Laboratory evaluation of commercial interferon preparations

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Summary

The antiviral, antiproliferative and natural killer-cell (NKC) stimulatory activities of four commercial therapeutic interferon preparations were assayed in our laboratory. The antiviral and antiproliferative activities of each preparation were relatively similar, but an unexpectedly high NKC stimulatory activity was found in one of them. In-house determination of antiviral activity and evaluation of the antiproliferative and NKC stimulation potential of interferon preparations are essential before rational clinical trials of this agent are carried out.

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The exact therapeutic role of the interferons remains to be established and defined. Preliminary clinical trials of exogenously administered interferons have shown that they have undoubted therapeutic value in the treatment of a variety of viral infections, and it is probable that they may well play some significant role in the treatment of certain forms of cancer.[1]

The interferons are a group of proteins which have potent and varied biological activity, including a broad-spectrum antiviral efficacy.[2-4] There are three types of interferons: HuIFN-α (previously called leucocyte interferon), HuIFN-ß (previously called fibroblast interferon), and HuIFN-γ (previously called immune interferon). The first two types are produced by most cells in the body in response to viral infection or exposure to foreign polynucleotides. The third type, HuIFN-γ, is produced by sensitized lymphocytes in response to specific antigenic or mitogenic stimulation. These three types of interferons may be distinguished from each other by their physicochemical, biological, pharmacological and immunological properties, although they share most, if not all, of their biological effects to a greater or lesser degree. Of these biological effects the three that are of greatest importance are: (*i*) the broad-spectrum antiviral activity; (*ii*) inhibition of cell proliferation; and (*iii*) stimulation of natural killer-cells (NKCs). Because of technical problems in the production and purification of HuIFN-γ, this substance is as yet unavailable in sufficient amounts for evaluation and clinical trials. The clinical evaluation of the interferons has therefore centred mainly on three products: HuIFN-α, processed from buffy coats of donated blood units; HuIFN-ß, produced from fibroblast cell cultures; and lymphoblastoid interferon, derived from a line of continuously growing lymphoblastoid cells transformed by Epstein-Barr virus, the so-called Namalwa cells. The latter product consists of a mixture containing predominantly HuIFN-α with a smaller volume of HuIFN-ß.[5]

Samples of the three types of interferon products were obtained from commercial sources for laboratory evaluation of their three major biological effects. The potency of the preparation is usually specified with reference to its antivirial activity, and is expressed in international units. A unit of interferon is defined as that amount which produces a 50% inhibition of replication of a test virus, measured either by inhibition of plaque formation or infectivity in tissue culture. For this purpose international standards are provided for the calibration of in-house laboratory standards.[6] A microtitre radio-immunoassay technique has been developed in our laboratory to assay antiviral activity.[7] This was shown to correlate with the plaque reduction assay, while at the same time having the advantages of being very much less cumbersome and utilizing smaller amounts of reagents. The subjectivity inherent in standard antiviral assays is thus avoided. This technique was used to quantitate the antiviral potency of the commercial preparations of interferons in terms of international unitage. International standards have not as yet been established for antiproliferative and NKC stimulatory activities; these were assayed by conventional techniques and the results obtained for the commercial preparations compared with our in-house laboratory standard.

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Materials and methods

Sources of interferon

The following four interferon preparations were kindly provided to us for laboratory evaluation:

Frone (Inter-Veda Ltd, Rehovot, Israel). This is a preparation of HuIFN-ß produced by stimulation of human foreskin fibroblast cell cultures with a synthetic polyribonucleotide (poly I:C) and superinduced with cycloheximide and actinomycin. This preparation has a stated value of 1 x 10^6 IU/ml on reconstitution as directed.

Namalvin (Heriff ApS; Omme, Denmark) —lymphoblastoid interferon prepared by stimulation of Namalwa cells with Sendai virus. The stated potency was 4 x 10^6 IU/ml.

