Clin. exp. Immunol. (1983) **51**, 369–374.

In vitro and *in vivo* effects of interferon on the response of human lymphocytes to mitogens

S. EINHORN, H. BLOMGREN, NINA EINHORN & H. STRANDER *Radiumhemmet, Karolinska Hospital, Stockholm, Sweden*

(Accepted for publication 3 September 1982)

SUMMARY

Previous studies have shown that addition of IFN to the assay *in vitro* inhibits the proliferative response of lymphocytes to mitogens, whereas long term treatment by IFN *in vivo* has no major effect on the mitogen responsiveness of tumour patient's lymphocytes. Possible reasons for the discrepancy between the results obtained following treatment by IFN *in vitro* and *in vivo* were investigated. It was observed that the proliferative response of tumour patients lymphocytes to various mitogens was not affected to any major extent 24 hr after a single injection of 3 million units of interferon-α (IFN-α). Lymphocytes from tumour patients and healthy donors were found not to differ in their susceptibility to IFNs' anti-proliferative effect *in vitro*. Pure IFN-β, present in the assay throughout the incubation period, inhibited the response of lymphocytes to polyclonal mitogens and PPD showing IFN and not contaminants in the preparations to be responsible for this effect. Although the presence of IFN in the assay throughout the incubation period inhibited the proliferative response of lymphocytes, pre-treatment of these cells with IFN-α *in vitro* was found to have no major effect on their response to mitogens. We conclude that the lack of effect on the proliferative response of lymphocytes following treatment by IFN *in vivo*, is probably due to the fact that the lymphocytes were only treated with IFN prior to the assay.

INTRODUCTION

Interferon (IFN) affects the immune system in a variety of ways (Epstein, 1977). One aspect of the immunomodulatory action of IFN is its ability to inhibit the proliferative response of human lymphocytes to polyclonal mitogens and antigens when IFN is present in the cultures. (Blomgren, Strander & Cantell, 1974). This effect is exerted in a dose-dependent fashion (Blomgren *et al.*, 1974). However, long term treatment of osteosarcoma patients with IFN-α has no major effect on the response of their lymphocytes to mitogens (Einhorn *et al.*, 1979). In this paper we have extended our previous studies in an attempt to explain the discrepancy between the results obtained following IFN treatment *in vitro* and *in vivo*. We have posed four major questions.

(1) Does short term treatment by IFN-α *in vivo* (a single injection) have any impact on the proliferative response of lymphocytes to polyclonal mitogens?

(2) Do tumour patients lymphocytes differ from those of healthy donors in regard to their susceptibility to IFNs anti-proliferative effect?

(3) Does completely pure IFN-β affect the *in vitro* response of lymphocytes to mitogens?

(4) Does pre-treatment of lymphocytes with IFN *in vitro* affect their proliferative response to polyclonal mitogens?

Correspondence: Stefan Einhorn, Radiumhemmet, Karolinska Hospital, S-104 01 Stockholm, Sweden.

0009-9104/83/0200-0369$02.00

MATERIALS AND METHODS

Patients and healthy donors. Sixteen patients receiving IFN-α therapy are included in the study; five with ovarian carcinoma, four with osteosarcoma, three with multiple myeloma, three with prostatic carcinoma and one with generalized warts. The patients received 3 million units of IFN-α daily by intramuscular injection. Due to shortage of lymphocytes, the response to some of the mitogen concentrations were not tested in all patients.

The *in vitro* influence of IFN on the response of lymphocytes to polyclonal mitogens was compared between a group of 12 patients with carcinoma of the uterine cervix and a group of 12 healthy donors. Lymphocytes from patients and healthy donors were tested in parallel. The groups were matched for age (mean age 50 and 51 years for patients and healthy donors respectively) and sex. The response of the patient's lymphocytes to mitogens was measured prior to initiation of treatment.

In other experiments where IFN was added *in vitro*, healthy members of the hospital staff served as blood donors.

