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Type I interferons spontaneously expressed during human pregnancy

Paulette Duc-Goiran,[1] Brigitte Robert,[1] Sébastien Navarro,[2] Jacqueline Lopez, Jacques Chavinié,[3] Françoise Ferré,[1] Charles Chany,[3] Janine Doly[2]

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Our previous studies have shown the presence of Interferons (IFNs) in human fetal annexes, in the absence of any apparent induction. The characterization of the IFN proteins was performed and their analysis by SDS-PAGE revealed the presence of α and β IFNs and unusually large IFN components. These results were confirmed by the demonstration of unusually large IFN-α transcripts, by means of Northern blot analysis. The large IFN-α transcript (4.3 kb) has been characterized and correlated with the presence of functionally active IFN protein. It may be specifically expressed during fetal development. In human species, the IFN-α proteins are detected throughout fetal gestation, particularly in the amniotic fluid. However, early embryos do not produce significant levels of IFN. In contrast, in domestic ruminants, some IFNs are only expressed in early embryos. A relation between the type of placentation and the IFN expression is suggested.

Key words: pregnancy; placenta-analysis; interferon type I

Introduction

During gestation, development of the uterus and of the fetal-placental unit is specially intense. It is mediated and regulated by a number of endogenous hormones, growth factors and cytokines. Cytokines are a heterogeneous family of proteins. They comprise interferons (IFNs), colony-stimulating factors, and cytokines produced by activated cells in inflammatory situations or in immune responses. Cytokines are protein mediators of cell-to-cell communication. They have pleiotropic activities, in a variety of cellular processes. Their action is usually local, unlike that of hormones, and is restricted to the micro-environment of the producer cells, specially at the fetal-maternal interface.

Interferons were the first cytokines identified. Their name was originally derived from their ability to interfere with viral replication. IFNs are now known to be involved in cell proliferation and tumor growth inhibition. They may alter immune functions and modulate a variety of physiological responses. IFNs are inducible proteins. Their production is usually subject to stringent control. However, a low spontaneous

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expression has been reported in a variety of tissues.[1, 2] In humans, endogenous IFNs have been detected in human fetal annexes, in the absence of any apparent induction. Spontaneous IFN-α has been detected in the amniotic fluid withdrawn by puncture at selected periods after the 15th week of pregnancy.[3] Some IFNs (α/β) were also found to be diffusing from the amniotic membrane maintained in a tissue culture medium, in placentae obtained after caesarean section,[4, 5] in fetal blood and in decidua, while they cannot be detected in maternal blood.[4, 5] In addition, an immuno-reactive IFN-α protein has been localized in the syncytiotrophoblast of chorionic villi[6] and in spindle shaped cells, which were thought to be macrophages.[7] There is also a low level of IFN-α in human embryo culture media.[8] Moreover, specific binding sites for human IFN-α have been described in human placental membranes.[9]

Patients and methods

Human placentas were obtained between the 35th and 40th week of pregnancy. Biological preparations, affinity chromatography, NaDod SO4/PAGE, RNA preparations and Northern blot hybridization were described previously.[4, 10]

Results

IFN activity

IFN activity was found in 29/37 placentae, 14/29 amniotic membranes and 3/10 umbilical cord blood samples.[4]

Characterization of IFN proteins

In order to characterize the IFNs present in human fetal annexes, we have previously analyzed a set of twenty placentas, in the third trimester of pregnancy.[4]

First, we performed affinity chromatography on Concanavalin A-Sepharose. IFN-α did not bind to the immobilized Concanavalin A and was found in the breakthrough fractions. After elution with methyl-α-D-mannoside and with ethylene glycol, IFN-β was recovered.[4]

We also performed chromatography using as a ligand, specific polyclonal Immunoglobulins against Interferon-α. Most IFN bound to specific immunoglobulins was eluted with pH 2.2 buffer.[4]

Analysis by SDS-PolyAcrylamide-Gel-Electrophoresis, under denaturing and reducing conditions, revealed the presence of α and β IFNs. Besides these typical IFNs, three IFN components of unusual size and antigenic properties have been detected, reminiscent of those previously found in the human amniotic membrane after viral induction. These three components are recognized by antibodies against IFN-α. The 43 kDa component is neutralized by antibodies against IFN-α or IFN-β to about the same extent.[4]

Type I IFN transcripts

We investigated the presence of type I IFN transcripts in placental samples by Northern blot analysis and showed the results of four cases.[10]

IFN-α transcripts have been identified, using an antisense riboprobe, complementary to the human IFN-α C gene. This probe gives a strong signal with the 1.1 kb IFN-α transcript which is usually detected in leukocytes after viral induction. This species is lacking in all placental samples. In contrast, in 3 out of 4 polyadenylated RNA samples, we found transcripts of 4.3, 2.8 and 2.1 kilobases, and a minute signal at 4.3 kb in placenta 3. The intensity of the signals displayed on the autoradiogram was estimated by densitometer scan. The area of the peak corresponding to the 4.3 kb transcript is shown in Figure 1A. The hybridization of the placental mRNAs to the IFN-α C gene appears to be specific. No artefactual signal with the 28 S ribosomic RNA could be detected with the corresponding non-polyadenylated RNAs. However, this hybridization is observed in moderate or low stringency conditions and the signals disappear after stringent washes and after

RNAse A treatment. These results suggest that the mRNAs detected here are only partially homologous to the IFN-α C gene. As IFN-α genes belong to a multigene family clustered on the same chromosome, we have suggested that the mRNA detected in the fetal tissue annexes, belongs to one of the IFN-α family genes, but it is not the IFN-α C gene.

The presence of IFN proteins was studied in the same placentas, by immune affinity chromatography for IFN-α. In placentas 1, 2 and 4, IFN-α was bound to the specific IgG and eluted with pH 2.2 buffer (Figure 1 B).

The IFN-α-like transcripts correlate with the presence of the corresponding functional IFN-α proteins, purified by immuno-affinity. The levels of both IFN-α-like transcripts and IFN-α proteins differ from one placenta to another; being high in placentas 1 and 4, moderate in placenta 2 and absent or minimal in placenta 3.

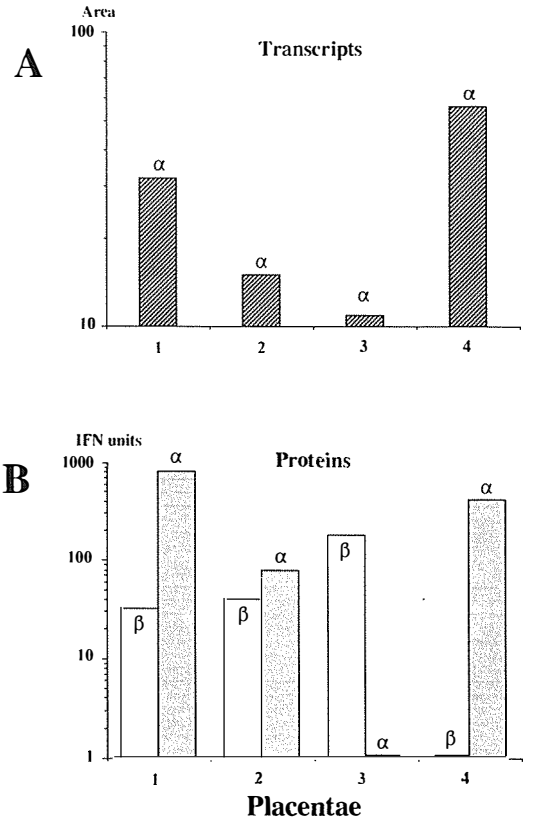

Figure 1. IFN-α expression in human placentae. A) IFN-α transcript signals, measured by densitometer scan (area). B) IFN proteins (International Units) isolated by immune affinity chromatography for IFN-α.

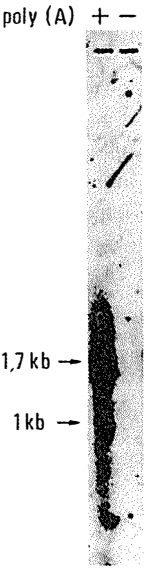

Figure 2. Hybridization of an ovine trophoblast-IFN cDNA to poly $(A)^+$ and poly $(A)^-$ RNAs from a placenta, analyzed by a Northern blot.

IFN β. In placentas 1, 2 and 3, some IFN was excluded from the immuno-absorbent IFN-α column and was found in the flowthrough fractions (Figure 1 B). The antiviral activity appeared to be related to an IFN-β protein and could be purified by a second affinity chromatography, using as a ligand, antibodies against IFN-β. However, we could not show the presence of IFN-β transcripts in any placental sample. The absence of IFN-β transcripts might be due to either minute amounts or to a rapid turnover of mRNAs.

Trophoblast-Interferon. In order to study the transcripts of a human trophoblast-IFN gene in placentas, we used an ovine Trophoblast-IFN cDNA probe,[11] in low stringency conditions of hybridization: Two mRNA species of 1.7 kb and 1 kb were detected. No signal was detected with the non-polyadenylated RNA (Figure 2). These preliminary results suggest the presence of mRNA transcripts possibly related to a trophoblast-IFN gene.

Discussion

Type I Interferons form a group of three distinct gene families: α, β and ω, based on sequence divergence and antigenic differences. Recently, trophoblast proteins from ovine and bovine species have been identified as type I IFNs and cloned.[11, 12, 13] These trophoblast IFNs appear to be immunologically distinct from IFN-ω and IFN-α and form a new class of IFNs, the "trophoblast-IFNs" or "IFN-TAU". These IFNs are poorly virus-inducible.

In ruminants, trophoblast IFNs are only expressed in early embryos, secreted by the extra-embryonic trophoblast. Their primary amino-acid structure ranged from 40 to 55% identity with IFNs-α and 70% with IFNs-ω. These Interferons play a key role in maternal recognition of pregnancy and maintain the corpus luteum by inhibiting the synthesis of the prostaglandin F2α, which is a luteolytic factor by endometrium. A type II IFN is also expressed by the early porcine trophectoderm.[14]

In contrast, in human species, early embryos do not produce significant levels of IFN. IFN-α proteins seem to appear later and are detected throughout gestation, being found in the amniotic fluid from the fifteenth week of pregnancy onwards.

Thus, Interferons seem to be expressed universally in mammalian species during gestation. However, the nature and amount of the Interferons released differ markedly, according to the species and placentation. We have observed variations both in the yield and in the nature of the type I Interferon expressed in human placenta. It could be suggested that the synthesis of these IFNs and the extent of their action could only take place at a specific time under certain conditions.

The situation seems to be the same in rodents:

1. Endogenous IFNs, which were first identified in murine placenta,[15] have been found throughout fetal murine gestation but not in significant amounts in early embryos.[16] In humans as in rodents, the initiation of implantation occurs early. In contrast, in ungulates with an epitheliochorial placenta, embryos which express a high level of trophoblast IFN, attach to the uterine epithelium later and penetration into this maternal layer does not occur.