HuIFN-α (Israel Institute for Biological Research: Ness-Ziona, Israel) (IIBR) — this preparation is derived from buffy coats of donor blood by stimulation of the mononuclear cells with Sendai virus. The potency was given as 3 x 10^6 IU/ml.

Lymphoblastoid interferon (IIBR) — a further preparation of lymphoblastoid interferon also produced by stimulation of Namalwa cells with Sendai virus and with a given potency of 2 x 10^6 IU/ml.

International human leucocyte standard G023-901-527

This was obtained from the Research Resources Branch of the National Institute of Allergy and Infectious Diseases, Bethesda, Md, USA, and had a unitage of 20 000 IU/ml.

In-house standard

An in-house standard of lymphoblastoid interferon was prepared from Namalwa cells kindly donated by Professor M. Revel of the Weizmann Institute of Science, Rehovot, Israel. The cells were grown in roller bottles in RPMI 1640 supplemented with 2% fetal calf serum (FCS). A suspension of the cells at 0,5 x 10^6/ml was distributed in 100 ml amounts into flasks of 75 cm^2 and *N*-butyric acid was added to a final concentration of 1 mM. After incubation at 37°C for 48 hours the suspensions were centrifuged and resuspended to a concentration of 2 x 10^6/ml with fresh RPMI 1640 with 2% FCS. The E82 strain of Sendai virus, used for large-scale production of buffy-coat HuIFN-α, was obtained from Dr K. Cantell of the Central Public Health Laboratory at Helsinki, and was grown in the allantoic cavity of embryonated chick eggs. The inducer virus was added to the Namalwa cells at a titre of 100 haemagglutinating units per 10^6 viable cells, and the suspensions incubated overnight at 35°C. The cells were then pelleted and the supernatant used as crude Namalwa interferon. The in-house standard was calibrated against the international standard and its unitage was calculated at 16 000 IU/ml.

Mock interferon

This was prepared as for the in-house standard, but the inducer virus was omitted. The mock interferon preparation is not devoid of all antiviral activity, as Namalwa cells have been shown to produce low basal levels of interferon even in the absence of any inducing condition.[8]

Antiviral radio-immunoassay

This technique has been described in greater detail elsewhere.[7] The test is based on the quantitation by radio-immunoassay of the reduction of growth of challenge Sindbis virus due to the protection of cells by the interferon samples.

Briefly, confluent monolayers of Vero cells were grown in microtitre plates and dilutions of the relevant interferon preparations in Eagle's minimum essential medium with 2% FCS were added. Initially, serial 10-fold dilutions ranging from 10^{-1} to 10^{-4} were tested, followed by 2-fold dilutions for finer adjustment. After an incubation period of 18 - 24 hours at 37°C in a 5% CO_2/air mixture, the medium was removed and the cells washed twice. The cells were then challenged with a dilution of Sindbis virus, usually 1:1 000, previously calculated to produce optimal sensitivity for the detection of interferon. After further overnight incubation the medium was removed, the monolayer washed 3 times and fixed with methanol at −20°C for 2 hours. The methanol was removed and the plates air-dried. The cells were then washed 3 times and 50 μl of murine anti-Sindbis ascitic fluid was added at a predetermined optimal titre, usually 1:100. The cells were then incubated for 1 hour at 37°C, washed 3 times and 100 μl of ^{125}I-protein A (Radiochemical Centre Ltd; Amersham, UK) at 300 cpm/μl was added to each well. After further incubation at 37°C for 1 hour the cells were washed 4 times and solubilized by adding 100 μl of a mixture of 0,1N NaOH and 0,1% sodium dodecyl sulphate. Aliquots of 50 μl were transferred to tubes for counting in a gamma counter.

The endpoint was determined as the highest dilution of interferon giving a 50% reduction of counts, and the unitage calculated by relating this titre to that obtained for the international or the in-house standard.