IFN preparations. Partially purified human leucocyte IFN-α preparations were derived from human peripheral blood leucocytes exposed to Sendai virus and were purified as previously described (Mogensen & Cantell, 1977). These preparations, with concentrations of approximately 6×10^6 IFN units/ml and specific activities of 10^6 IFN units/mg of protein, were kindly provided by Professor K. Cantell, Central Public Health Laboratory, Helsinki. Pure fibroblast IFN (IFN-β) was kindly provided by Dr E. Knight, Central Research and Development Department, E.I. du Pont de Nemours and Company, Wilmington, Delaware, USA and was produced, purified and characterized as previously described (Knight, 1976). This IFN preparation had a concentration of approximately 2×10^8 IFN units/mg protein. It contained no polypeptides except IFN according to amino acid analysis (Knight *et al.*, 1980).

The anti-viral activities of the preparations were determined by assaying inhibition of plaques induced by vesicular stomatitis virus in U-cells (Strander & Cantell, 1966). The anti-viral activities are expressed in international units by comparison with the international reference preparation 69/19.

Mitogens. The contents of vials containing phytohaemagglutinin (PHA, Phytohaemagglutinin M, DIFCO Lab., Detroit, USA) and pokeweed mitogen (PWM, GIBCO, New York, USA) were dissolved in 5 ml of MEM (Eagle's minimal essential medium). These solutions will be referred to as 100% of PHA and PWM respectively. Concanavalin A (Con A, Sigma Chemical Co., St Louis, USA) and purified protein derivative of tuberculin (PPD, RT 22, Statens Seruminstitute, Copenhagen, Denmark) were dissolved in MEM. Different batches with varying potencies were used for the different experiments.

Separation of lymphocytes. Lymphocytes were separated from heparinized venous blood by centrifugation on a layer of Ficoll-Isopaque (Jondal, Holm & Wigzell, 1972) and washed twice by centrifugation in medium (MEM supplemented with 10% of heat-inactivated human serum). Approximately 90% of the cells were classified as lymphocytes after crystal violet staining, the rest as monocytes and granulocytes.

IFN pre-treatment of lymphocytes in vitro. Lymphocytes, at a concentration of 5×10^6/ml medium, were incubated for 24 hr at 37°C in a humidified 5% CO_2 atmosphere in the absence or presence of different concentrations of IFN-α. After incubation the cells were washed four times by centrifugation. The viability of the cells, as assessed by trypan blue exclusion, was not altered to any detectable extent by the IFN pretreatment.

Culture conditions. The proliferative response of lymphocytes to mitogenic stimuli was measured using a microassay as previously described (Lilliehöök & Blomgren, 1979). In brief, 10^5 lymphocytes were cultured in wells of microtest plates containing medium. Mitogens were added to some of the cultures, whereas others received no stimulants. In some experiments IFN, at concentrations indicated in the text, was added in the beginning of the culture period. The final volume of the wells was always 0·2 ml. The cells were incubated in a humidified 5% CO_2-air atmosphere and after 4 days the cultures received 1·0 μCi of ^{3}H-thymidine (5 Ci/mM, The Radiochemical Centre, Amersham, England). Twenty-four hours later the cultures were terminated

and incorporated radioactivity, expressed as counts per minute (c.p.m.), determined by liquid scintillation counting. Mean values of quadruplicate cultures were calculated and expressed as $\log_{10}$ c.p.m.

Statistical analyses. Mean (M) and standard error (s.e.) was calculated on a geometric basis and expressed as either $\log_{10}$ c.p.m. or as percentage relative proliferative response. Statistical differences were evaluated using the Student's *t*-test.

RESULTS

IFN treatment in vivo

The response of lymphocytes to PHA, Con A and PWM was measured in 16 patients prior to and 24 hr after the first injection of IFN-α (Table 1). The IFN injection caused no significant change in the response of the lymphocytes to any of the mitogens.

Comparison between tumour patients and healthy donors

The *in vitro* influence of IFN on the response of lymphocytes to mitogens was compared between a group of patients with carcinoma of the cervix uteri and a group of healthy donors, matched for age and sex. The mean values for the response of lymphocytes to mitogens was lower for the tumour patients as compared to healthy donors (Table 2). The difference was, however, not statistically significant. The presence of IFN in the cultures seemed to cause a similar inhibition in the proliferative response of tumour patient's lymphocytes as compared to lymphocytes from healthy donors (Table 2).