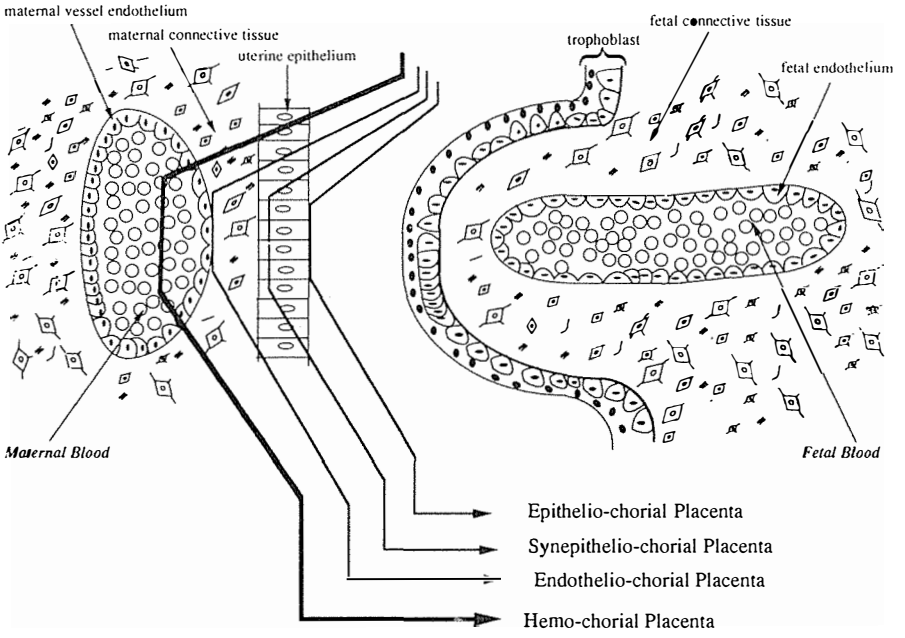

Figure 3. Diagrammatic representation of comparative placentation in relation to invasion of the uterine layers.

2. Like humans, rodents have a hemochorial placenta, but some differences are observed: the murine trophoblastic epithelium is trilaminar (and not monochorial) and is arranged in a labyrinthic pattern, without forming villi. Once the implantation of the blastocyst has been initiated, the fetal trophoblast invades all maternal layers, penetrating as far as the maternal blood, in these placentae (Figure 3). IFNs may be considered as negative growth factors,[17] inhibiting cell proliferation by inducing cellular cycle arrest in the G_0/G_1 phase and the decrease of c-*myc* expression.[18] Therefore, we suggest that the early cytotrophoblast, which is highly proliferative and invasive, with a high expression of proteases, is not controlled by a molecule which has properties of an IFN.

3. One of the IFNs detected in murine fetal annexes seems to be an atypical molecule.[19, 20] In humans, the RNA population of the same placental sample might contain different IFN transcripts and, among them, some high molecular weight transcripts related to an IFN-α gene. These high molecular weight placental IFN-α like transcripts could be those of a new IFN gene involved in fetal development.

Conclusion

Presently, functions of a human IFN-α in pregnancy are almost speculative. As previously suggested:

– Human placental IFN-α could protect the fetus against viral infections.

– IFN-α could inhibit the lymphocytes proliferative response to Interleukin-2 and thus protect the fetus against rejection by maternal cytolytic cells.

– IFN could also down-regulate tissue growth previously promoted by growth-factors, and thus limit tissue invasion.

– As IFN is a powerful inducer of class I antigen, it may induce expression of a non polymorphic class I antigen of the Major Histocompatibility Complex, the HLA-G antigen in cytotrophoblast cells.[21]

– Moreover, it could enhance production by syncytiotrophoblast of Human Chorionic Gonadotropin (hCG) which is presumed the main luteotrophic factor in early human pregnancy. This increase of hCG by IFN-α has been shown in ectopic bladder tumor cells.[22]

IFNs are immunoregulatory molecules. They might serve as intercellular communication signals between the immune and reproductive systems, and seem to be required for successful pregnancy.

The presence of IFNs in human and murine fetal annexes, the discovery of the trophoblast Interferon in ruminants, the expression of a type II Interferon gene and that of a new type I Interferon gene in porcine trophoblast[23] provide a considerable insight into interactions between the embryo and mother during pregnancy thus opening a new area of Interferon biology.

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Changes in the quantity of cathepsin D in irradiated human cells following treatment with hyperthermia and interferon α

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In the present work, the changes in quantity of cathepsin D, an aspartic proteinase, in proliferating human nonmalignant (HEF) and malignant (HEp2) cells after combined treatment by gamma irradiation, hyperthermia and by interferon α-2b (IFNα) were followed. Correlation between the antiproliferative effect of these combined agents and changes in the concentrations of this cathepsin D were expected. Evidently, the treatment of cells in culture by IFNα, combined with irradiation and elevated temperature, produces an increased quantity of cathepsin D. In nonmalignant HEF cells these effects are more expressed than in malignant HEp2 cells.

Key words: cathepsin D; cell, cultured-radiation effects; hyperthermia, induced; interferon-alfa-2B

Introduction

Intracelular proteinases participate in the vital cellular processes such as growth and multiplication, response to DNA damage and radiation response.[1, 2, 3, 4] Recent studies suggest that the aspartic proteinase cathepsin D, may also be implicated in the process of tumor invasion and metastasis,[5] Several *in vitro* observations showed that this proteinase may facilitate the spread of neoplastic cells through different mechanisms releated to its proteolytic activity, by acting at different levels of the metastatic cascade. Cathepsin D was also shown to be able to degrade *in vitro* the extracellular matrix, and to activate latent precursor forms of other proteinases involved in the invasive steps of the metastatic process.

Interferon α-2b (IFNα) initially recognized for its antiviral effects, has also been shown to have antiproliferative, immunoregulatory and antitumor activities.[6] There is some experimental evidence supporting the concept that modest levels of hyperthermia might be beneficial to the action of interferons. This concept is supported by *in vivo* experiments showing that modest levels of hyperthermia enhanced the action of interferon.[7] *In vitro* it was found that hyperthermia acted synergistically by enhancing the proliferative effects of IFNα.[8] There is definitive experimental evidence *in vivo* for synergistic effect of this combined treatment.[9] Similar synergistic effects were also observed, when interferon was applied in combination with irradiation.[10]

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Such results may have clinical importance, because they suggest that hyperthermia could be used in combination with IFNα to provide a synergistically enhanced antitumor action.[9, 10] Therefore, it is possible that the combination of hyperthermia and IFNα therapy may have clinical application in cases when technically feasilbe. Such combined treatment can, however, induce an increase in proteolitic enzymes in cells, which might enhance metastasing of the treated tumor.

In this work we determined the changes in the quantity of the aspartic intracellular proteinase – cathepsin D in irradiated human nonmalignant (HEF) and in malignant (HEp2) cells after combined treatment with hyperthermia and/or IFNα. Correlation between the antiproliferative effect of these combined agents and the changes in concentrations of this cathepsin was found.

Materials and methods

Cell cultures and experimental procedure

Human embrional fibroblasts (HEF) and human laryngeal carcinoma (HEp2) cells, were cultured as monolayers in Eagle's minimal essential medium, supplemented with 10 % foetal calf serum. Cell cultures were prepared by plating 10^5 of 10^6 cells per Petri dish of 10 cm in diameter (3 dishes per experimental point) and after two days of growth, cells were irradiated, treated by IFNα and by hyperthermia in the following combinations: irradiation only, IFNα only, irradiation plus IFNα, irradiation plus IFNα plus hyperthermia.

Following the mentioned treatment, cell cultures (10^6 per dish) were kept at 37°C and samples taken after one hour were stored at –20°C until proteinase quantity assay. The number of proliferating cells (10^5 per dish) following irradiation and combined treatment were counted 24 and 96 hours after treatmen.

Interferon

Recombinant interferon α-2B (INTRON-A, Schering-Plough-Baltimore-USA) was added to the growth medium to reach final concentration of 1×10^4IU/ml. Cells were incubated in the IFNα-containing medium at 37°C for 1 hour and then incubated until additional treatment or harvesting.

Hyperthermia

Heat treatment was conducted by submerging the Petri dishes in a water bath at 44°C for 20 min. HEF and HEp2 cells were exposed simultaneoulsy to IFNα and to hyperthermia.

Irradiation

For gamma irradiation, a Gamma Cell 220 (Atomic Energy of Canada, αtd) unit was used. The dose rate was 4,13 Gy/min, with the total dose 15 Gy/sample for cathepsin D, or 5 Gy for the growth inhibing effect.

Cathepsin D concentration

Following treatment, cell cultures were incubated in the growth medium at 37°C. Samples were taken after different time intervals, placed on ice and washed three times with cold phosphate buffered saline. The cells were harvested by a rubber policeman, concentrated by centrifugation (10 min at 1000 rpm), lysed in distilled water and frozen at –20°C until assay.

The concentrations of cathepsin D were determined using specific enzyme immunometric assay (ELSA-CATH-D kit, CIS Bio International, Solid phase two-site immunoradiometric assay), for the quantitative determination of total cathepsin D in cytosol.

Results

The combined effects of IFNα, hyperthermia and irradiation on cell proliferation, expressed as the number of growing HEF cell population, are shown in Table 1. The antiproliferative effects were expressed as percentage of cell numbers in control samples. While irradiation or IFNα alone, moderately inhibited cell growth, the treatments, by IFNα plus irradia-

tion, and in particular, when IFNα was combined with irradiation and hyperthermia, the antiproliferative effect was markedly enhanced.

Table 1. Cell numbers (x10^5 HEF cells) after combined treatment with irradiation and/or interferon α and/or hyperthermia.

Group of treatment	Incubation time after treatment		Percent of control	
	24^h	96^h	24^h	96^h
Controls	10,9 ± 2,6	20,4 ± 3,1	–	–
Irradiation	5,0 ± 0,9	7,5 ± 1,1	46	37
Interferon α	6,6 ± 0,2	8,7 ± 1,7	61	43
Irradiaton + Interferon α	3,7 ± 0,1	4,0 ± 0,4	34	20
Irradiation + Interferon α + Hyperthermia	1,8±0,1	1,9 ± 0,2	16	9

The combined effects of IFNα, hyperthermia and irradiation on cell proliferation, expressed as the numbers of growing HEp2 cell population, are shown in Table 2. The antiproliferative effects were expressed as percentage of cell numbers in control samples. While irradiation or IFNα alone, moderately inhibited HEp2 cell growth, the treatmens by IFNα plus irradiation, and in particular when IFNα was combined with irradiation and hyperthermia, the antiproliferative effect was markedly enhanced.

