INTERFERON

Properties, Mode of Action, Production, Clinical Application

Proceedings of the 3rd International Expert Meeting of the
Deutsche Stiftung für Krebsforschung,
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Drug Dosage

The author and publisher have exerted every effort to ensure that drug selection and dosage set forth in this text are in accord with current recommendations and practice at the time of publication. However, in view of ongoing research, changes in government regulations, and the constant flow of information relating to drug therapy and drug reactions, the reader is urged to check the package insert for each drug for any change in indications and dosage and for added warnings and precautions. This is particularly important when the recommended agent is a new and/or infrequently employed drug.

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Preface

The Deutsche Krebshilfe was founded by Dr. Mildred Scheel as a private foundation. The reaction and response of the German people to this foundation, which is dedicated to finding solutions for this urgent medical problem, has been very great. The Deutsche Krebshilfe received, throughout the years, money from individual private donors, legacies, and many other sources. The enthusiastic donations by the population have never ceased since the start of the foundation and continue in a way which gives reason to admire the goodwill of the people.

However, in regard to this money donated to the Deutsche Krebshilfe, there are, of course, great demands placed upon these persons, particularly on Dr. Scheel and her scientific and organizational advisors, and suggestions on how to make the best use of these donations. The people expect, of course, this money to be given to the discovery of solutions to the most urgent problems in the medical care of cancer and to the most fruitful projects in cancer research. In regard to this challenge, the Deutsche Krebshilfe always followed one main goal, that is the idea that a private foundation should always be innovative. It should always start and support projects which are new, which cannot be started because of the lack of official initiative, or because of the lack of official financial support or because there are administrative barriers which inhibit or retard the tackling of urgent projects.

These goals, however, require the persistent efforts and the expertise of those who are asked to advise the Deutsche Krebshilfe. In addition, since the cancer problem is international and the fight against

cancer needs a combined international effort, a comprehensive, world-wide group of experts is needed. The Deutsche Krebshilfe always sought the advice of international experts in order to help the foundation work best for the benefit of the cancer patients of today and tomorrow.

The meetings of experts, of which this was the third one, are examples for the international and comprehensive discussions on the aims and tasks of the Deutsche Krebshilfe. The idea to devote this meeting to interferon is Dr. Scheel's because she was frequently approached by patients to supply the financial means for individual interferon therapies. Since the interferon problem is, at present, extensively discussed within the community of scientists as well as the laypress, Dr. Scheel again sought the advice of internationally recognized experts in this field. She expects, from the results of this meeting, the best possible clarification and an expert evaluation of the significance of interferon therapy, particularly in comparison with other forms of cancer treatment. During the past year, the interferon field has exploded scientifically. Some of the dynamite workers involved were at this meeting.

The reasons for the enormous progress in interferon research have been basically threefold:

First, there have been breakthroughs in the protein chemistry and the molecular biology of interferon. Thus, it is now proven that interferons are a class of different proteins, and the amino acid sequence of some has been identified.

We want to stress the following: Interferons are a group of defined proteins with exceedingly high biologic activities in different systems.

Secondly, it has been possible to introduce and express the gene of human interferon in *E.coli*. This may be a promising way towards producing interferon in sufficient quantities. Most excitingly, the *E.coli* product seems to share many of the known properties of human interferon.

Thirdly, there has been a tremendous clinical interest in interferon due to the possibility that interferon may be of some use in the treatment of neoplastic disease.

Please note that we have used the term *may*. It was obviously one of the purposes of this meeting to achieve a critical evaluation of the available clinical data.

When a research field explodes, as it has happened to interferon research, people working in the field may have a twofold reaction, and this applies to those who have worked in the field for ten or more years, particularly our distinguished guests.

On the one side, it is a good feeling to realize that the scientific community finally acknowledges the importance of something of which we have been convinced for quite a while.

On the other side, one is afraid when there is too much uncritical enthusiasm, particularly when something as serious as the treatment of cancer is involved and when the lay-press begins to be interested.

Now, in this situation, we feel that we owe the public clear-cut statements about the state of affairs. Dr. Scheel is very concerned about these matters.

As it has happened often in cancer medicine, so-called new forms of therapy have raised much optimism, then failed, and subsequently seriously blocked future developments. In such situations, short-cuts to practical medicine have turned out to be disasters.

