Eur J Cancer Clin Oncol, Vol. 24, No. 9, pp. 1505–1510, 1988.
Printed in Great Britain

0277–5379/88$3.00 + 0.00

Interferon Exerts a Cytotoxic Effect on Primary Human Myeloma Cells

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Abstract—*A dye exclusion method was used for testing the sensitivity of primary human myeloma and normal bone-marrow cells to interferon (IFN). Following 4 days of incubation with 5000 units/ml of natural (n) IFN-α, there was a >90% decrease in the number of viable myeloma cells in cultures from some patients, whereas myeloma cells from other patients showed intermediate or no sensitivity to IFN. The number of viable non-malignant bone-marrow cells (from the same patients) following a 4-day exposure to nIFN-α (5000 units/ml) decreased by 10–60%. Exposure of malignant and non-malignant bone-marrow cells to natural β- and recombinant α- and γ-IFN, also induced a decrease in the number of viable cells. The decreased number of viable myeloma cells could be observed already after 1 day of exposure to IFN-α in vitro. To test whether inhibition of proliferation could account for the observed effects, proliferation, as measured by [^{3}H]thymidine uptake, was studied in some experiments. Only approx. 1–2% of the myeloma cells were labeled with [^{3}H]thymidine during the 4 days of culture in vitro, whereas the proportion of labeled non-malignant cells was approx. 40%. Thus, the IFN-induced reduction of cell number in normal bone-marrow cells could possibly be attributed to a cell multiplication inhibitory effect of IFN, whereas the effect observed in myeloma cells cannot be attributed to cell multiplication inhibition. To test the possibility that the reduction in the number of myeloma cells could be attributed to the activity of autologous cytotoxic T-cells, NK-cells or macrophages, these cells were depleted in some experiments. Depletion of these cells did not, however, influence the IFN-induced decrease in the number of viable myeloma cells. We thus conclude that IFN can reduce the number of viable tumor cells by a cytotoxic effect, unrelated to cell multiplication inhibition.*

INTRODUCTION

INTERFERONS (IFNs) have the capacity to inhibit the multiplication of malignant and non-malignant cells *in vitro* [1, 2]. The direct cell multiplication inhibitory effect of IFN has been suggested to be the mechanism by which IFN induces remissions in human malignancies [3]. However, in some patients with B-cell malignancies, such as myeloma, remissions have been shown to occur within a few weeks after initiation of IFN therapy [4]; effects occur too fast to be explained simply by an inhibition of the multiplication of tumor cells. Possible explanations for this rapid effect of IFN on malignant tumors are, for instance, stimulation of an antitumor immune response by IFN, or an induction of differentiation by IFN. In this paper another possibility is suggested, based on work using a dye exclusion assay [5], namely that IFNs exert antitumor effects by a direct cytotoxic effect on primary human myeloma cells.

Accepted 4 May 1988.

MATERIALS AND METHODS

Patients

Bone-marrow samples were obtained from 19 patients with multiple myeloma. Data on these patients are shown in Table 1.

IFN preparations

The natural α-IFN (nα-IFN) was prepared from Sendai virus induced human Namalwa cells and purified by an anti-IFN-antibody affinity system [6]. The specific activity of this preparation was 2×10^8 units/mg of protein and the purity approx. 90%. Recombinant α_2-IFN (rα-IFN; from Ernst-Boehringer-Institut für Arznemittelforschung) was derived from *E. coli*. The specific activity of this preparation was 3.2×10^8 units/mg of protein and the purity was >99%. *E. coli* derived recombinant IFN-γ (rγ-IFN; from Ernst-Boehringer-Institut für Arznemittelforschung) had a specific activity of 2×10^7 units/mg of protein. Natural β-IFN (nβ-IFN) was produced from fibroblasts by induction with poly(I) poly(C). The specific activity of the