Effect of completely pure IFN-β on the in vitro *response of lymphocytes to mitogens*

To study whether the inhibitory effects of IFN *in vitro* on lymphocytes response to mitogens are due to IFN itself or to contaminants in the preparations, pure IFN-β was tested for its capacity to modify these responses. Pure IFN-β diminished the response of lymphocytes from healthy donors to polyclonal mitogens and to PPD showing IFN to be the factor responsible for these effects (Table 3).

Pre-treatment of lymphocytes with IFN in vitro

To study whether pre-treatment of lymphocytes with IFN *in vitro* modifies their response to mitogens, 13 experiments were performed in which lymphocytes from healthy donors were pre-treated with IFN-α for 24 hr, washed extensively and subsequently tested for their proliferative response. Pre-treatment with IFN *in vitro* did not markedly change the response of the lymphocytes to any of the mitogens (Table 4). The presence of IFN throughout the incubation period, however, significantly decreased the responsiveness of the lymphocytes to all mitogens (Table 4). In two experiments, the lymphocytes were also treated with pure IFN-β for 24 hr prior to the assay.

Table 1. Influence of a single injection of 3 million units of IFN-α on the response of lymphocytes to mitogens. M ± s.e. of $\log_{10}$ c.p.m. prior to injection and percentage of pre-injection value after 24 hr are presented. No significant changes were observed

	PHA		Con A		PWM	
	3%	1·5%	200γ/ml	50γ/ml	1%	0·1%
Before injection	4·76 ±0·07	4·73 ±0·08	4·22 ±0·13	4·64 ±0·05	4·40 ±0·04	4·48 ±0·11
24 hr after injection	85% ±17	83% ±17	138% ±32	106% ±18	103% ±19	98% ±12
	15	14	26	16	16	11
No. of patients	12	12	14	15	13	15

Table 2. *In vitro* influence of IFN-α (1,000 units/ml), present in the cultures, on the response of lymphocytes to mitogens. Comparison between patients with carcinoma of the cervix uteri and healthy donors. M ± s.e. of 12 donors are presented. The stimulation in cultures containing IFN are expressed in per cent of the values obtained in cultures without IFN

	PHA			Con A		PWM	
	3%	1·5%	0·75%	200γ/ml	50γ/ml	1%	0·1%
Medium only							
Patients	5·02 ±0·05	4·88 ±0·06	4·69 ±0·07	4·72 ±0·06	4·61 ±0·08	4·45 ±0·06	4·40 ±0·11
Healthy donors	5·07 ±0·03	4·99 ±0·04	4·79 ±0·04	4·78 ±0·06	4·70 ±0·04	4·50 ±0·04	4·57 ±0·04
IFN							
Patients	64% ±3	60% ±6	18% ±5	60% ±3	40% ±4	64% ±2	45% ±4
Healthy donors	69% ±4 3	61% ±3 2	21% ±4 3	60% ±3	47% ±4	68% ±3	53% ±4 3

Table 3. Influence of IFN (100 units/ml), present in the cultures, on the response of lymphocytes to polyclonal mitogens and PPD. The stimulation in cultures containing IFN are expressed in percentage of the values obtained in cultures without IFN

	PHA		Con A		PWM		PPD	
	0·75%	0·4%	200γ/ml	50γ/ml	1%	0·1%	1γ/ml	0·1γ/ml
Expt. 1								
Medium only	4·29	4·33	4·46	4·35	4·27	4·24	2·90	2·95
IFN-α	58%	40%	95%	77%	91%	98%	88%	65%
Pure IFN-β	56%	18%	86%	70%	87%	81%	63%	50%
Expt. 2								
Medium only	4·24	4·13	4·54	4·45	4·41	4·47	4·20	3·90
IFN-α	64%	8%	81%	56%	79%	86%	76%	84%
Pure IFN-β	39%	7%	73%	55%	70%	72%	56%	31%

Pre-treatment with this IFN preparation had no effect either on the proliferative response of lymphocytes to mitogens (data not presented).