All of us are convinced that interferons are of tremendous biologic significance. Interferons may turn out to be of clinical value but we are far from having a sound evaluation on this point. We want to stress, however, that most likely no progress will be made unless the way is paved by thorough research in the laboratories. Clinical application of interferons would probably have never occurred if scientists did not treat mice with interferons about ten years ago. Similarly, we believe that future therapeutic improvements will depend on the progress made in laboratories. Furthermore, in regard to clinical studies, the *very least* we have to postulate is that therapeutical trials are accompanied by thorough clinical investigations so that we may learn more about the pharmacokinetics of interferons and many other things. Otherwise, the therapeutic trials may turn out to be useless and extremely costly.

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Heidelberg, 1981

Klaus Munk
Holger Kirchner

Contents

Mechanisms of Interferon Action on Cell Growth and on Murine Leukemia, Vesicular Stomatitis, and Encephalomyocarditis Viruses

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The basis for the antiviral activity of interferons in the case of a lytic virus like encephalomyocarditis virus (EMC) appears to be the inhibition of viral protein synthesis [1]. In contrast, the interferon-induced suppression of retroviruses, such as murine leukemia virus (MLV) in chronically infected cells, appears to occur at a late stage of virus growth [1]. In interferon-treated cells infected with mouse mammary tumor virus [2, 3] or MLV [4, 5, 6], virus yields are significantly inhibited. Inhibition of retrovirus production was not correlated with inhibition of at least some intracellular steps in virus replication [4, 5]. In interferon-treated AKR cells in which there was a marked decrease in the production of both endogenous MLV particles and infectious MLV, the intracellular concentration of viral p30 (group-specific) antigen was unaffected or even increased [5]. Synthesis of the viral proteins p30, gp69/71, and p15 was not inhibited in interferon-treated mouse 3T3 fibroblast cells infected with MLV; synthesis and cleavage of the precursors of these proteins were also unaffected [7]. In interferon-treated AKR cells, scanning electron and transmission

micrographs, in fact, clearly indicated that in mouse systems the number of surface-associated MLV particles was increased [8, 9].

RNA synthesis by retroviruses has also been studied. In interferon-treated mouse cells infected with Moloney-MLV or AKR-MLV, the concentration of virus-specific RNA was approximately the same as in untreated cells. The rates of viral RNA synthesis were also about equal [6, 10]. Substantially the same findings have been made in chronic mouse mammary tumor virus infection after interferon treatment [3]. During inhibition by interferon of exogenous or endogenous infection, the findings were similar to those previously discussed in that the interferon block appeared to occur at or just before virus assembly so that, although there was no inhibition of viral p30 (group-specific) antigen, there was a decrease in released virus [6]; however, although the interferon-induced inhibition of assembly seemed to result in a relatively small decrease in the number of virus particles released, there was a marked inhibition in the infectivity of the MLV particles produced in one AKR-MLV system [6]. This observation has been confirmed in both chronic and acute Moloney MLV infection in TB cells [11]. It would seem, therefore, that, although in some systems interferon treatment resulted in marked inhibition of virus release, in others, particle production was almost normal, but the virus released was quite deficient in infectivity [1].

One possible basis for the low infectivity of the MLV produced by interferon-treated TB cells was a decrease in the amount of viral envelope glycoprotein gp69/71 associated with the virions. This conclusion was based on the reduced molar ratio of gp69/71 relative to other structural proteins in ^{35}S-methionine-labeled defective particles, as well as the reduced amount of ^{3}H-glucosamine incorporated into the glycoprotein (gp69/71) of these virions. The deficiency in gp69/71 may account for part, if not all, of the decrease in infectivity of these particles, since it has been shown that gp69/71 is responsible for the recognition and binding of the virion to the receptor sites on the cell membrane [12].

Apart from its antiviral activity, IFN has been shown to alter cellular parameters, in particular, those involved in cell growth. There may be a correlation between this property of interferons, termed the anticellular activity, and their antiviral activity since cells which respond to the anticellular activity of interferons also appear to be sensitive to the anti-lytic virus effect [14]. It is not clear, however,

whether similar correlations exist between the interferon-induced inhibition of MLV production and the anticellular or anti-lytic virus activities of interferons. One could argue for instance, that the inhibition of MLV is mediated through the anticellular effect since production of MLV has been shown to be affected by the cell cycle [15] and extends both the G_1 and $S + G_2$ phases [16, 17, 18].