Table 1. *Patient data*

Patient	Age	Sex	Phenotype	Previous treatment	Percentage plasma cells in the bone marrow
1	75	F	IgGk	No	69
2	71	F	IgAl	Chemotherapy	12
3	55	F	IgAk	Chemotherapy	33
4	76	F	IgAl	No	10
5	65	M	IgGk	Radiotherapy	14
6	69	F	IgGk	No	31
7	81	M	BJl	No	75
8	58	F	Non-S	No	17
9	69	F	IgAl	No	35
10	72	M	IgAl	Chemotherapy	90
11	65	M	BJk	No	95
12	64	M	BJk	Chemotherapy	17
13	51	M	IgGk	Chemotherapy	22
14	89	M	BJk	Chemotherapy	33
15	63	M	BJl	No	30
16	65	M	IgAk	Chemotherapy	31
17	59	F	IgAk	Chemotherapy	50
18	62	F	IgAl	Chemotherapy	35
19	69	M	IgGk	Chemotherapy	34

partially purified preparation was 1.6×10^6 units/mg of protein [7]. The antiviral activities of the preparations were determined by assaying inhibition of the cytopathogenic effect of vesicular stomatitis virus in human fibroblasts as previously described [8]. The antiviral activities are expressed in international units by comparison with international reference preparations.

Preparation and culture of cells

Heparinized bone marrow samples and peripheral blood were centrifuged on a layer of Ficoll-Hypaque [9]. The interphase cells were collected and washed twice by centrifugation in medium (RPMI 1640 with 1% L-glutamine, 10% fetal calf serum, 50 μg/ml of penicillin and 50 μg/ml of streptomycin). In some experiments monocytes and granulocytes were depleted by plastic adherence during 30 min as well as by iron powder and a magnet. High- as well as low-avidity T-cells were depleted by E-rosette sedimentation using neuraminidase treated sheep red blood cells as previously described [10]. Following counting, 10^6 cells were added to 5-ml plastic tubes together with medium with or without IFN and incubated at 37°C for 1–9 days.

Dye exclusion assay

A dye exclusion assay originally reported by Weisenthal *et al.* [5] and later modified by Bird *et al.* [11] was used with some additional modifications. Briefly, a defined number of permanently fixed duck red blood cells (DRBC) were added to the cell suspensions after incubation, after which the cells were stained with 2% fast green and 1% nigrosin dye solutions; 0.1 ml of both. After 10 min the cells were cytocentrifuged onto slides at 500 rpm for 10 min after which the slides were air-dried and fixed in methanol for 20 s. All slides were counterstained with May–Grunwald–Giemsa (MGG) to enable differentiation between cell types.

Assay interpretation

The DRBSs served as an internal standard in this assay, minimizing the problems with uneven distribution of cells on the slides, cell autolysis and proliferation of cells during incubation, as discussed by Weisenthal *et al.* [5]. Live tumor cells as well as live normal bone marrow cells were calculated in relation to DRBC in all slides. The number of viable cells in the cultures with IFN were expressed as a percentage of the number of viable cells in control cultures.

Autoradiography

DNA synthesis in the myeloma cells and in the normal bone-marrow cells was determined by the following procedure: a suspension of 10^6 cells/ml was incubated with 10 μCi [^{3}H]thymidine/ml medium at 37°C. The incubation time was 1–4 days. Following staining with MGG, autoradiography was performed with liquid emulsion technique using Ilford K2 film. The labeling index was calculated by counting 500 cells and determining the percentage of cells containing more than 10 grains over the nucleus.

Statistical analyses

Statistical significances were evaluated by Student's *t*-test and by linear regression analyses.

RESULTS

In 19 myeloma patients the number of viable myeloma cells was tested following exposure to nα-IFN for 4 days. In relation to control cultures, nα-IFN caused a more than 90% decrease in the number of viable myeloma cells in cultures from three patients (Table 2). In cells from some patients, IFN-α had no major effect on the viability of the myeloma cells, whereas cells from other patients showed an intermediate sensitivity to IFN (Table 2). In contrast to the results obtained with myeloma cells, non-malignant cells from none of the patients exhibited a similar high sensitivity to nα-IFN, i.e. the greatest decrease in viable cells observed was 63% (Table 2). As can be seen in Fig. 1, the myeloma cells exhibited a more heterogeneous picture in regard to IFN sensitivity as compared to non-malignant cells. There was no correlation between the IFN sensitivity of non-malignant cells and the IFN sensitivity of the myeloma cells, for individual patients.

We studied whether the effects of IFN on the number of myeloma and non-malignant cells could be correlated to clinical parameters. There was a statistically significant correlation between sex and the sensitivity of the myeloma cells to IFN; cells from female patients were more sensitive to IFN than cells from male patients ($P < 0.001$). Apart from this, there were no statistically significant correlations between IFN sensitivity and clinical parameters such as Ig type, age, previous treatment and the proportion of plasma cells in the bone marrow.