DISCUSSION

Presence of human IFN-α in lymphocyte cultures stimulated by mitogens inhibits the proliferative response (2,3, Tables 2–4). This is probably attributable to the direct cell multiplication inhibitory effect of IFN (Paucker, Cantell & Henle, 1962). On the other hand long-term treatment of tumour patients with 3×10^6 units of IFN-α three times weekly, causes no major change in the response of their lymphocytes to mitogens (Einhorn *et al.*, 1979). In the present study we have investigated four possible reasons for the observed discrepancy between the *in vitro* and the *in vivo* effects of IFN on the proliferative response of lymphocytes to mitogens.

(1) In the previous study (Einhorn *et al.*, 1979) only long term effects of IFN treatment were

Table 4. Influence of pre-treatment by different doses of IFN-α on the response of lymphocytes to mitogens. M ± s.e. from 13 experiments. In nine experiments IFN was present in some of the cultures throughout the incubation period. The stimulation in cultures treated by IFN are expressed in percentage of the values obtained in cultures treated with medium only

	PHA		Con A		PWM	
	1·5%	0·75%	200γ/ml	50γ/ml	1%	0·1%
Medium only	5·03 ±0·03	4·99 ±0·04	4·24 ±0·17	4·83 ±0·06	4·37 ±0·06	4·54 ±0·06
IFN pre-treatment						
10 IFN units/ml	99% ±5	96% ±4	116% ±18 16	110% ±8 7	115% ±11 10	108% ±12 11
100 IFN units/ml	97% ±5	98% ±5	107% ±20 17	113% ±9 8	116% ±10 9	110% ±9
1,000 IFN units/ml	95% ±5	92% ±4	93% ±17 14	103% ±12 11	115% ±12	107% ±11
IFN present						
1,000 IFN units/ml	70% ±8 7 ($P<0·05$)	68% ±7 6 ($P<0·01$)	50% ±5 4 ($P<0·001$)	64% ±6 ($P<0·001$)	68% ±9 8 ($P<0·05$)	54% ±6 5 ($P<0·001$)

measured and we may have failed to detect possible IFN effects, due to the relatively long time periods between the tests (3–6 months). We therefore tested whether a single injection of IFN-α had any impact of the response of lymphocytes to mitogens, as measured 24 hr after the injection. We found that the mitogen responsiveness of tumour patients lymphocytes was not affected to any major extent by a single injection of IFN-α (Table 1). A single injection of 3 million units of IFN-α gives rise to serum titres of around 100 IFN units/ml (Cantell, Pyhälä & Strander, 1974), which is a concentration sufficient to inhibit the proliferative response of lymphocytes to mitogens (Table 3). This type of injection has previously been shown to enhance the natural killer cell activity of human lymphocytes (Einhorn, Blomgren & Strander, 1980a) and to increase the proportion of lymphocytes expressing receptors for sheep red blood cells (E rosettes) (Einhorn, Blomgren & Strander, 1980b).

(2) The discrepancy could be due to the fact that the *in vitro* studies were performed using lymphocytes from healthy donors, whereas the *in vivo* studies were performed using lymphocytes from tumour patients. To investigate this possibility lymphocytes from healthy donors were compared to lymphocytes from tumour patients in regard to their susceptibility to IFNs' anti-proliferative effect *in vitro*. It was observed that the influence of IFN on the response of lymphocytes to mitogens did not differ to any major extent between tumour patients and healthy donors (Table 2).

(3) The *in vitro* effects observed could be due to contaminants in the more or less crude IFN preparations used. To test this possibility a completely pure IFN-β preparation was tested for its capacity to modify these reactions (Table 3). Since pure IFN-β was found to cause an inhibition in the response of lymphocyte to polyclonal mitogens and PPD, we conclude that these effects are due to IFN itself and not to contaminants in the preparations.