In comparing the anti-MLV, anti-EMC virus and anti-cellular activities of interferons, we have used three parameters for measuring the anti-cellular effects: inhibition of (i) cell division; (ii) DNA synthesis; or, (III) ornithine decarboxylase (ODC) induction. Inhibition of ODC induction has been shown to be an independent parameter for the anticellular activity of interferons [19, 20]. Indeed, the anti-

Table I. Effects of interferon on virus production in two Swiss 3T3-sublines, D-8 and H-2

		Concentration of interferon (U/ml)			
		10	25	50	100
Inhibition of:					
MLV (%)[a]	D-8	70	85	90	100
	H-2	65	95	95	100
EMC ($\log_{10}$)[b]	D-8	0	not done	1.0	1.0
	H-2	0.7	not done	4.8	4.8

[a] Subconfluent cultures of M-MuLV-infected cells, D-8 and H-2 (1×10^5 cells per 100 mm petri dish in 10 ml of Dulbecco modified Eagles' medium containing 10% fetal calf serum) were incubated for 24 h with L-cell interferon (specific activity 2×10^7 units per mg protein). Culture fluids were replaced with fresh medium containing interferon and after 24 h, MLV released into culture fluids was measured by assay of reverse transcriptase activity. Fluids were clarified by low speed centrifugation and virus was pelleted by sedimentation for 1 h at 105,000 × g. Pellets were resuspended in 100 ul of 20 mM Tris HCl pH 7.5, 100 mM NaCl, and 1 mM EDTA. Reverse transcriptase activity in the virus pellet was determined by incorporation of ^{3}H-TMP in the presence of poly A oligo dT template primer. Since cell number changed with the interferon concentration, results were corrected for original fluid volume and cell number at time of harvest. MLV from control cultures of D-8 and H-2 without interferon (100% activity) catalyzed the incorporation of 0.27 and 0.53 pmoles ^{3}H-TMP per min respectively.

[b] Parallel cultures of D-8 and H-2 were infected with EMC virus (MOI of 0.1) after 24 h treatment with interferon. After an additional 24 h, fluids were harvested; clarified by low speed centrifugation; and EMC virus was titered by infecting L-cells in 96-well microtiter plates with serial virus dilutions. CPE was recorded at 24 and 48 h post-infection. Results are given as inhibition of virus growth in $\log_{10}$.

MLV effect of interferons can be dissociated from both its anticellular and antiviral activities. This was shown in the following manner: Swiss 3T3-cells, susceptible to the anticellular and antiviral effects of mouse interferon, were infected with Moloney-MLV at a multiplicity of one. The chronically infected cells were cloned by plating in a microtiter dish. Two of the clones were tested for susceptibility to interferon by assaying three parameters: (i) anti-MLV effect; (ii) anti-EMC virus activity and (iii) anticellular effects. Table I shows a dose-response effect of interferons on the production of MLV in two cell clones, D-8 and H-2. The virus released into the medium was assayed for reverse transcriptase activity and activity was corrected for cell number in the cultures. The sensitivity of the clones was similar. Virus production was also monitored by focus formation on S^+L^--cells [21] and parallel reduction curves were obtained (results not shown), indicating that the residual virus released from IFN-treated cells was fully infectious. In contrast to the anti-MLV effect, EMC virus was inhibited by interferon differentially in D-8 and H-2 cells; 50 U/ml reduced EMC virus yield by over 4 logs in H-2 but only 1 log in D-8 cells.

Next, the anticellular effects curve of interferon on: (i) cell division; (ii) DNA synthesis, as measured by thymidine incorporation; and, (iii) induction of ornithine decarboxylase (ODC) enzyme activity. All of the above activities were inhibited (table II). Comparison of D-8 and H-2 sensitivities indicated that for D-8 cultures, the amount of interferon required for 50% reduction was about 5-fold more for cell number, 45-fold more for DNA synthesis, and 3-fold more for ODC activity, as compared to H-2 cultures. Thus, the H-2 subclone appeared to be more sensitive than the D-8 subclone in terms of both the anti-EMC virus and anticellular activities of interferon; however, MLV inhibition in the two subclones was similar. These results suggested that interferon may affect cell growth functions and EMC replication through pathways different from those by which MLV production is inhibited.