In seven patients with myeloma the effect of different IFN preparations on the viability of myeloma cells and non-malignant cells was tested. In most patients, rγ-IFN had the most pronounced effect on cell viability (Table 3), whereas IFN-α (natural as well as recombinant) was the least efficient in inhibiting the number of viable cells. The difference between the α-IFN preparations and the β- and γ-IFNs in reducing the number of myeloma cells was in some cases statistically significant ($P < 0.01$ between rα-IFN and nβ-IFN; $P < 0.01$ between rα-IFN and rγ-IFN).

The effect of various concentrations of nα-IFN on the viability of bone marrow cells was tested in five patients with myeloma. The effect of IFN on myeloma cell viability was dose-dependent (Fig. 2). In some of the patients 0.5 units/ml of nα-IFN was sufficient to cause a reduction in the number of viable myeloma cells (Fig. 2). Optimal effects were seen using 500–5000 IFN-α units/ml. In non-malignant bone-marrow cells, the reduction in cell number by IFN-α was also found to be dose-dependent (Fig. 2).

In two patients, time–kinetic studies were performed on the influence of nα-IFN (5000 units/ml) on myeloma cell viability. In one of the patients, no major effect on cell viability was seen until day 4 of culture with natural IFN-α (Fig. 3). In the other patient, who showed a weaker susceptibility to IFN-α, a minor decrease in myeloma cell viability was observed already after 1 day of culture with IFN.

The possibility that IFN reduced the number of viable myeloma cells by a stimulation of cytotoxic

Table 2. Influence of natural IFN-α (5000 units/ml; 4 days of culture) on the number of viable malignant and non-malignant cells in bone-marrow from 19 myeloma patients. The data are expressed as a percentage of control cells cultured in medium only

Patient	Percentage viable myeloma cells	Percentage viable non-malignant cells
1	0	48
2	28	90
3	4	67
4	7	58
5	30	65
6	25	92
7	93	42
8	53	40
9	22	37
10	60	N.D.
11	65	N.D.
12	106	73
13	88	N.D.
14	63	83
15	116	88
16	105	76
17	63	86
18	30	51
19	92	52
Mean ± S.E.	55 ± 9	66 ± 5

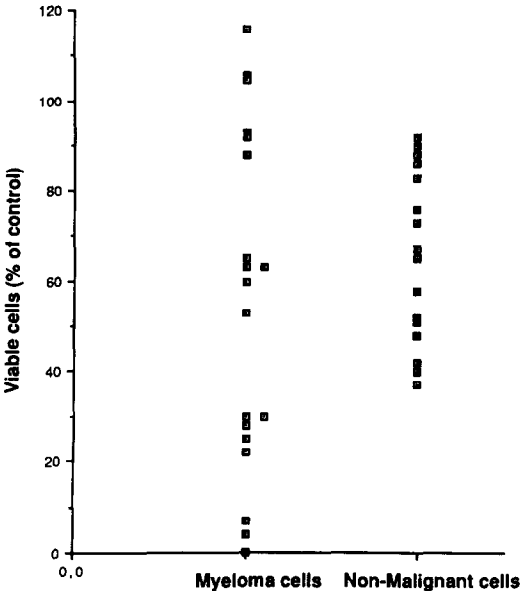

Fig. 1. Percentage viable cells following incubation in the absence or presence of nα-IFN (5000 units/ml) for 4 days ⊡ denotes different patients.

monocytes or lymphocytes, active against the autologous myeloma cells was tested by depletion of monocytes, granulocytes as well as high- and low-avidity T-cells from the bone-marrow preparations. Depletion of these cells from the preparations did not abrogate the IFN-induced decrease in the number of viable myeloma cells, which indicates that IFN acts by a direct effect on the myeloma cells (Table 4).

By the use of autoradiography, the number of myeloma cells and non-malignant cells labeled with [^{3}H]thymidine during a 4-day culture without IFN was determined in bone marrow cultures from some of the patients. Only 1–2% of the myeloma cells were labeled with [^{3}H]thymidine during the culture period. During the same time-period nα-IFN caused a reduction in myeloma cell number by up to 40% in cultures from these patients. During the same period of time, approx. 40% of the non-malignant cells entered S-phase, whereas nα-IFN caused a reduction in the number of viable non-malignant cells by 12–24% during four days of culture *in vitro*.