Changes in the concentrations of cathepsin D measured in irradiated, proliferating human nonmalignant (HEF) and human malignant (HEp2) cell lines following combined treatment by interferon α and by hyperthermia are shown in Table 3.

Table 2. Cell numbers (x10^5 HEp2 cells) after combined treatment with irradiation and/or interferon α and/or hyperthermia.

Group of treatment	Incubation time after treatment		Percent of control	
	24^h	96^h	24^h	96^h
Controls	16,4 ± 1,4	41,0 ± 4,2	–	–
Irradiation	12,0 ± 0,9	18,6 ± 1,6	73	45
Interferon α	11,0 ± 2,1	21,8 ± 4,0	67	53
Irradiation + Interferon α	8,0 ± 1,1	10,0 ± 0,7	48	24
Irradiation + Interferon α + Hyperthermia	3,6 ± 0,8	7,7 ± 0,7	22	18

The changes in the concentrations of cathepsin D were dependent on the agent used. Gamma iradiation alone revealed little change in the concentration of cathepsin D in the malignant cell line (1.1), whereas the same changes were more evident in the nonmalignant cells (1.3). Interferon α increased the levels of cathepsin D in both cell strains. These effects are more expressed in malignant cells (1.5), than in nonmalignant cells (1.2). Interferon α plus irradiation increased the concentration of cathepsin D significantly more than in the former two cases, when the agents were applied separately. The increased concentrations of the enzyme were similar in malignant (1.6) as in nonmalignant (1.8) cells. Combined application of all three agents together was most effective in increasing the amounts of cathepsin tested. Most evident effects were achieved in both cell lines after combined treatment with all three agents and again, with more expressed effects

Table 3. Changes in the quantity of Cathepsin D in irradiated HEF and HEp2 cells following combined treatment.

Group of treatment	HEF cells		HEp2 cells	
	Cathepsin D ng/mg proteins	T/C*	Cathepsin D ng/mg proteins	T/C*
Controls	105 ± 11	–	562 ± 14	–
Irradiation	138 ± 7	1,3	604 ± 12	1,1
Interferon α	127 ± 9	1,2	875 ± 22	1,5
Irradiation + Interferon α	198 ± 26	1,8	856 ± 31	1,6
Irradiation + Interferon α + Hyperthermia	332 ± 16	3,1	1407 ± 53	2,5

* T/C = Treated/Control

on the nonmalignant HEF cell line (3.1), the in the malignant HEp2 cells (2.5).

Discussion

Cathepsin D, as mentioned before, plays an important role in tumor invasion and metastasis. Correlation between elevated levels of the enzyme in tumor cells and their ability to metastasise were found.[5] On the other hand in our previous experiments we found that agents used in tumor therapy can influence the concentrations of various intracellular proteolytic enzymes, either by increasing or decreasing their concentrations[11] or activities.[12] This raises the question, particularly when cathepsin D is concerned, whether a particular tumor treatment could perhaps, apart of its cell killing potential, have some unwanted effects due to possibly elevated levels of cathepsin D.

Our results show that such agents (irradiation, interferon α and heath), when given in amounts that evidently produce cell growth inhibition, can significantly increase the intracellular concentrations of cathepsin D, and therefore could consequently enhance the potential of the tumor cells to infiltrate the neighbouring tissues or to metastasise. If this occurs in a tumor bearing organism, this should also be kept in mind, when predicting the outcome of a particular tumor therapy. This may be even more important at combined modality therapy regimens.

Acknowledgement

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Radiol Oncol 1993; 27: 275-9.

Interactions of interferon and vinblastine on experimental tumor model melanoma B-16 *in vivo*

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In the study, we investigated the in vivo *interaction of two antitumor agents, that have different sites and different mechanisms of action. Vinblastine (VLB) in combination with human recombinant interferon α A/D (rHuIFN-α A/D) and in combination with human leukocyte interferon α (HuLIFN-α) was tested on intraperitoneal (i.p.) melanoma B-16 tumor model. The effect of the combination was determined with follow-up of animals' survival and the interaction defined by means of Spector's formula. Only subadditive enhancement of interferon's (IFN's) antitumor activity was observed when rHuIFN-α A/D was combined with VLB and supraadditive, but not synergistic, interaction when HuLIFN-α was combined with VLB. Synergism between VLB and rHuIFN-α A/D on B-16 melanoma* in vitro, *that had been observed in our previous study, did not come true* in vivo.

Key words: melanoma, experimental-drug therapy; vinblastine; interferon alpha, recombinant;

Introduction

Chemotherapy and biotherapy are the two systemic modalities available for cancer treatment. However, because it is apparent that neither one nor the other are perfect treatments for cancer, the combination of cytotoxic drugs and cytokines offers a new approach to increase the therapeutic index in the treatment of neoplastic diseases.[1,2]

Interferons (IFNs) are a complex group of cytokines with antiviral, antibacterial, antitumor and immunomodulatory activities.[3,4] They exert antiproliferative effect on tumor cells, while IFNs β and γ also have a direct cytotoxic activity.[5] Antitumor activity of VLB is a consequence of its binding to microtubular proteins of the mitotic spindle, which causes metaphase arrest of cells in mitosis.[6,7] VLB is, in higher concentrations, also directly cytotoxic for interphase cells.[8]

While *in vitro* studies have demonstrated both direct cytotoxic and cytokinetic effects of IFNs, a more interesting role derives from their ability to sinergistically potentiate the wide variety of cytotoxic agents against multiple human and rodent tumors, both *in vitro* and in animal models.[9] The broad spectrum of cytotoxic drugs whose activity can be enhanced by cytokines argues for multiple levels of drug interaction *in*

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vitro: alteration of cellular drug uptake, modulation of drug target enzymes, and changes in metabolism or disposition of a drug. *In vivo* interaction between cytokines and cytotoxic drugs involves an additional layer of complexity because of the effects of cytokines on the host immune system and on drug-metabolising enzymes.[2]

The ability of IFNs to directly modulate the biochemical effects of cytotoxic agents independent of immunomediated or host-protective effects has been evaluated in a variety of *in vitro* systems.[9] Since synergistic cytotoxicity has been observed *in vitro* for IFN-α combination with VLB on BG-1 human ovarian carcinoma line,[10] on RPMI 8226 human myeloma line, on MCF-7 human breast carcinoma line, on WiDr human colon carcinoma line[11] and on murine B-16 melanoma line,[12] we wanted to define the interaction of VLB with rHuIFN-α A/D or HuLIFN-α *in vivo* on i.p. B-16 melanoma tumor model.

Materials and methods

Reagents

Recombinant HuIFN-α A/D was provided by Hoffmann-LaRoche (Nutley, New Jersey) and HuLIFN-α by Immunological Institute (Zagreb, Croatia). Both were diluted with phosphate buffered saline (PBS).

Vinblastine sulfate (Lymphomed, Deerfield, Illinois) was used in combination with rHuIFN-α A/D and Velbe (Lilly, Firenze, Italy) with HuLIFN-α. Both were diluted with PBS.

Animals

Six to eight weeks old pathogen-free female C57Bl/6 mice were purchased from Jackson Laboratories, Bar Harbor, USA. Animals were maintained in a pathogen-free state in animal rooms with alternating cycles of 12 h light and 12 h darkness. Each experimental group consisted of 10 to 11 mice. These animals were used for experiments with rHuIFN-α A/D and Vinblastine sulfate.

Female C57Bl/6 were purchased from Rudjer Bošković Institute, Zagreb, Croatia. Animals were maintained at a natural day/night cycle in a standard animal colony. Eight to ten weeks old mice in good condition without any signs of fungal or other infections were used in the experiments. Each experimental group consisted of ten mice. These animals were used for experiments with HuLIFN-α and Velbe.

Tumor cells

Murine B-16 melanoma cells (clone F1, American Type Culture Collection, Rockville, Maryland) were grown in Eagle's Minimal Essential Medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and gentamycin (11 μg/ml). These cells were used for experiments with rHuIFN-α A/D and Vinblastine sulfate.

Murine B-16 melanoma cells (clone B6, Rudjer Bošković Institute, Zagreb, Croatia) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, gentamycin (500 μg/ml) and 7,5% sodium bicarbonate (27 ml/l). These cells were used for experiments with HuLIFN-α and Velbe.

Experimental procedure

Mice were i.p. injected with 10^6 B-16 melanoma (clone F1 or B6) cells on day 0 and randomly divided into four groups. All treatment was intraperitoneal and was administered as follows:

– control group - PBS for five consecutive days, starting day 1

– vinblastine group - Vinblastine sulfate or Velbe (30 μg per animal) on day 4 only

– interferon group - rHuIFN-α A/D (1×10^5 I.U. per animal) or HuLIFN-α (5×10^5 I.U. per animal) for five consecutive days, starting day 1

– combination group - Vinblastine sulfate or Velbe (30 μg per animal) on day 4 only + rHuIFN-α A/D (1×10^5 I.U. per animal) or HuLIFN-α (5×10^5 I.U. per animal) for five consecutive days, starting day 1.

The mice were monitored for the day of death and the average day of death was determined.

Statistical analysis

The Mantel - Cox test (BMDP Statistical Software, Los Angeles, California) was employed for comparison of the animals' survival and Spector's formula[13] to define the interaction of rHuIFN-α A/D or HuLIFN-α with VLB.

Results

Vinblastine sulfate and rHuIFN-α A/D as single agents or in combination were tested for their effect on survival of animals with i.p. B-16 melanoma (F1). Intraperitoneal application of 30 μg of Vinblastine sulfate on day 4 had a moderate ($p = 0{,}054$) antitumor effect, while treatment with 1 x 10^5 I.U. of rHuIFN-α A/D for 5 consecutive days showed a more pronounced statistically significant ($p<0{,}001$) effect on survival of mice with i.p. B-16 melanoma. The combination of both agents had a statistically significant ($p<0{,}001$) antitumor effect, but there was no significant difference ($p = 0{,}497$) in survival between the "interferon group" and the "combination group" of animals (Figure 1). According to Spector's formula the interaction of rHuIFN-α A/D with VLB was merely subadditive and the antitumor effect of the combination was 90% of the one expected on the basis of their separate activities.

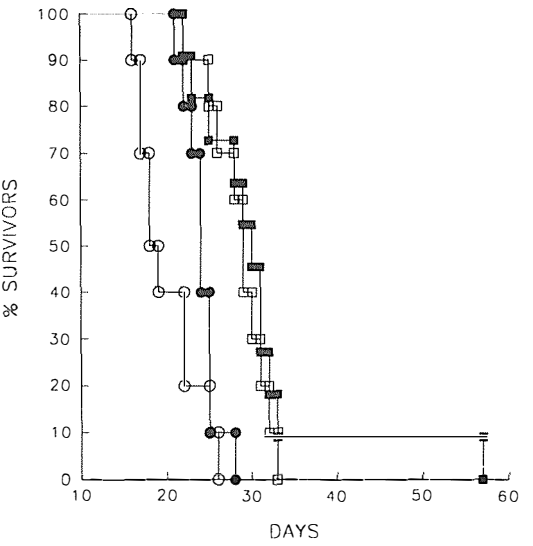

Figure 1. Survival of mice with i.p. B-16 melanoma (clone F1) treated with Vinblastine sulfate (●), rHuIFN-α A/D (□) or combination of both agents (■); control (○). The antitumor effect of the combination was merely subadditive in comparison to the one expected on the basis of separate activities of VLB or IFN.