Antiproliferative assay

Human amnion cells, which are in the routine diagnostic laboratory for the isolation of viruses, were employed to determine the antiproliferative activities of the various interferon preparations. The cells were seeded at 1,5 x 10^5/ml in 24-well tissue culture plates (Linbro, Flow Laboratories). The interferon preparations were incorporated into the medium at the same dilutions as for the antiviral assay and the cells were then incubated for 72 hours at 37°C in a 5% CO_2/air mixture. Mitotic activity was then measured by pulsing with ^{3}H-thymidine at 2 μCi/ml in minimum essential medium with 10% FCS for 1 hour. The cells were then washed with phosphate-buffered saline, pH 7,4, and 5% trichloro-acetic acid (TCA) was added for 30 minutes at 37°C. After 2 further washings with 5% TCA a mixture of 0,1N NaOH and 0,1% sodium dodecyl sulphate was added and the cells incubated at 37°C for 1 hour. The samples were neutralized with 1M hydrochloric acid and then counted in a scintillation counter. The percentage inhibition of TCA precipitable counts was recorded at specific dilutions of the various interferon preparations.

NKC stimulation assay

Mononuclear cells, obtained by Ficoll-Hypaque density gradient fractionation of peripheral blood donated by healthy volunteers, were used as the effector cells for the NKC assay. These cells were washed 3 times in RPMI 1640 and resuspended to a concentration of 10^7/ml in the same medium. For the assay 0,8 ml of mononuclear cells (10^7/ml) were treated with 0,2 ml of the interferon preparations or mock interferon and incubated for 3 hours at 37°C. The cells were then washed 3 times and resuspended in RPMI 1640 with 10% FCS and were then used in the NKC assay.

The target cells used were from the myeloid line (K562), which was maintained as a suspension culture in RPMI 1640 with 10% FCS. These cells were labelled with ^{51}Cr by suspending approximately 3 x 10^6 cells in 0,2 ml medium containing 100 μCi of Na_2 ^{51}Cr-O_4 and incubating for 2 hours at 37°C with occasional agitation. They were then washed 3 times and resuspended in RPMI 1640 with 10% FCS.

The NKC was performed by adding 100 μl aliquots of 2 x 10^4 labelled target cells to each well of flat-bottomed microtitre plates. The effector mononuclear cells were likewise added in 100 μl aliquots to the plates to give an effector cell : target cell ratio of 80:1, 40:1, 20:1 and 10:1 respectively. The cultures were incubated at 37°C in 5% CO_2/air for 16 hours, after which 100 μl of the supernatants were removed from each well and the ^{51}Cr released was quantitated in a Packard 5320 gamma counter. The tests were all carried out three times. The percentage of cytotoxity was calculated as:

$$\frac{\text{experimental } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}}{\text{total } ^{51}\text{Cr counts} - \text{spontaneous } ^{51}\text{Cr release}} \times 100.$$

Results

Antiviral activity of interferon preparations (Table I)

The results of the antiviral assays carried out on the interferon preparations are given in Table I. For two preparations (the IIBR HuIFN-α and lymphoblastoid interferon) the values obtained by us corresponded with those given by the manufacturers. With the two other commercial preparations (Frone HuIFN-ß and Namalvin lymphoblastoid interferon) the laboratory-determined values were just under one-half of those given by the manufacturers.

Antiproliferative activity of interferon preparations (Table II)

The antiprolifertive activities of each of the commercial preparations were determined at varying dilutions in parallel with the

TABLE I. ANTIVIRAL ACTIVITIES OF INTERFERON PREPARATIONS

Interferon preparations	Activity (IU/ml)	
	Stated	Detected
A	1×10^6	$3{,}2 \times 10^5$
B	4×10^6	$1{,}8 \times 10^6$
C	3×10^6	$3{,}2 \times 10^6$
D	2×10^6	$1{,}6 \times 10^6$
HS	—	$1{,}6 \times 10^4$
IS	2×10^4	—
M	—	1×10^2

A = Frone; B = Namalvin; C = IIBR HuIFN-α; D = IIBR lymphoblastoid interferon; HS = in-house standard; IS = National Institutes of Health international standard G023 901 527; M = mock interferon.