DISCUSSION

IFN has previously been shown to inhibit the multiplication of normal and malignant cells *in vitro* [1, 2]. In some highly IFN-sensitive cell-lines, such as L1210, the cells have been shown to die after a period of culture *in vitro* [12]. In the case of L1210 this effect has, however, been shown to be secondary to the slowing in the multiplication rate of the cells [13]. In this work we show that IFN *in vitro* can reduce the number of viable primary myeloma cells. This is not due to an inhibition of cell multiplication, since only a few per cent of the cells are labeled with [^{3}H]thymidine, indicating DNA synthesis, during the period of *in vitro* culture. The possibility that IFN reduced the number of viable tumor cells indirectly, by an effect on immunologically active cells, is not likely, since depletion of monocytes, granulocytes and T-cells did not influence this IFN effect. We thus conclude that IFN can have a direct cytotoxic effect on myeloma cells at concentrations that are regularly obtained in the serum during IFN therapy [3]. A direct cytotoxic effect of IFN on malignant cells has previously been shown in established cell lines [14].

In contrast to the results obtained with myeloma cells, IFN usually caused only minor decreases in the number of non-malignant bone-marrow cells (Table 2). Approximately 40% of non-malignant bone marrow cells were found to be labeled with [^{3}H]thymidine during 4 days of culture *in vitro*. Thus, possibly the reduction in the number of viable normal bone-marrow cells could be explained by a cell multiplication inhibitory effect of IFN. Whether the cytotoxic effect of IFN is exclusive for myeloma cells or whether also other malignant cells are susceptible to this effect of IFN is presently being investigated.

α-, β- and γ-IFN differed in their capacity to reduce the number of viable myeloma cells; β- and γ-IFN are more efficient than α-IFN on a per

Table 3. Influence of various IFNs (500 units/ml) on the number of viable malignant and non-malignant cells from seven myeloma patients

Patient	Percentage viable malignant cells				Percentage viable non-malignant cells			
	nα-IFN	rα-IFN	nβ-IFN	rγ-IFN	nα-IFN	rα-IFN	nβ-IFN	rγ-IFN
Myeloma								
1	10	35	5	40	93	135	68	76
4	19	47	24	17	63	86	86	46
5	20	28	20	12	61	76	66	93
6	28	32	22	12	70	86	62	42
7	84	91	84	83	76	89	71	49
8	81	61	49	23	74	48	73	38
9	58	45	24	15	62	51	45	21
Mean ± S.E.	43 ± 12	48 ± 8	33 ± 10	29 ± 10	71 ± 4	82 ± 11	67 ± 5	52 ± 9

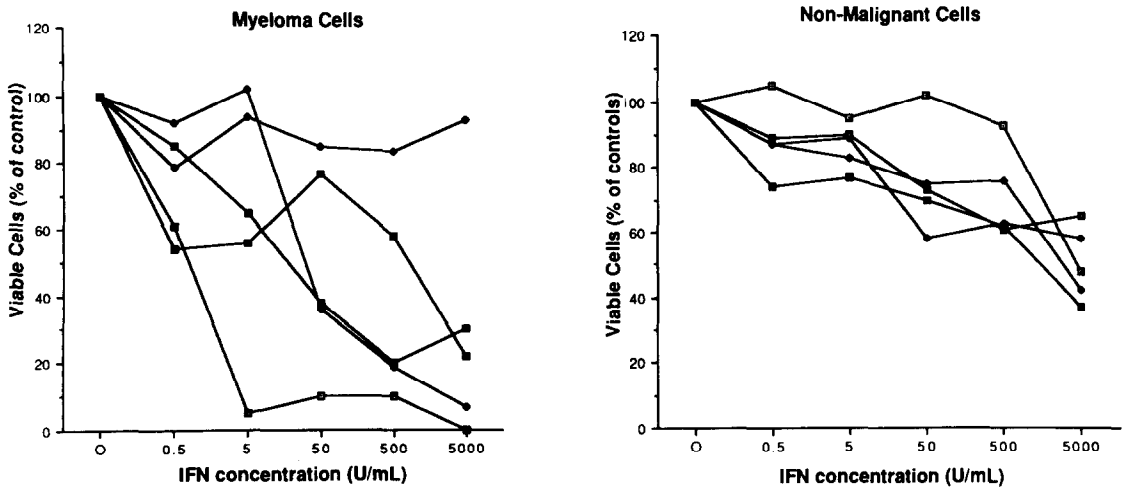

Fig. 2. Influence of various natural IFN-α concentrations on the number of viable myeloma and non-malignant bone-marrow cells. The lines denote different patients.

antiviral unit basis. As is well known, it is difficult to compare the activity of different IFNs on a per antiviral unit basis. Studies are therefore in process to evaluate whether different α-IFN subtypes differ in this function.