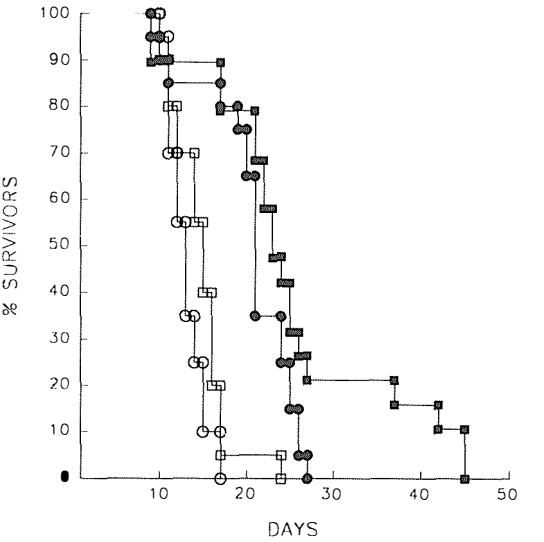

Figure 2. Survival of mice with i.p. B-16 melanoma (clone B6) treated with Velbe (●), HuLIFN-α (□) or combination of both agents (■); control (○). The effect of the combination on survival of the animals was supraadditive in comparison to the one expected on the basis of separate activities of VLB or IFN.

Survival of animals with i.p. B-16 melanoma (B6) treated with HuLIFN-α or Velbe alone or in combination is presented in Figure 2. Treatment with 5 x 10^5 I.U. of HuLIFN-α for five consecutive days had a moderate ($p = 0{,}059$) antitumor effect, while i.p. application of 30 μg of Velbe significantly ($p<0{,}001$) prolonged survival of the animals. The combination of both agents had a statistically significant ($p<0{,}001$) antitumor effect, but there was no significant difference ($p = 0{,}058$) in survival between the "vinblastine group" and the "combination group" of animals. The interaction of HuLIFN-α with VLB was supraadditive and the antitu-

mor effect of the combination was 115% of the one expected on the basis of their separate activities.

Discussion

The results of *in vivo* studies testing the combination of IFNs with VLB are controversial. Sidkey *et al.* report that murine IFN α/β increased survival in mice with P388 leukemia cells after treatment with VLB.[14] Harrison *et al.* on the other hand found no positive interaction when murine IFN α/β was combined with VLB on s.c. Meth A sarcoma tumor model and when recombinant murine IFN-γ was combined with VLB on s.c. Meth A sarcoma and s.c. B-16 melanoma.[15] Also Mitchell has pointed out that although type I IFN has been found to potentiate chemotherapy in cultured cells, "there is very little substantiation *in vivo*".[16]

Our results clearly demonstrate that *in vitro* synergism between rHuIFN-α A/D and VLB observed on F1 clone of B-16 melanoma cells[12] did not come true *in vivo*. However, there is an interesting difference between the antitumor activity of combination of rHuIFN-α A/D with VLB and combination of HuLIFN-α with VLB. Even though HuLIFN-α alone has only moderate antitumor activity, the interaction with VLB was supraadditive, in comparison with rHuIFN-α A/D that has significant antitumor activity, but demonstrated only subadditive interaction with VLB. In part this difference could be explained with the fact that different clones of B-16 melanoma were used in the experiments, but Sklarin *et al.* report that in most of the cases where potentiation was observed, human IFN-α alone had only weak antitumor activity; however, IFN-α seemed to be most effective in combination with drugs that alone possessed substantial activity against the specific tumor.[17]

The question of the mechanism of interaction between IFNs and VLB still cannot be resolved and it seems likely that multiple factors may be contributing to success or failure in these preclinical models. The interactions observed are not solely the consequence of the combined effect of two cytoreductive agents, since the enhanced activity of the drug-interferon combination was observed even in instances where IFNs alone lacked activity, and IFNs also failed to potentiate the activity of other efficacious drugs.[9] There is also a complex relationship between the timing of interferon (IFN) with a cytotoxic agent, the doses used, and the efficacy of the regimen. In combination with cytotoxic drug, sequence and duration of exposure to IFN may play as significant a role as dose and dose intensity, and the maximum tolerated dose of IFN may not be the most biologically effective dose. Up till now, the lack of understanding of the biochemical interaction of these agents has prohibited a rational approach to design of schedule and sequence that allow translation of the positive *in vitro* data into effective preclinical treatment regimens.

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Combined treatment of murine SA-1 tumors by human leukocyte interferon alpha and electrotherapy

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Antitumor effectiveness of human leukocyte interferon alpha (IFN-α) was assessed in combination with electrotherapy. Subcutaneous fibrosarcoma SA-1 tumors were treated with IFN-α for five consecutive days. The results indicate that IFN-α given either locally (peritumorally) or systemically (intraperitoneally) as a single treatment has moderate antitumor effect. In order to potentiate its effectiveness, IFN-α was combined with electrotherapy. Low level direct current ranging from 0.2 to 1.2 mA for 60 minutes was delivered via Platinum/Iridium electrodes placed subcutaneously, outside the tumor. Combined treatment with electrotherapy and IFN-α given intraperitoneally, proved to have more than additive antitumor effect, assessed by tumor growth delay. Interaction between the two treatment modalities increased at higher current levels used for electrotherapy. The results indicate that IFN-α and electrotherapy interact in local tumor growth control. Therefore, electrotherapy can be used to locally potentiate systemic IFN-α treatment or vice versa, the latter agent can potentiate the effect of electrotherapy.

Key words: fibrosarcoma-therapy; interferon alpha; electric stimulation therapy; mice

Introduction

Interferons (IFN-s) are the members of a big family of regulatory cytokines.[1] These molecules control the growth and differentiation of many cells in the organism. By positive and negative feedback loops they interact with growth factors, oncogenes and other regulatory molecules.[2] Studies on IFN-s have yielded an expanding list of bioactivities; besides anti-viral and microbicidal action, antitumor effectiveness has drawn much of attention.[3, 4, 5] Interferons exert antiproliferative effect on a number of malignant cells, have transformation-suppressing effect and can regulate their differentiation.[2, 5, 6] Also, immunoregulatory effect of IFN-s is very important, which has put these agents in place of immunoadjuvant settings.[7] In clinical trials IFN-s have shown significant activity against a wide range of human cancers. Hematological disorders proved to be the most responsive to IFN-α treatment, contrary to solid tumors, where response rates seldom exceed 20–30 %.[8] From the vast experience it is evident that treatment in low tumor burden is more effective than in advanced, bulky disease. In this respect combination with other cytotoxic

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treatments is possible, since they can reduce tumor burden and interact with IFN-s treatment.

One of the treatment modalities, which has recently proved effective in reducing tumor burden is electrotherapy.[9] It is effective as an anti-tumor agent which has been demonstrated on several tumor models as well as in clinic.[10-13] Application of electrotherapy is foreseen predominantly in combination with biological or cytotoxic treatments.[13-15] In our preliminary study combined treatment with human leukocyte IFN-α and electrotherapy demonstrated some positive interactions.[16] In the present study electrotherapy with electrodes placed outside the tumor, in order to avoid mechanical intrusion (field electrotherapy)[17] was combined with IFN-α treatment. Interaction of the two treatments was evaluated by tumor growth delay, according to the route of IFN-α treatment and direct current levels used for electrotherapy.

Materials and methods

Cell cultures

Fibrosarcoma SA-1 cell were grown in tissue culture flasks at 37° C in a humidified 5 % CO_2 atmosphere, using Eagle's MEM supplemented with 10 % fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml). To study the effect of IFN-α on cell growth, cells in petri dishes were treated continuously with 1, 5, 10 and 20×10^3 U IFN-α/ml. Three days after treatment viable cells in the cell cultures were counted in hemocytometer and the cell number/control (%) ratio was calculated. The statistical evaluation was done by means of Student-t test.

Animals

Female and male inbred A/J mice were purchased from the Institute Rudjer Bošković, Zagreb, Croatia. Animals were maintained in conventional animal colony at constant room temperature 24° C at natural day/night light cycle. Mice in good condition, without signs of fungal or other infection, eight to ten weeks old were included in the experiments. Experimental groups consisted of 8-10 animals.

Tumors

As a tumor model fibrosarcoma (SA-1) syngeneic to A/J mice was used. Single tumor cell suspension was obtained from an ascitic form of the tumor. Solid subcutaneous tumors, dorsolaterally in animals, were initiated by injection of 5×10^5 viable SA-1 cells. When the tumors reached 30–40 mm^3 in volume, animals were marked individually and on day 0 randomly divided into smaller groups, subjected to specific experimental protocol. On each consecutive day the tumor volume was calculated from the three mutually orthogonal diameters measured by vernier caliper gauge. Arithmetic mean (AM) and standard error of the mean (SE) were calculated for each day in all experimental groups. Tumor doubling time (DT) was determined for individual tumors and tumor growth delay calculated (GD) from mean DT of experimental groups.[14] The differences between the experimental groups were evaluated statistically by nonparametric Mann-Whitney Rank-Sum test, taking into account the Bonferoni adjustment when multiple comparisons were performed.

Electrotherapy

The direct current (DC) source for electrotherapy was designed and manufactured at the Faculty of Electrical and Computer Engineering, Ljubljana, Slovenia. Current and voltage were continuously monitored during electrotherapy with 0.2, 0.4, 0.6, 0.8 or 1.2 mA DC of one hour duration. Current was delivered through Pt/Ir (90/10 %) alloy needle electrodes (1.0 mm diameter, 22.0 mm long) with rounded tips and inserted subcutaneously 5–10 mm from the margin of the tumor on the two opposite sites.[17] The control group was treated in the same way as experimental groups, except that no current flowed.

Lymphokines and therapy protocol

Partially purified human leukocyte interferon alpha (IFN-α) was purchased from Immunological Institute, Zagreb, Croatia.[18] Animals were treated with 5×10^4 U IFN-α daily, for five consecutive days, starting one hour before electrotherapy. Therapy was performed either peritumorally with 0.1 ml IFN-α injected subcutaneously in the vicinity of the tumor, with precaution not to damage tumor capsule, or intraperitoneally with IFN-α dissolved in 0.5 ml phosphate buffer saline (PBS).