(4) The reason for the discrepancy between the *in vitro* and the *in vivo* effects could be that IFN *in vitro* was present throughout the incubation period, whereas the lymphocytes *in vivo* were exposed to IFN prior to the test. To investigate this possibility, lymphocytes were pre-treated with IFN-α for

24 hr *in vitro*, extensively washed and subsequently tested for their response to mitogens. It was observed that pre-treatment with IFN *in vitro* did not markedly change the response of the lymphocytes to mitogens, although presence of IFN in the cultures throughout the incubation period inhibited the lymphocytes proliferative response (Table 4).

This study thus indicates that IFN *in vitro* affects the proliferative response of lymphocytes to mitogens, only when IFN is present in the assay throughout the incubation period and that pre-treatment by IFN has no such effect. This is most probably the explanation for the discrepancy between IFNs effects *in vitro* and *in vivo*, since the lymphocytes, under the experimental conditions used, were pre-treated with IFN *in vivo*. It is possible that the proliferative response of lymphocytes is impaired during continuous exposure to IFN *in vivo* although our *in vitro* test system failed to detect it. To test this possibility *in vivo* test systems, such as skin testing, could be applied.

The excellent technical assistance of Mrs Elisabet Anderbring, Mr Ingvar Juhlin and Miss Lena Ödin is gratefully acknowledged. Our thanks are due to Drs Kari Cantell and Ernest Knight for providing the IFN preparations.

This study was supported by grants from the Swedish Cancer Society.

REFERENCES

BLOMGREN, H., STRANDER, H. & CANTELL, K. (1974) Effect of human leukocyte interferon on the response of lymphocytes to mitogenic stimuli. *Scand. J. Immunol.* **3,** 697.

CANTELL, K., PYHÄLÄ, L. & STRANDER, H. (1974) Circulating human interferon after intramuscular injection into animals and man. *J. gen. Virol.* **22,** 453.

EINHORN, S., BLOMGREN, H., CANTELL, K. & STRANDER, H. (1979) Effect of prolonged *in vivo* administration of human leukocyte interferon on the mitogen responsiveness of human lymphocytes. *Acta Med. Scand.* **206,** 345.

EINHORN, S., BLOMGREN, H. & STRANDER, H. (1980a) Interferon and spontaneous cytotoxicity in man. V. Enhancement of spontaneous cytotoxicity in patients receiving human leukocyte interferon. *Int. J. Cancer*, **26,** 419.

EINHORN, S., BLOMGREN, H. & STRANDER, H. (1980b) Effect of an intramuscular injection of human leukocyte interferon on blood leukocyte counts and the proportion of lymphocytes forming E, EA and EAC rosettes. *Int. Arch. Allergy appl. Immun.* **63,** 139.

EPSTEIN, L. (1977) The effects of interferons on the immune system *in vitro* and *in vivo*. In *Interferons and their actions* (ed. by W.E. Stewart) pp. 91, CRC Press, Cleveland.

JONDAL, M., HOLM, G. & WIGZELL, H. (1972) Surface markers on human T- and B-lymphocytes. I. A large population of lymphocytes forming non-immune rosettes with sheep red blood cells. *J. exp. Med.* **136,** 207.

KNIGHT, E. (1976) Interferon: purification and initial characterization from human diploid cells. *Proc. Natl. Acad. Sci. USA.* **73,** 520.

KNIGHT, E., HUNKAPILLER, M.W., KORANT, B.D., HARDY, R.W.F. & HOOD, L.E. (1980) Human fibroblast interferon: amino acid analysis and terminal amino acid sequence. *Science*, **207,** 525.

LILLIEHÖÖK, B. & BLOMGREN, H. (1979) Strong stimulation of CBA lymphocytes in the mixed lymphocyte interaction with cells from the h-2 identical strain C3H. *Scand. J. Immunol.* **3,** 627.

MOGENSEN, K.D. & CANTELL, K. (1977) Production and preparation of human leukocyte interferon. *Pharmacol. Ther.* **1,** 369.

PAUCKER, K., CANTELL, K. & HENLE, W. (1962) Quantitative studies on viral interference in suspended L-cells. III. Effect of interfering viruses and interferon on the growth rate of cells. *Virology*, **17,** 324.

STRANDER, H., & CANTELL, K. (1966) Production of interferon by human leukocytes *in vitro*. *Ann. Med. Exp. Fenn.* **44,** 365.