How does IFN cause a reduction in the number of viable myeloma cells? One possible explanation could be that IFN induces terminal differentiation of the myeloma cells, a terminal differentiation ultimately leading to the death of the affected cells. IFN has previously been shown to induce differentiation in malignant B-cells from patients with CLL *in vitro* [15, 16]. Another possibility is that IFN acts by influencing the expression of genes that are of importance for the viability of the malignant cells. IFN has been shown to influence the expression of a variety of genes [17] and amongst them, several oncogenes [18, 19].

Table 4. Influence of natural IFN-α (5000 units/ml) on the number of viable myeloma cells. Tests were done on separated cells and cells depleted of monocytes, granulocytes and T-cells

Patient	Percentage viable malignant cells	
	Unseparated	Separated
13	88	49
14	63	63

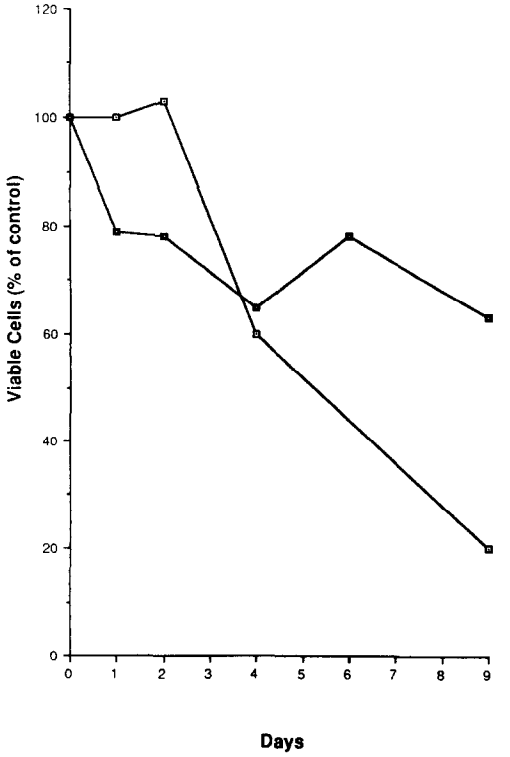

Fig. 3. Time-course study on the influence of natural IFN-α (5000 units/ml) on the number of viable myeloma cells. The lines denote two different patients.

Cells from different patients vary in their susceptibility to IFN (Table 2). In our relatively small material we found a close correlation between sex and the sensitivity of the myeloma cells to IFN; cells from female patients being more sensitive than cells from male patients ($P < 0.001$). Whether or not this holds true in a larger material is presently being investigated. A variability in the *in vitro* sensitivity to IFN has also been shown for primary myeloma cells using an agar-colony assay and an autoradiographic method measuring inhibition of ^3H uptake [20]. In the latter study a correlation between *in vitro* sensitivity and the clinical response to IFN-α therapy was found. Whether the observed reduction in colony formation was due to the cell multiplication inhibitory effects of IFN, or whether it was due to the direct cytotoxic effect of IFN is not known.

A major drawback in optimizing IFN therapy is that we do not know how IFN exerts antitumor

effects. Several different mechanisms have been suggested to explain how IFN can induce remissions in tumors. Apart from indirect effects mediated by, for instance, the immune system, stromal cells or endocrine organs, IFN may act by direct effects, such as cell multiplication inhibition or induction of differentiation..Based on the results obtained in this work, we now suggest that IFN may also act by another mechanism, namely a direct cytotoxic effect on the tumor cells. Whether or not this is a means by which IFN induces remissions in multiple myeloma and/or in other malignancies, can only be speculated on.

Acknowledgements—The excellent technical assistance of Miss Karin Burvall, Mrs Inger Foskolos, Mrs Lena Odin and Mr Dimitrios Papadimas is acknowledged. We thank Drs Ernest Knight Jr, Otto Adolf and David Secher for providing the IFN preparations used. This work was supported by grants from The Swedish Cancer Society, The Cancer Society of Stockholm and The King Gustav V Jubilee Fund, Stockholm.