TABLE III. NKC ACTIVITY OF MONONUCLEAR CELLS TREATED WITH INTERFERON PREPARATIONS*

Interferon preparations	Effector cell : target cell ratio			
	80:1	40:1	20:1	10:1
A	**1,9**	**2,1**	**2,4**	**1,8**
B	**2,1**	**2,6**	**4,2**	**2,0**
C	**1,7**	**1,9**	**2,3**	**2,0**
D	**1,5**	**1,9**	**2,4**	**1,5**
HS	**2,1**	**2,2**	**3,2**	**2,2**
M	**0,8**	**0,4**	**0,4**	**0,2**

*NKC activity expressed as ratio of: $\frac{\%\ ^{51}Cr \text{ released by interferon-treated mononuclear cells}}{\%\ ^{51}Cr \text{ released by untreated mononuclear cells}}$

A = Frone; B = Namalvin; C = IIBR HuIFN-α; D = IIBR lymphoblastoid interferon; HS = in-house standard; M = mock interferon.

in-house standard and were expressed as the percentage inhibition of ^{3}H-thymidine uptake (Table II). The commercial preparations all produced higher levels of inhibition of cellular proliferation than the in-house standard at all dilutions tested. This is consistent with the antiviral results. With all the preparations it was noted that the antiproliferative activities remained elevated on serial dilution and then dropped substantially when a specific dilution was reached. It was thus possible to define a 'titre' as with antibody activity. A convenient cut-off value for antiproliferative activity was taken as a percentage ^{3}H-thymidine uptake value of greater than double that of the mock interferon. Thus, the titre for each preparation was determined as the highest dilution giving a percentage ^{3}H-thymidine uptake value greater than 30%. The titres for the Frone, Namalvin, IIBR HuIFN-α, IIBR lymphoblastoid interferon and the in-house standard were found be 10^3, 10^3, 10^4, 10^4 and 10^2 respectively.

TABLE II. ANTIPROLIFERATIVE ACTIVITIES OF INTERFERON PREPARATIONS

Interferon preparations	% inhibition of ^{3}H-thymidine uptake by dilution of preparation				
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A	**60**	**55**	**43**	**17**	**ND**
HS	**39**	**42**	**19**	**13**	**ND**
B	**46**	**44**	**44**	**18**	**ND**
HS	**37**	**37**	**16**	**13**	**ND**
C	**ND**	**63**	**55**	**40**	**25**
HS	**ND**	**33**	**14**	**ND**	**ND**
D	**ND**	**64**	**57**	**40**	**27**
HS	**ND**	**33**	**16**	**ND**	**ND**
M	**15**	**ND**	**ND**	**ND**	**ND**
HS	**42**	**ND**	**ND**	**ND**	**ND**

ND = not done; A = Frone; B = Namalvin; C = IIBR HuIFN-α; D = IIBR lymphoblastoid interferon; HS = in-house standard; M = mock interferon.

NKC stimulatory activity (Table III)

The degree to which the interferon preparations stimulated the NKC activity of peripheral mononuclear cells was expressed as the ratio of the lytic activity of interferon-treated cells for the target cell (K562) relative to untreated mononuclear cells. This index was determined at varying ratios of effector (mononuclear) cells to target cells, as shown in Table III. Optimal results were obtained at the effector cell : target cell ratio of 20:1, although the relative efficacies of the different interferon preparations were the same at all of the ratios tested. It is noteworthy that the Namalvin preparation stimuated NKC activity far more than the other commercial preparations. It is also of interest that the in-house standard stimulated NKCs more than the commercial preparations other than Namalvin, even though its antiviral and antiproliferative activities were considerably lower.