To test this hypothesis further, several additional cell lines were infected with Moloney-MLV and analyzed for their ability to develop antiviral states against EMC virus and MLV after interferon treatment. Of the cells tested, the results obtained with NIH-3T3 cells [22], chronically infected with MLV, were most intriguing. Table III demonstrates that 1500 u/ml of interferon inhibited MLV

Table II. Anticellular effects of interferon in D-8 or H-2 subclones

Growth parameter	Concentration of interferon (U/ml)		
	10	50	100
Cell number (%)[a]			
D-8	100	92	48
H-2	65	38	23
Ornithine decarboxylase[b] induction (%)			
D-8	100	71	52
H-2	63	40	37
^{3}H thymidine incorporation[c]			
D-8	91	52	45
H-2	45	11	8

[a] Inhibition of cell multiplication. Cultures were treated with interferon for 48 h as described in table II. After fluids were harvested for reverse transcriptase assay, the monolayers were trypsinized for determination of cell number. Control cultures of D-8 and H-2 without IFN (100%) gave cell counts of 3.2×10^5 and 6.5×10^5, respectively.

[b] Inhibition of ornithine decarboxylase (ODC) induction. Quiescent cultures were prepared by serum depletion The cultures were stimulated with 10% serum in the presence of increasing concentrations of interferon and ODC activity in the cells was measured at the end of 6 h following serum addition. Values are expressed as percent of control cultures stimulated in the absence of interferon. The level of ODC activity in unstimulated cultures was less than 0.1 nmoles $^{14}CO_2$ released per mg cell protein per h. ODC activities in serum-stimulated D-8 and H-2 subclones were 2.58 and 2.26 nmoles $^{14}CO_2$/mg/h, respectively.

[c] Inhibition of DNA synthesis. The incorporation of ^{3}H-thymidine in quiescent cultures stimulated with serum was measured. After 30 h incubation, incorporation into acid-precipitable DNA was determined and is expressed as percent of control cultures which were stimulated in the absence of IFN. Quiescent and serum-stimulated D-8 cultures incorporated 5×10^5 cpm per culture while similar values for H-2 were 2×10^3 and 2.7×10^5 cpm per culture, respectively.

production by over 95%, while this high interferon concentration had no effect on EMC virus replication. Next, we tested the anticellular activity of interferon on these cells (NIH 3T3-MuLV). Interferon treatment had no inhibitory effects on cell division, DNA synthesis, or ODC enzyme induction. Similar resistance to interferon in terms of these parameters was also observed in NIH-3T3 cells not infected with MLV and obtained from a different source (results not shown). This cell line (NIH 3T3) thus showed a complete dissociation of the anti-MuLV activity from the anti-EMC or anticellular effects induced by interferons.

Table III. Effect of interferon on virus production in NIH-3T3 cells

	Concentration of interferon (U/ml)					
	0	10	30	500	1000	1500
Inhibition of[a]						
MLV (%)	0	60	75	80	85	90
EMC (%)	0	0	0	0	0	0

[a] Subconfluent NIH-3T3 cells, chronically infected with MLV, were incubated with interferon as described in table II. MLV production and EMCV replication in these cultures were assayed as described in table II. MLV production in control cultures without interferon (100% activity) catalyzed the incorporation of 1.16 pmoles ^{3}H-TMP per min. Inhibition of replication of EMCV and MLV are listed as percent inhibition.

The finding, that two subclones, isolated from a single culture of Swiss-3T3 cells chronically infected with MLV, exhibited different ranges of sensitivity to IFN which suggested variability among cells in a population. While variability was not observed with the anti-MLV activity, the clones differed in their sensitivities to both the anti-EMC virus and anticellular activities. Such a variability could have developed at one of two stages: (i) during cell passage; or, (ii) subsequent to infection by MLV. Since MLV infection of fibroblasts in a culture appears to have no effect on the cellular phenotype, the latter possibility is less likely.