Results

Antitumor effectiveness of IFN-α was tested on fibrosarcoma SA-1 *in vitro* and *in vivo*. The effect of IFN-α *in vitro* on the growth of SA-1 cells is presented in Figure 1. Lower concentrations seemed to enhance tumor cell proliferation, but the effect was not statistically significant. At the highest concentration (2×10^4 U/ml) moderate antiproliferative effect was demonstrated which was statistically significant ($p < 0.05$).

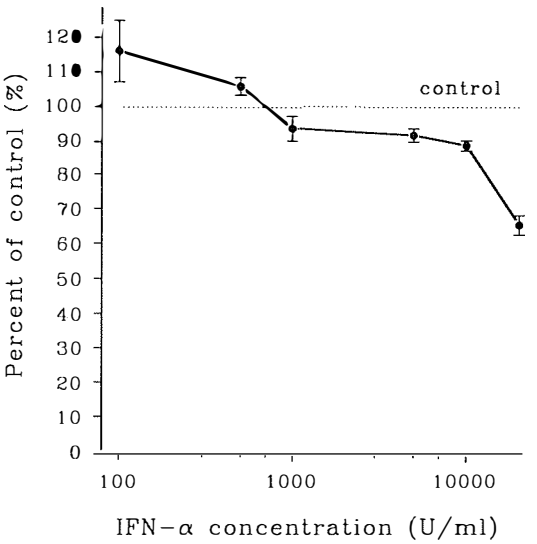

Figure 1. Effect of IFN-α on the growth of SA-1 cells *in vitro*. Cells were grown in different IFN-α concentrations for three days and thereafter their growth rate was determined.

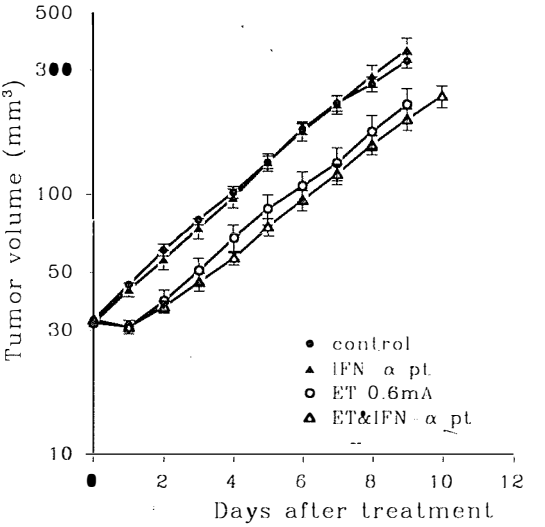

Figure 2. Antitumor effect of IFN-α and electrotherapy on subcutaneous SA-1 tumors. IFN-α (5×10^4 U) was injected peritumorally five consecutive days, starting on day 0. Electrotherapy was performed with 0.6 mA one hour after IFN-α treatment on day 0. Experimental groups comprised 8–10 animals.

Anti-tumor effect of IFN-α was tested also on SA-1 tumors *in vivo*. Solid subcutaneous SA-1 tumors were treated for five consecutive days with 5×10^4 U IFN-α daily. Different routes of IFN-α administration were tested; i.e. intraperitoneal and peritumoral application. Both, peritumoral (GD = 0.4 ± 0.2 days) and intraperitoneal (GD = 0.6 ± 0.3 days) treatment did not significantly delay tumor growth ($p > 0.05$) (Figure 2, 3). Also, there was no statistical difference between the effectiveness of IFN-α after peritumoral and intraperitoneal application ($p = 0.6$).

In order to test for interaction of IFN-α treatment with electrotherapy, both treatment modalities were combined. Electrotherapy (0.6 mA for 1 hour) as a single treatment statistically significantly delayed tumor growth ($P < 0.001$) (Figure 2, 3). In combined modality treatment electrotherapy was performed one hour after the first IFN-α application. The interaction was better when electrotherapy was combined with intraperitoneal IFN-α treatment than with peritumoral application (Figure 2, 3). Additive antitumor effect was obtained with peritumoral

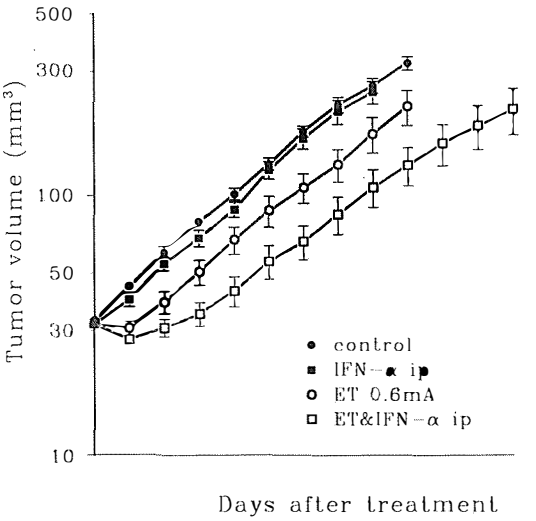

Figure 3. Antitumor effect of IFN-α and electrotherapy on subcutaneous SA-1 tumors. IFN-α (5×10^4 U) was injected intraperitoneally five consecutive days, starting on day 0. Electrotherapy was performed with 0.6 mA one hour after IFN-α treatment on day 0. Experimental groups comprised 8-10 animals.

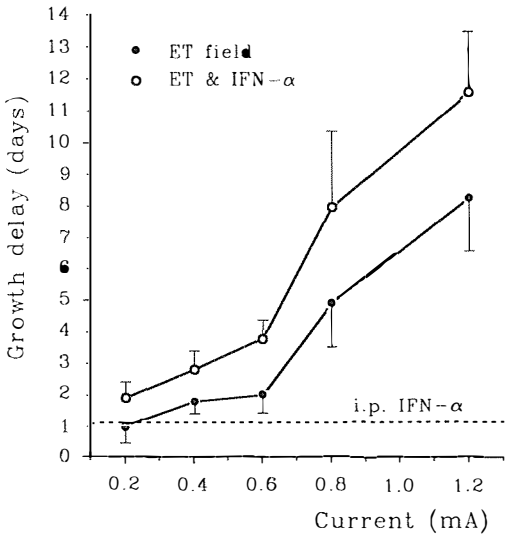

Figure 4. Tumor growth delay after electrotherapy or combined modality treatment with electrotherapy and IFN-α. Electrotherapy was performed with different current levels one hour after IFN-α treatment on day 0. The data are presented as arithmetic means and standard deviations of the mean. The tumor growth delay after treatment with IFN-α alone is presented as an average tumor growth delay. Experimental groups consisted of 10 animals.

treatment and more than additive with intraperitoneal treatment.

To determine how IFN-α therapy interacts with escalating electrotherapy doses, combined modality treatment was performed with different direct current levels, ranging from 0.2 mA to 1.2 mA. IFN-α treatment schedule remained the same as in the previous experiment. Relationship between the effectiveness of therapy, presented as tumor growth delay, in relation to electrotherapy at different current levels, is presented in Figure 4. The interaction of IFN-α with electrotherapy was additive up to 0.4 mA and more than additive from 0.6 mA on (Table 1).

Table 1. Tumor growth delay after electrotherapy (ET) alone or in combination with IFN-α.

	Growth Delay (days) ET only	ET + IFN-α[1]
ET 0.2 mA	1.0 ± 0.5 ($\upsilon = 15$)[2]	1.9 ± 0.5 ($\upsilon = 15$)
ET 0.4 mA	1.8 ± 0.4 ($\upsilon = 15$)	2.8 ± 0.6 ($\upsilon = 16$)
ET 0.6 mA	2.0 ± 0.6 ($\upsilon = 18$)	3.8 ± 0.6 ($\upsilon = 18$)
ET 0.8 mA	4.9 ± 1.4 ($\upsilon = 14$)	8.0 ± 2.4 ($\upsilon = 16$)
ET 1.2 mA	8.3 ± 1.7 ($\upsilon = 15$)	11.6 ± 1.9 ($\upsilon = 16$)

[1] Tumor growth delay after intraperitoneal IFN-α treatment was 1.1 ± 0.5 ($\upsilon = 15$)
[2] Tumor growth delay in days (AM ± SD), υ degree of freedom

Discussion

The study shows that electrotherapy and IFN-α treatment interact in control of fibrosarcoma SA-1 tumor growth. More than additive antitumor effect was obtained when electrotherapy was combined with intraperitoneal IFN-α treatment. The interaction between the two treatment modalities increased by escalating current levels.

Electrotherapy is a new treatment modality used in local control of tumor growth.[9, 11, 19] Its antitumor mechanisms are probably multiple: biochemical reactions in the vicinity of the electrodes and influences of electric current directly on tumor cells.[17, 20, 21] Among biochemical reactions are changes of pH and changes of ion composition in extra cellular matrix which all exert influence on cell growth and

survival.[17] Effectiveness of electrotherapy is predominantly dependent on electric current intensity.[11, 19] With currents 1.8 mA a growth delay of approximately 12 days can be achieved on SA-1 tumors, while on B-16 melanoma tumor model even tumor cures can be induced.[19] Nevertheless, after the treatment viable tumor cells remain, which again give rise to a tumor. In order to potentiate effectiveness of electrotherapy, and eradicate the remaining tumor cells, attempts were made to combine electrotherapy with radiotherapy,[22] chemotherapy.[13, 15] and biological response modifiers.[14, 16, 23] In most cases additive or supra-additive effects were obtained.

Our interest was focused on combinations of electrotherapy with biological response modifiers. The studies combining interleukin-2 (IL-2),[14] tumor necrosis factor alpha (TNF-α)[23] and human leukocyte interferon alpha (IFN-α)[16]demonstrated that stimulation of host's defence mechanisms contributes to antitumor effectiveness of electrotherapy. Depending on the biological response modifier used, different arms of the cytokine network are stimulated, but in all cases the effectiveness of electrotherapy was increased.

In our preliminary study we have already tested the combined modality treatment of human leukocyte interferon alpha (IFN-α) with electrotherapy on SA-1 tumor model.[16] In that study IFN-α treatment protocol was the same as in the present study, however, electrotherapy protocol was different. Repetitive electrotherapy treatment was not very effective, therefore, according to later experience we applied the "field" electrotherapy as a single treatment.[17] As demonstrated in the present study, the effect is dose dependent resulting in a moderate antitumor effect at 0.2mA current level, and a significant growth delay at 1.2 mA. Comparison of the IFN-α antitumor effects according to the route of application demonstrated that IFN-α is moderately effective at the doses used. No difference in the antitumor effectiveness of IFN-α was observed, given either locally or systemically. But when combined with cytoreductive electrotherapy, systemic treatment was more effective than local treatment. Although IFN-α was demonstrated to be cytostatic to SA-1 cells *in vitro,* it is very unlikely to reach sufficiently high concentrations in the tumor to exert such an effect, when injected locally or systemically *in vivo.* Therefore, enhancement of the antitumor mechanisms of the organism must be contributing to the supra-additive effect of electrotherapy combined with systemic IFN-α treatment.