Discussion

Over the past few years there has been a dramatic upsurge in interest in the interferons among basic scientists such as cell biologists, biochemists and virologists, as well as among clinicians. The tremendous commercial pressure to find economic ways of producing large amounts of interferon for clinical investigation has undoubtedly played a substantial role in the recent extremely rapid developments in our understanding of the biochemistry and molecular biology of the interferons. A number of the genes have been successfully cloned in bacteria[9] and yeasts,[10] and effective interferon products produced from them.[11] The polypeptide sequence of HuIFN-α has been elucidated[12] and one of its genes was recently totally synthesized.[13] There is now little doubt that soon large amounts of interferon derived from recombinant DNA techniques or chemically synthetized interferons will become available for therapeutic use.

Somewhat less dramatic have been the advances in the study of the biology of interferons. The complex activities of these remarkable agents have been extensively reviewed elsewhere.[2-4] Their two most striking properties are their potency and the wide range of biological activities. Interferons are the most potent biological substances known, having demonstrable activity at 10^{-14}M.[2] The spectrum of their activities stretches from the inhibition of insulin-activated conversion of mouse fibroblasts to adipocytes[14] to inhibition of myocardial contractility.[15] From a clinical point of view, however, the three most important activities of the interferons are their broad-spectrum antiviral activity, their antiproliferative activity for both normal and transformed cells and their stimulation of cytotoxic cells, especially NKC. The best understood and most thoroughly studied of these is the antiviral activity, and this is used to quantitate the potency of the interferons in terms of unitage.

Least understood are their clinical activities and therapeutic potential. It was felt that in order to obtain a greater degree of understanding of their clinical effectiveness, parameters other than their antiviral potency would need to be determined before meaningful clinical trials could be planned and carried out. Samples of four commercial preparations of therapeutic interferons were therefore evaluated in terms of their three major activities in order to characterize their potency more accurately.

The antiviral activities of two of the preparations correlated well with the values provided by the manufacturers, but in the other two there was a drop of approximately 50% when the antiviral potency was tested in our laboratory. It should be noted, however, that one of these preparations was an HuIFN-ß and was calibrated against an HuIFN-α standard. This could

possibly have resulted in a falsely low result. The test is, in any event, a semiquantitative one, and a 50% variation is relatively insignificant. At this stage of clinical trials dosages are based on antiviral unitage, and it would therefore seem reasonable that the dosage provided by the manufacturers should be complemented by in-house evaluation. This is especially of importance in the monitoring of the dynamics of distribution and effective levels in blood or body fluids during therapy.

The antiproliferative activity is most conveniently expressed as a titre and correlated closely with the antiviral activity. Interferon preparations purified by affinity chromatography using monoclonal anti-interferon antibodies have displayed antiviral and antiproliferative activities running in parallel with each other. However, as regards Namalvin and the in-house standard, the NKC stimulatory activities showed discrepancies when compared with the antiviral and antiproliferative results. Both preparations were very potent stimulators of NKCs, this being out of proportion to their antiviral and antiproliferative activities. The in-house standard is a crude unpurified preparation, and impurities present in this preparation and possibly also in the Namalvin preparation may be responsible for a substantial proportion of this significant increase in NKC stimulation. Alternatively, it may be the case that an interferon component with pronounced NKC stimulatory activity has been partially lost or denatured in the purification process of the other preparations. Whatever the explanation for this discrepancy, it remains essential that parameters other than antiviral activity should be taken into account when clinical trials of the interferons are planned and when the results are evaluated. This is critical not only for the planning of dosage schedules and determination of pharmacodynamics and stability, but also for a better understanding of the mechanisms of clinical action and the effectiveness of these remarkable agents.

Addendum

A sample of recombinant leucocyte A interferon produced by the cloning of the human leucocyte interferon (HuIFN-α) gene in *Escherichia coli* and subsequent purification of the product on an affinity column using monoclonal antibody[16] was provided for assay by F. Hoffmann-La Roche & Co., Basle, Switzerland. This product was examined for antiviral activity and was found on our assay to yield a value of $1,6 \times 10^6$ IU/ml. The stated value on the product was 3×10^6 IU/ml.

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