$
[c] Interferon yields expressed as reference research units/ml/10^6 cells
[d] Superinduction schedule as described in table 1. $CaCl_2$ additions as for [a] above and maintained throughout superinduction regime
[e] n.d., not done

Table 3
Effect of ribonuclease on poly(IC) inductions of interferon in human MG63 cells in the presence or absence of added $CaCl_2$

RNAase treatment		$CaCl_2$ (10 mM)[b]	Interferon yields[a] stimulated by poly(IC) preparation
(i)	None	–	6700
		+	27 000
(ii)	Poly(IC) predigested with RNAase 30 min at 37°C	–	5
		+	2700
(iii)	RNAase present with poly(IC) on cells 1 h at 37°C	–	240
		+	1870
(iv)	RNAase digestion of poly(IC) bound to cells after induction 30 min at 37°C	–	5300
		+	10 700

[a] Yields expressed as reference research units/ml/10^6 cells
[b] +, added; –, not added

Several experiments designed to determine the mechanism by which the interferon yield is increased have been carried out. Does $CaCl_2$ act directly on the poly(IC), on the interaction of poly(IC) with the cell or on the cell itself? When poly(IC) was treated with RNAase in the presence and absence of 12 mM $CaCl_2$, it appeared that the extra $CaCl_2$ stabilized the poly(IC) to degradation (table 3).

Calcium chloride is known to increase the uptake of infectious adenovirus DNA [12] and when cells were induced with poly(IC) containing poly(I) · poly([5-^{203}Hg]C) more radioactivity was found to be associated with the cells when 12 mM $CaCl_2$ was present (210 cpm) than when the normal medium level of 2 mM $CaCl_2$ was present (53 cpm).

Experiments designed to detect the inhibition of protein and RNA synthesis by 12 mM $CaCl_2$ indicated that no such inhibition occurred (data not shown). The kinetics of production of interferon in MG63 cells in the presence of 12 mM $CaCl_2$ were similar to those of untreated cells except that the amount of interferon was greater at each time (fig.1).

Thus, it is possible that $CaCl_2$ may act by stabilizing the poly(IC) or by increasing cellular binding and/or uptake of poly(IC). Clearly it does not act by inhibiting protein or RNA synthesis, which has been suggested as a general mechanism for interferon induction [5], or by prolonging the period of interferon production.

Whatever the mechanism by which $CaCl_2$ acts, it is clear that this method of increasing interferon yields will prove useful for large scale production of human fibroblast interferon. In a pilot experiment 20 winchesters of MG63 were induced in 12 mM $CaCl_2$, and the total amount of interferon produced was 2.5×10^7 reference research units (1.25×10^6 units/winchester). This was of the same order achieved with MG63 cells in a superinduction schedule [9] and is certainly cheaper. Calcium salts are more rapidly dialysed than cycloheximide and actinomycin D, the use of which complicates the purification procedure, especially if the final product is to be used in clinical trials.

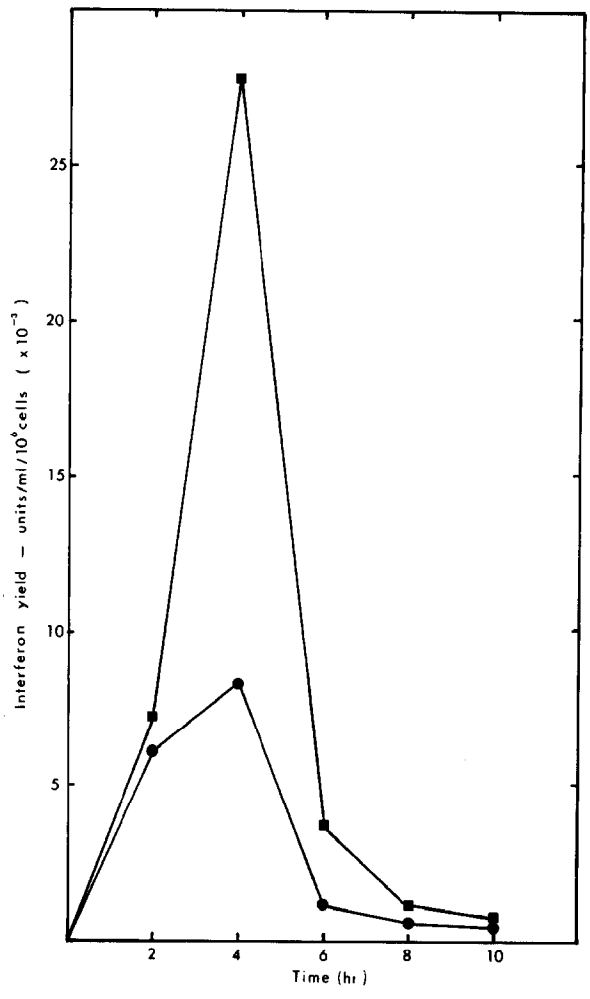

Fig.1. Kinetics of interferon production by MG63 cells after poly(IC) stimulation in the absence and presence of added $CaCl_2$ (10 mM). Cell monolayers were pretreated with $CaCl_2$ (12 mM) for 12 h at 37°C, induced with poly(IC) (50 μg/ml) and $CaCl_2$ (12 mM) for 1 h at 37°C, then incubated with 2 ml maintenance medium with 12 mM $CaCl_2$. At 2 h intervals the medium was harvested and fresh maintenance medium with 12 mM $CaCl_2$ put back on the cells. Control cultures, where no $CaCl_2$ was added into the medium, were similarly treated. Control (●). + $CaCl_2$ (■).

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References

[1] Ho, M. (1973) in: Interferons and Interferon Inducers, Frontiers of Biology, (Finter, N. B. ed), vol. 2, p. 29, North-Holland, Amsterdam.
[2] Merigan, T. C. (1973) in: ibid, p. 45.

[3] Tan, Y. H., Armstrong, J. A., Ke, Y. H. and Ho, M. (1970) Proc. Natl. Acad. Sci. USA 67, 464–471.
[4] Vilcek, J. and Ng, M. N. (1971) J. Virol. 7, 588–594.
[5] Tan, Y. H. and Berthold, W. (1977) J. Gen. Virol. 34, 401–411.
[6] Billiau, A., Joniau, M. and De Somer, P. (1972) Proc. Soc. Exp. Biol. Med. 140, 485–491.
[7] Billiau, A., Joniau, M. and De Somer, P. (1973) J. Gen. Virol. 19, 1–8.
[8] Rosenberg, N., Schaeffer, W. I. and Diamond, L. (1975) Cancer Res. 35, 1970–1974.
[9] Billiau, A., Edy, V. G., Heremans, H., Van Damme, J., Desmyter, J., Georgiades, J. A. and De Somer, P. (1977) Antimicrob. Ag. Chemother. 12, 11–15.
[10] Atkins, G. J., Johnston, M. D., Westmacott, L. M. and Burke, D. C. (1971) J. Gen. Virol. 25, 381–390.
[11] Dale, R. M. K., Martin, E., Livingston, D. C. and Ward, D. C. (1975) Biochemistry 14, 2447–2457.
[12] Graham, F. L. and Van Der Eb, A. J. (1973) Virology 54, 536–539.