The interaction of IFN-α treatment with electrotherapy was dependent on antitumor effectiveness of electrotherapy. With escalating electrotherapy doses also combined modality treatment was more effective. This demonstrates that adjuvant IFN-α treatment was more effective on a smaller tumor burden. The doses used in both treatment modalities were low and no treatment related side effects were observed.

Our study shows that IFN-α and electrotherapy interact in antitumor effectiveness on fibrosarcoma in mice. Combined use of IFN-α and electrotherapy resulted in effective tumor control. Thus, electrotherapy can be used to locally potentiate systemic IFN-α treatment. Further studies are required for possible implementation of the investigated treatment approach in clinical practice.

Acknowledgment

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Antitumor effect of interferon-α administered by different routes of treatment

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Besides the fact that interferons were identified as factors capable of inhibiting viral infections, they have proved to be antiproliferative, immunomodulatory and differentiation-inducing factors. On the basis of these activities, they have been employed clinically for treatment of various tumors. The study was performed to determine whether there was different antitumor effect of recombinant human interferon-α A/D (rHuIFN-α A/D) when it was given as a local or systemic therapeutical agent. Two different tumor models, i.e. subcutaneous (s.c.) and intraperitoneal (i.p.) B-16 melanoma on C57Bl/6 mice, were employed in these experiments. Experimental mice were treated locally or systemically with different doses of rHuIFN-α A/D; the treatment was begun 24 hours after tumor cell inoculation and continued through five consecutive days. Intraperitoneal treatment of animals with i.p. tumors resulted in significantly longer survival time in comparison with control group or with subcutaneously treated animals ($p<0.001$). Similarly, the delay of tumor detection and tumor growth in mice with s.c. tumors treated subcutaneously with rHuIFN-α A/D was significantly greater than in intraperitoneally treated animals ($p<0.01$). According to these results we can conclude that rHuIFN-α A/D is much more potent antitumor agent when it is used locally. However, systemic treatment with higher doses was effective in both tumor models and it is still more convenient for treatment of some tumor lesions which are not accessible for local treatment.

Key words: melanoma, experimental-drug therapy; interferon-alpha; drug administration routes

Introduction

Interferons are glycoproteins which were identified as factors capable of inhibiting viral infections.[1, 2] Besides, interferons have proved to be antiproliferative, immunomodulatory and differentiation inducing factors.[3, 4, 5] Other putative functions include antioncogene activity and mobilisation of energy stores during sickness.[6,7]

Three subtypes of interferons (IFN α, β and γ) have been identified, differing in terms of their cell surface receptors, their acid stability, their primary sequence and their chromosomal location and organisation.[3, 8] Interferon-α and interferon-β produced by leukocytes and fibroblasts, respectively, are acid stable and share the same receptor, while interferon-γ is produ-

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ced by T lymphocytes, is acid labile and has a different receptor.[4, 8]

The precise mechanisms of action for the antitumor effects of interferons are not fully explained. They involve both direct (antiproliferative effects, cytotoxic effects and enhancement of cell surface antigen expression on tumor cells) and indirect antitumor action (activation of macrophages/monocytes, activation of T cells, activation of NK cells and modulation of antibody production).[9, 10]

More then 20 subtypes of interferon-α are known, but only few of them are used systemically or locally in the treatment of neoplasms as hairy cell leukemia, AIDS - related Kaposi sarcoma, Hodgkin's disease, non - Hodgkin's lymphomas, oral cancer, malignant melanoma, renal cell carcinoma and bladder cancer.[11-16]

In our experiments we investigated the relative capability of local versus systemic treatment with rHuIFN-α A/D as an antitumor agent against B-16 melanoma. To address this question we used two different tumor models: i.p. and s.c. B-16 melanoma tumors.

Materials and methods

Animals

Six to eight weeks old female C57Bl/6 mice were used in the experiments. Mice were purchased from Jackson Laboratories (Bar Harbor, USA) and held in a pathogen free animal colony. The adaptation period before use was two to three weeks. At least nine healthy animals with normal body weight were included in each experimental group.

Tumor models

Subcutaneous (s.c.) and intraperitoneal (i.p.) tumors were employed as tumor models. Subcutaneous tumors were induced subcutaneously in the left lower abdomen with 10^6 B-16 melanoma cells in 0.1 ml EMEM (Eagle's minimal essential medium) supplemented with 2% fetal calf serum (FCS), while mice for i.p. tumors were inoculated with the same number of viable cells intraperitoneally. In the experiments with s.c. tumors the day of tumor detection was monitored and tumor growth was followed by measuring two tumor diameters with a vernier caliper. The tumor burden was calculated by the standard formula for a prolate sphere $V = \pi/6 \times d_1 \times d_2^2$ ($d_2 < d_1$). Mice with i.p. tumors were monitored for the day of death and the increased life span (ILS) was calculated. Also, mice with s.c. tumors were monitored for the day of tumor development and the increased tumor detection span (ITDS) was determined as shown below.

$$\text{ILS} = \frac{\text{av. day of death for IFN treated mice - av. day of death for control}}{\text{average day of death for control}} \times 100$$

$$\text{ITDS} = \frac{\text{av. day of tu. det. for IFN treated mice - av. day of tu. det. for control}}{\text{average day of tumor detection for control}} \times 100$$

Tumor cells

Murine B-16 melanoma cells (clone F1)[17] were grown in EMEM supplemented with 10% FCS, penicillin (100 units/ml), streptomycin (100 μg/ml) and gentamycin (11 μg/ml). The final cell suspension for inoculation (10^6 viable tumor cells per 0.1 ml) was prepared with EMEM supplemented with 2% FCS and antibiotics as indicated above.

Interferon

Recombinant human interferon-α A/D (rHuIFN-α A/D) used in this study was generously provided by Dr. Michael Brunda of Hoffman-La Roche (Nutley, New Jersey) and had a specific activity of 6.4×10^7 U/mg of protein. Recombinant human IFN-α A/D is a recombinant molecular hybrid of two subtypes of HuIFN-α which exerts antiviral, antitumor and myelotoxic activities in mice.[18,19] The interferon was diluted in phosphate buffered saline (PBS) supplemented with 0.3% bovine serum albumin (BSA, Sigma Chemical Company) and frozen at -70°C until used for treatment.

Treatment

Interferon treatment was started 24 hours after tumor cell inoculation and continued daily through five consecutive days. Animals with i.p. tumors were treated with different IFN doses (3000 U/0.2ml, 10000 U/0.2ml or 30000 U/0.2ml per animal per day) intraperitoneally (locally) or subcutaneously (systemically). Subcutaneous tumor bearing animals were treated with the same doses as mentioned above, but in this case subcutaneous treatment was performed as local and intraperitoneal as systemic treatment. The animals in the control group were injected subcutaneously or intraperitoneally with 0.2 ml of PBS supplemented with 0.3 % BSA.

Statistical analysis

The data were evaluated for significance using Student's T-test.

Results

Experiments were performed to determine whether the antitumor effect of rHuIFN-α A/D was different when the agent was administered locally or systemically. Two different tumor models were employed: s.c. B-16 melanoma and i.p. B-16 melanoma.

Antitumor effect on s.c. tumors

Mice were inoculated s.c. with B-16 melanoma tumor cells and randomly divided in eight groups:

– control group treated subcutaneously with PBS/BSA;

– control group treated intraperitoneally with PBS/BSA;

– group treated subcutaneously with 3000 U (3 KU) of rHuIFN-α A/D;

– group treated intraperitoneally with 3000 U (3 KU) of rHuIFN-α A/D;

– group treated subcutaneously with 10000 U (10 KU) of rHuIFN-α A/D;

– group treated intraperitoneally with 10000 U (10 KU) of rHuIFN-α A/D;

– group treated subcutaneously with 30000 U (30 KU) of rHuIFN-α A/D and

– group treated intraperitoneally with 30000 U (30 KU) of rHuIFN-α A/D.

Table 1. Average day of tumor detection for s.c. B-16 melanoma bearing mice treated subcutaneously or intraperitonealy with rHuIFN-α A/D.

	Average day of tumor detection	SD*	p-value (comparing to the control)	p-value (comparing the same doses)
i.p. control	9.9	2.1	-----	-----
i.p. 3 KU	12.7	2.9	0.0039	-----
i.p. 10 KU	12.2	2.5	0.0087	-----
i.p. 30 KU	14.7	4.8	0.001	-----
s.c. control	10.4	2.2	-----	-----
s.c. 3 KU	16.9	5.2	0.0001	0.005
s.c. 10 KU	19.9	3.9	0.0001	0.0001
s.c. 30 KU	20.2	3.8	0.0001	0.001

*SD - Standard deviation

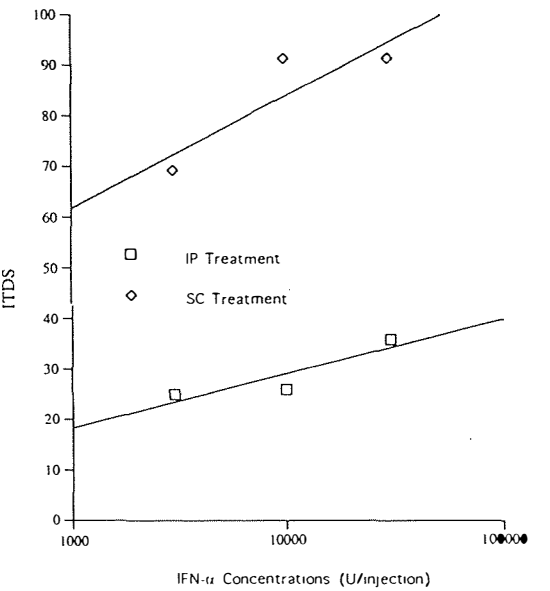

Figure 1. Increase in tumor detection span (ITDS) for subcutaneous B-16 melanoma tumors. Tumors were implanted using 10^6 viable tumor cells, and 24 hours later treated subcutaneously or intraperitoneally with different concentrations of rHuIFN-α A/D during five consecutive days.

Starting 24 hours after tumor cell inoculation and continuing for five days, mice were injected s.c. or i.p. with either rHuIFN-α A/D (different concentrations) or PBS/BSA.

The day of tumor detection and tumor growth were monitored. Table 1 presents the average results of two identical experiments that gave similar results. Both subcutaneous (local) and intraperitoneal (systemic) treatments caused a significant antitumor effect at all interferon concentrations employed. However, it can be seen that local treatment was more effective than systemic treatment.

Locally treated mice with 3 KU of rHuIFN-α A/D developed tumors in 16.9 days on average, with 10 KU in 19.9 days, and with 30 KU in 20.2 days; these periods being 62.5%, 91.3% and 94.2% longer than those in the control group (Table 1, Figure 1).