Three different parameters were assayed for the anticellular effect of interferons; of the three, cell division appeared to be the most sensitive. This may be explained by the fact that interferons affect not only the G_1 and S phases (analyzed by ODC induction and DNA synthesis), but also the G_2 phase of the cell cycle [16]. In the two subclones D-8 and H-2, DNA synthesis was more sensitive to the inhibitory effect of interferon than induction of ODC. This is consistent with the observation that the inhibition of DNA synthesis in IFN-treated Swiss-3T3 cells is not a direct consequence of the inhibition of ODC induction [19].

Resistance of cells to interferons might be attributed to either a lack of receptors for interferon or to a deficiency in an intracellular factor required for establishment and/or maintenance of the antiviral and anticellular activities. NIH-3T3 cells appeared to be completely resistant to interferon in terms of the anti-EMC and anticellular activities, but they retained sensitivity to the anti-MLV activity. There

are at least two possible explanations for the differential inhibitions of MLV and EMC virus in NIH-3T3 cells: (i) two different receptors for IFN are responsible for the establishment of antiviral states against EMC and MLV, and NIH-3T3 cells are devoid of the receptors necessary to initiate the anti-EMC viral state; or, (ii) there is only one type of receptor for interferon, but NIH-3T3 cells are deficient in an intracellular factor needed for the expression of the anti-EMC virus activity. Several enzymatic systems have been implicated in the anti-EMC viral state induced by interferons. One pathway involves induction of a synthetase activity which catalyzes the polymerization of 2'5'-oligo-A and this oligonucleotide induces a latent endonuclease activity in the interferon-treated cells [1]. Preliminary observations of *Epstein et al.* (personal communication) have indicated that 2'5'-oligo-A synthetase is induced in the same NIH-3T3 cell line in which EMC-virus replication is not inhibited after interferon treatment. This observation suggested the existence of receptors for interferon for the initiation of what is thought to be an anti-EMC virus activity. Work in progress indicates this pathway in NIH-3T3 cells is aborted at a later step, the level of the 2'5'-oligo-A-dependent endonuclease. This enzyme is lacking in NIH-3T3 cells.

Finally, it has been shown that retroviruses as well as lytic viruses can be used for assaying the antiviral activity of interferons [23]. In view of the present results, use of the standard assays (e. g. EMC virus inhibition) may not be sufficient for the determination of cell sensitivity to interferons, since cells, resistant to the anti-lytic virus activity of interferons, may still respond to the anti-MLV effect.

We are also in the process of studying VSV produced by interferon-treated L-cells. We have so far observed that, in L-cells treated with 30 U of interferon/ml, there was an approximately 200-fold reduction in the titer of infectious VSV production; however, virus particle production, as measured by VSV particle-associated RNA, N-protein, or transcriptase was inhibited by a maximum of 10-fold by this concentration of interferon. In addition, there was biochemical and morphological evidence of a significant reduction in glycoprotein (G) and membrane protein (M) content of VSV particles released from interferon-treated cells. The results in some respects resemble those previously reported for interferon-treated TB-cells infected with MLV. We concluded, therefore, that such findings are not limited to murine RNA tumor virus systems [24].

When the effects of tunicamycin (TM), and inhibitor of glycosylation of proteins, and IFN on VSV released from mouse cells were compared, they were both found to reduce the production of infectious VSV by 80- to 100-fold; to decrease the amounts of G and M viral proteins in VSV released from IFN-treated cells; and to inhibit an early step in the formation of asparagine-linked oligosaccharide chains, so that the incorporation by membrane preparations from interferon-treated cells of N-acetylglucosamine into glycolipid with the properties of dolichol derivatives was inhibited. It is possible that this effect of interferon-treatment is related to the deficiency in glycosylation of MLV and VSV protein, but there is as yet no biological data linking these findings [25].

The inhibition of some membrane-associated viruses by treatment with relatively moderate concentrations of interferon may be a widespread phenomenon that is closely related to functional abnormalities in the proteins incorporated into noninfectious virions produced by such cells. Since many studies have demonstrated that VSV particles with reduced amounts of glycoprotein are low in infectivity, it is likely that at least some of the reduced infectivity of VSV particles, produced by interferon-treated cells, was due to the reduced amount of this protein incorporated into such particles. It is possible that induced changes, that have been reported to occur in the plasma membrane of interferon-treated cells, may account for the alterations in infectivity of both VSV and murine RNA tumor viruses, since these viruses bud from the cell surface as a terminal step in the replication process [26].

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