REFERENCES

1. Paucker K, Cantell K, Henle W. Quantitative studies on viral interference in suspended L cells. III. Effect of interfering viruses and interferon on the growth rate of cells. *Virology* 1962, **17**, 324–334.
2. Einhorn S, Strander H. Interferon therapy for neoplastic diseases in man. *In vitro* and *in vivo* studies. In: *Human Interferon. Production and Clinical Use.* A Symposium Workshop, W. Alton Jones Cell Science Center, Lake Placid, U.S.A., 19–20 May 1977, 159–174.
3. Strander H. Interferon treatment of human neoplasia. *Adv Cancer Res* 1986, **46**, 1–265.
4. Mellstedt H, Ahre A, Björkholm M, Holm G, Johansson B, Strander H. Interferon therapy in myelomatosis. *Lancet* 1979, **1**, 245–247.
5. Weisenthal LM, Marsden JA, Dill PL, Macaluso CK. A novel dye exclusion method for testing *in vitro* chemosensitivity of human tumors. *Cancer Res* 1983, **43**, 749–757.
6. Finter NB, Fantes KH. The purity and safety of interferons prepared for clinical use. The case for lymphoblastoid interferon. In: Gresser I, ed. *Interferon.* New York, Academic Press, 1980, 65–80.
7. Obert HJ. Clinical trials and pilot studies with beta-interferon in Germany. In: *UCLA Symposium on Molecular and Cellular Biology.* 1982, Vol. XXV, 426–432.
8. Einhorn L, Einhorn S, Wahren B. Interferon induction in human leukocytes after *in vitro* exposure to cytomegalovirus or Epstein-Barr virus. Influence of interferon on the expression of viral antigens. *Intervirology* 1985, **23**, 10–149.
9. Böyum A. Separation of leukocytes from blood and bone marrow. *Scand J Clin Lab Invest* 1968, **21**, Suppl 97, 1–109.
10. Wasserman J, Einhorn S, Petrini B, von Stedingk L-V. Further studies on the influence of interferon on pokeweed mitogen induced Ig-synthesis *in vitro*. *J Clin Lab Immunol* 1985, **18**, 187–189.
11. Bird MC, Bosanquet AG, Gilby ED. *In vitro* determination of tumour chemosensitivity in hematological malignancies. *Hematol Oncol* 1985, **3**, 1–9.
12. Gresser I, Brouty-Boyé D, Thomas M-T, Macieira-Coelho A. Interferon and cell division. II. Influence of various experimental conditions on the inhibition of L1210 cell multiplication *in vitro* by interferon preparations. *J Natl Cancer Inst* 1970, **45**, 1145–1153.
13. Tovey M, Brouty-Boyé D. The use of the chemostat to study the relationship between cell growth rate, viability and the effect of interferon on L1210 cells. *Exp Cell Res* 1979, **118**, 383–388.
14. Le J, Yip YK, Vilcek J. Cytolytic activity of interferon-gamma and its synergism with 5-fluorouracil. *Int J Cancer* 1984, **34**, 495–500.
15. Einhorn S, Robèrt K-H, Ostlund L, Juliusson G, Biberfeld P. Interferon induces proliferation and differentiation in fresh chronic lymphocytic leukemia cells. *Antiviral Res* 1984, **3**, 40.
16. Ostlund L, Einhorn S, Robèrt K-H, Juliusson G, Biberfeld P. Chronic B-lymphocytic leukemia cells proliferate and differentiate following exposure to interferon *in vitro*. *Blood* 1986, **67**, 152–159.
17. Friedman RL, Manly SP, McMahon M, Kerr IM, Stark GR. Transcriptional and posttranscriptional regulation of interferon-induced gene expression in human cells. *Cell* 1984, **38**, 745–755.
18. Jonak GJ, Knight E Jr. Selective reduction of c-myc mRNA in Daudi cells by human β interferon. *Proc Natl Acad Sci USA* 1984, **81**, 1747–1750.
19. Einhorn S, Showe L, Ostlund L, Juliusson G, Robèrt K-H, Croce C. Influence of interferon-α on the expression of cellular oncogenes in primary chronic lymphocytic leukemia cells. *Oncogene Res* (in press).
20. Brenning G, Åhre A, Nilsson K. Correlation between *in vitro* and *in vivo* sensitivity to human leukocyte interferon in patients with multiple myeloma. *Scand J Haematol* 1985, **35**, 543–549.