Systemically treated mice with 3 KU developed tumors in 12.7 days in average, with 10 KU in 12.2 days, and with 30 KU in 14.7 days; those periods being 28.3%, 23.2% and 48.5% longer than those in the control group (Table 1, Figure 1). Tumor growth kinetics was the same as in the control group, while local treatment slowed down the tumor growth in all treated groups (Figure 2).

Statistically significant differences in the day of tumor detection were observed between the two routes of interferon administration for all treatment doses ($p = 0.005$ with 3 KU, $p = 0.0001$ for 10 KU, and $p = 0.001$ for 30 KU of rHuIFN-α A/D).

Antitumor effects on i.p. tumors

It was important to consider previous data from s.c. tumor model in order to asses whether the differential responsiveness of the tumors would be observed on i.p. tumor model after different routes of treatment with rHuIFN-α A/D. To address this point, mice were inoculated i.p. with B-16 melanoma tumor cells and randomly distributed (as mice with s.c. tumors), into eight groups. Mice were also treated locally (intraperitoneally) and systemically (subcutaneously) with different doses of rHuIFN-α A/D or PBS/BSA. Treatment schedule was the same as the one described above for s.c. tumors. Mice were monitored for the day of death.

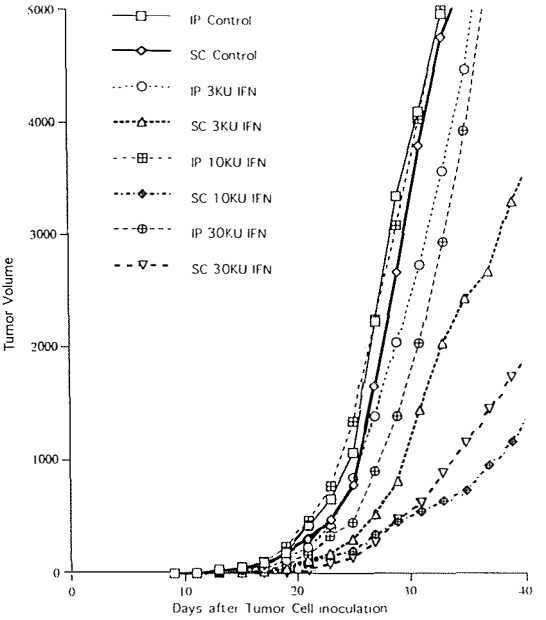

Figure 2. Growth kinetics of subcutaneous B-16 melanoma implanted in the left lower abdomen using 10^6 viable tumor cells, and treated subcutaneously or intraperitoneally with rHuIFN-α A/D.

Table 2 presents the average results of two identical experiments which gave similar results. It can be seen that also in i.p. tumors local treatment was more effective than systemic

Table 2. Average day of death for i.p. B-16 melanoma bearing mice treated subcutaneously or intraperitonealy with rHuIFN-α A/D.

	Average day of death	SD*	p-value (comparing to the control)	p-value (comparing the same doses)
s.c. control	19.9	1.8	-----	-----
s.c. 3 KU	19.6	1.7	0.5688	-----
s.c. 10 KU	20.9	1.7	0.0714	-----
s.c. 30 KU	21.5	1.8	0.0098	-----
i.p. control	19.3	1.3	-----	-----
i.p. 3 KU	22.6	2.5	0.0001	0.0001
i.p. 10 KU	23.3	1.9	0.0001	0.0004
i.p. 30 KU	24.8	3.1	0.0001	0.0002

*SD - Standard deviation

treatment (Table 2, Figure 3). Percent of increase in life span (%ILS±SE) for locally treated mice with rHuIFN-α A/D was 17.3±2.99 (3 KU), 20.5±2.27 (10 KU) and 28.7±3.68 (30 KU); for systemically treated mice the %ILS was -1.7±1.96 (3 KU), 5.2±1.98 (10 KU) and 7.9±2.08 (30 KU) (Figure 4). Owing to a higher agressivness of i.p. tumors, systemic (subcutaneous) treatment did not statistically significantly affect the average day of death in comparison with control mice, except when the mice were treated with the highest dose (30 KU) (Table 2, Figure 3).

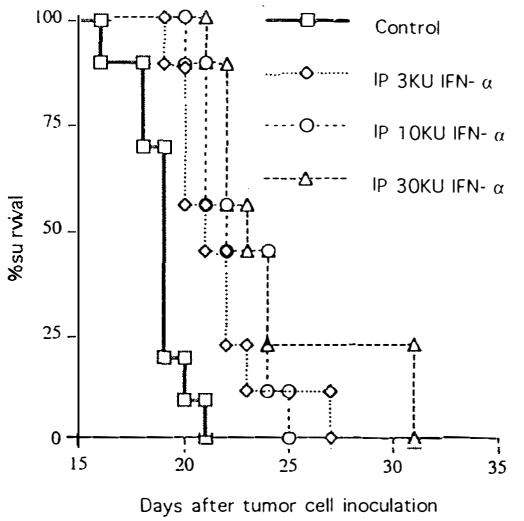

Figure 3. Survival curves for intraperitoneal B-16 melanoma bearing mice (C57Bl/6); tumors were induced intraperitoneally with 10^6 viable tumor cells and 24 hours later treated intraperitoneally (upper figure) or subcutaneously (lower figure) with different concentrations of rHuIFN-α A/D during five consecutive days.

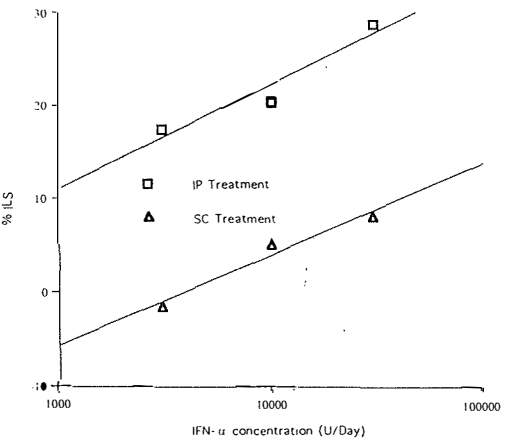

Figure 4. Increase in life span (ILS) for intraperitoneal B-16 melanoma bearing mice treated systemically or locally with rHuIFN-α A/D.

However, statistically significant increase in life span was observed when we compared locally (intraperitoneally) treated animals to the ones treated systemically (subcutaneously) with the same dose of rHuIFN-α A/D; the p-value for mice treated with 3 KU was 0.0001, for mice treated with 10 KU 0.0004 and for mice treated with 30 KU 0.0002. Moreover, the animals that received a threefold lower dose (10 KU) of rHuIFN-α A/D locally (intraperitoneally) survived significantly longer than those treated systemically (subcutaneously) with 30 KU ($p = 0.005$).

Based on the results obtained in both tumor models, it is clear that maximal antitumor activity occurred when rHuIFN-α A/D was given locally. Systemic treatment was moderately effective: more effective in s.c. tumors when rHuIFN-α A/D was administered intraperitoneally than on i.p. tumors when it was administered subcutaneously.

Discussion

Previous experimental findings demonstrated that IFN-α has reproducible antiproliferative effects in vitro[20-22] and in *vivo*.[23] On the basis of these findings, IFN-α has been employed clinically for treatment of various tumors.

Today, IFN-α is approved as an antiproliferative agent for the treatment of hairy cell leukemia and AIDS related Kaposi sarcoma.[5,10] Nevertheles, IFN-α as a single agent has been reported to induce clinical remission in many hematological malignancies and solid tumors.[16,24,25] Moreover, IFN-α has reproducible activity against malignant melanoma, a tumor for which conventional chemotherapy has poor efficacy.[10,25]

The present study was undertaken to assess which route of administration is more suitable for IFN-α treatment. Therefore, we chose s.c. and i.p. B-16 melanoma tumor models. Mice were treated locally and systemically for five consecutive days with different doses of rHuIFN-α A/D. Systemic treatment of s.c. tumors was performed in the form of intraperitoneal injection, while locally treated animals were injected subcutaneously. In contrast, in the i.p. tumor model intraperitoneal administration was performed as a local and subcutaneous as a systemic treatment. In both cases local treatment proved to be significantly superior to systemic. An interesting observation was that systemic (intraperitoneal) treatment of s.c. tumors resulted in a statistically significant delay in tumor detection at all interferon concentrations examined, while systemic treatment (subcutaneous) of i.p. tumors did not significantly increase the life span of treated animals (except 30 KU). The fact that developed tumors in systemically treated animals continued growing at the same rate as tumors in control mice, suggests that systemically administered rHuIFN-α A/D exerts antitumor effect only on a very small tumor burden.

In accordance with our observations, rHuIFN-α A/D is more effective when given as a local therapeutical agent. Nevertheless, when we have to use rHuIFN-α A/D systemically, it is much more advisable to administer it intraperitoneally than subcutaneously. This is also in agreement with previous pharmacokinetic findings that intraperitoneal administration of IFN-α has good bioavailability (30 times higher) compared to the intravenous route.[26]

The role of IFN-α in the treatment of malignancies has not yet been fully established. Many questions remain to be answered concerning the optimal strategy for incorporating IFN-α into anticancer therapy, and one of them is the optimal route of its administration. However, the future of IFN-α usage in oncology seems to be in its local (and also systemical) use as adjuvant therapy after the tumor burden has been reduced by other therapeutic modalities.

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Anti-tumor effect of interferon alpha in combination with cisplatin – animal experiments

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Anti-tumor effect of human interferon-α and cisplatin was studied on B-16 melanoma bearing C57Bl/6 syngeneic mice. When the tumors reached 1 mm in diameter, applications of cisplatin were started at a dosage of 0.001 mg/g of animal's body weight, or human interferon-α was given at a dosage of 5×10^4 IU. A control group of mice received normal saline solution. The treatment was applied every next day, altogether 12 times. Tumor volumes were measured every next day, and their mean values calculated; all 12 measurements confirmed that the mice treated with human interferon-α and cisplatin had smaller mean value than the control group, or the groups receiving either interferon or cisplatin alone, respectively ($p < 0.05$). The mean tumor volumes of cisplatin treated mice were lower than those of the controls or interferon-treated animals ($p < 0.05$). The obtained results indicate that human interferon-α enhances the antitumor effect of cisplatin.

Key words: melanoma, experimental drug therapy; interferon-alpha; cisplatin

Introduction

Melanoma represents less than 5 % of all malignant diseases, though its incidence in the last few decades has been rapidly increasing.[1, 2, 3]

Chemotherapy in melanoma has not been particularly effective. Although several drugs have a low order of antitumor activity, combination chemotherapy has not produced better results that those observed with the use of single agents such as dacarbazine, nitrosoureas, vinca-alkaloids, and cisplatin (CDDP). Moreover, none of these drugs as well as their combinations have been proved to definitely increase the survival of patients with metastatic melanoma.[4, 5]

A number of pre-clinical and clinical trials studying the effects of interferon alpha were consistent in confirming its antitumor activity against melanoma.[6, 7] Even if the mechanism of antitumor effects of interferon are still insufficiently understood, it has become clear that in the majority of the experimental systems investigated, interferons act in a very different way from chemotherapy. Thus, the idea of using interferons in combination with other agents has interested investigators for a long time.[8]

Different experimental data have shown that combined treatment can improve the response rate and prolong the duration of response due

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to different actions of drugs on the tumor, interactions between the drugs and, perhaps, influences on the host immune system.[9, 10]

Therefore, the aim of this study was to investigate the possibility of enhancing the cisplatin antitumor effect by the application of human interferon alpha in suboptimal doses.

Materials and methods

Experimental animals

The animals, 8–10 week old C57Bl/6 mice were obtained from Rudjer Bošković Institute, Zagreb. Mice used in the experiment were of the same sex and age. Animal colonies were maintained in accordance with the recommendations issued by the National Cancer Institute in Bethesda, USA. B-16 melanoma was used as an experimental tumor model. Tumors were implanted to the animals by subcutaneous injection of 5×10^5 viable tumor cells given dorsolaterally. Tumor cell suspension was prepared by mechanical decomposition of viable tumor tissue.

Treatment

Mice were divided into four experimental groups as follows: 1) control group, 2) group treated with CDDP, and 3) group receiving human interferon-α (IFN-α) and 4) group receiving combined CDDP and IFN-α treatment. Each group consisted of 7 animals. Intraperitoneal applications of the cytotoxic agent and/or IFN-α and normal saline solution were started when the tumors reached 1 mm in diameter, or a volume of 0.5 mm^3. The injections of active substances were administered every next day, and the experiment was completed on the 25th day from the beginning of application.

The solution of CDDP (Bristol-Myers Co.) and normal saline was injected at a dose of 0.001 mg/g b.w. or 0.01 ml of the solution per gram of the animal's body weight.

IFN-α (human interferon alpha from the Institute of Immunology, Zagreb, Croatia) dissolved in normal saline was injected at a dose of 5×10^4 IU which equalled to 0.25 ml of the solution per application.

In one group CDDP injections were followed after one hour by IFN-α application; drug dosage was the same as in groups receiving either of the agents alone. Animals in the control group had the same quantity of normal saline solution injected intraperitoneally every next day.

Tumor measurement and statistical analysis

Tumor growth was followed up daily by the evaluation of tumor diameter and thickness. Tumor volume was calculated using the following formula: $0.523 \times a \times b \times c$, where a, b, c were tumor diameters.

Mean volumes, as well as standard deviation and standard error of the mean values were calculated from the results of measurements performed on the same day. The data were statistically analysed by means of CIA software.[11]

Results

Mean values of tumor volumes (MTV) expressed in mm^3, measured every next day during the treatment with normal saline solution, IFN-α, CDDP, or combination of both are presented in Table 1.

In all measurements MTVs of CDDP-treated animals were found to be lower that those of the control group ($p<0.05$), and the values obtained after the 5th measurement were also statistically significantly lower that those of the IFN-α treated animals.

In all 12 measurements MTV of the animals receiving combined CDDP and IFN-α treatment were statistically significantly lower than the relevant values obtained in all other experimental groups ($p < 0.05$).

Figure 1 shows MTV values and 95 % confidence interval resulting from all 12 measurements performed in all experimental groups.

In the groups receiving combined CDDP and IFN-α treatment or CDDP alone 2 animals died immediately after drug application. The

Table 1. B16 melanoma volume in mm^3 in all 4 groups of C57Bl/6 mice.

Measurement	Controls MTV (m^3) 95-CI	IFN-α MTV (mm^3) 95-CI	CDDP MTV (mm^3) 95-CI	IFN-α and CDDP MTV (mm^3) 95-CI
1	0.5	0.5	0.5	0.5
2	3.9	3.1	2.1	1.3
	2.7–4.1	2.7–3.5	1.8–2.4	0.9–1.8
3	13	10.4	9.9	6.2
	11–15	9.6–11.2	9.2–10.7	5–7.4
4	57.7	46.9	49.1	35.2
	55–61	44–50	44.8–53.3	29–42
5	131	122	119	59.3
	127–135	116–128	113–125	48–71
6	181	179	145	91.4
	173–189	170–188	137–153	79–104
7	324	326	240	219
	316–332	314–338	231–249	205–233
8	444	438	369	299
	433–455	428–448	349–389	281–317
9	538	524	417	371
	525–551	510–538	295–439	349–393
10	622	613	498	443
	608–636	600–626	473–523	421–465
11	696	689	602	523
	683–709	676–702	573–631	496–550
12	789	798	711	611
	775–803	784–812	672–750	576–646

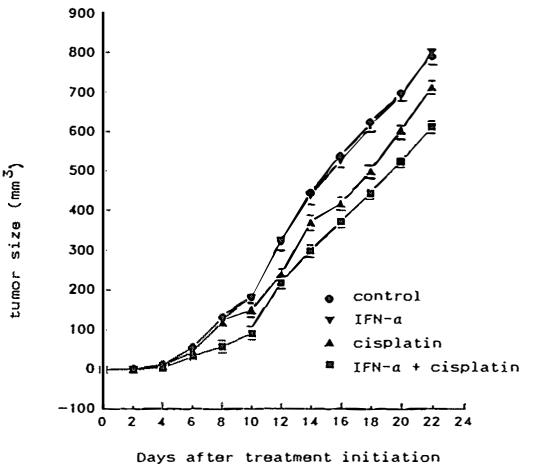

Figure 1. The effect of IFN-α and CDDP on the growth of B-16 melanoma. Mice were treated every next day after the tumors reached 1mm in diameter. IFN-α (5×10^4 IU) and CDDP (0.001mg/g) were injected intraperitoneally. Each experimental group consisted of seven mice. The vertical bars represent 95% CI.

animals in CDDP-treated group had for 1g lower body weight on average, whereas the body weight of animals in other three groups did not differ from that of the control animals.

Discussion

According to the obtained results, IFN-α alone does not exert any direct statistically significant effect on the tumor.

The antiproliferative effect of IFN-α depends on the dose applied, as well as on the mode of application, tumor size and the type of metastases. An indirect, immunomodulatory effect of interferon can be achieved at doses lower than those required for a direct antitumoral effect. In experimental animals human IFN-α does not influence the immune cells such as T-lymphocytes and NK cells.[14]

Balkwill has reported on the interactions between human IFN-α and chemotherapeutic agents in human tumours grown in mice. The efficacy of sub-optimal doses of cyclophosphamide and doxorubicin was greatly increased by interferon in a human breast cancer xenograft growing in nude mice. Even low doses of interferon, which alone had no effect on tumour growth, were able to potentiate the activity of anticancer drugs.[15]

Numerous preclinical and clinical trials have shown synergistic or additive effects between

interferon and at least 20 different cytotoxic agents including doxorubicim, vinca alkaloids, 5-fluorouracil and CDDP.[9, 16]

The most striking synergy was demonstrated when low doses of IFN were used and, and it was associated predominantly with lymphoma cell lines.[17]

However, not all studies have established positive interaction between IFNs and chemotherapy. Antagonistic effects between some cytotoxic drugs and IFN have also been reported.[18]

Little is known about the mode of IFN interaction with CDDP and other cytotoxic drugs. IFNs could potentially alter drug metabolism or act independently of the other agent. The cytochrom P-450 system was inhibited, thus significantly influencing the cell level of glutathione transferase. The decreased levels of cell glutathione result in increased cytotoxic effect of CDDP. IFN slows down the cell cycle by inhibiting the production of nucleic acids in its postmitotic G1 phase. IFN also slows down catabolism and elimination of some cytotoxic agents such as cyclophosphamide and doxorubicin, and influences cell membrane fluidity, i.e. the transport system for cytostatics.[9, 19]

Combined IFN and chemotherapy has resulted in clinical benefit in many patients with solid tumours.[20] In general, however, significantly improved response rates were not observed.

In many regimens, IFN are combined with cytotoxic drugs with different rationale: biochemical modulation, immunopotentiation, immunostimulation or host protection. Each approach is valid; however, the complexity of potential interactions requires close considerations.[9, 22]

Our study showed that IFN, even at a dose insufficient to influence the growth of B-16 melanoma when given as monotherapy, statistically significantly increased the antitumor effect of CDDP (95% CI). Additional prospective clinical trials are of paramount importance for further explanation of the interaction mechanisms involved.

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Serum interleukin-2 levels in malignant melanoma patients

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In a majority of human neoplasms a mitogen mediated decrease in the production of interleukin 2 (IL-2) in vitro can be observed. Poor resolution of the available tests does not enable the evaluation of spontaneous and in vivo IL-2 production in cancer patients. Using ELISA method (Genzyme), serum IL-2 concentrations were determined in malignant melanoma patients and healthy controls. The mean value (±SE) of serum IL-2 in 30 healthy donors was 269 U/ml (269 ± 66). In patients with malignant melanoma this value was lower - 37 U/ml (37 ± 16). The difference between mean values of both groups was significant ($p < 0.05$). The mean level of serum IL-2 in 11 patients treated with human leukocyte interferon was higher than the mean value of all melanoma patients (53 U/ml); the small number of patients and variability of the obtained results, however, render the difference statistically insignificant. The results indicate that the decreased in vitro production of IL-2 in malignant melanoma is associated with a decrease in in vivo IL-2 production in comparison with healthy persons.

Key words: melanoma, interleukin-2

Introduction

Solid malignant tumors are frequently accompanied by suppression of cell-mediated immunity and associated with that impaired survival.[1-3] The effects of specific antitumor responses, such as generation of cytotoxic T-cells, activated macrophages, and antitumor antibodies, are probably most relevant to immunologic control by the host. Besides the initial maturation effects of thymic hormones,[4] the most important is the so-called interleukin cascade which represents a sequence of events involving the citokines of monocyte lypmhoid origin helping to drive the cellular response to target antigens. Current studies focus on interleukin-2 (IL-2) which is a glycoprotein produced by activated T lymphocytes.

According to the results of some studies[5] it is known that most antitumor immune reactions, such as proliferation of T helper and cytotoxic cells, NK activity and generation of LAK cells, are IL-2 dependent. Acting on specific IL-2 cell surface receptors, expressed by activated though not resting immune cells, IL-2 stimulates immunity. Though activated T cells, B cells, and macrophages express IL-2 receptors, the most important source of serum IL-2 has still not been clearly explained. Also,

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the results of some studies[6] suggest that serum IL-2 is involved in regulation of some IL-2-dependent immune functions.

The central question is whether IL-2 function is abnormal in patients with solid tumors who commonly exhibit depression of cell-mediated immunity.

Several studies have demonstrated a reduced in vitro IL-2 production after mitogenic stimulation in most patients with metastatic cancer. On account of too low sensitivity of previous assays, little data were available on IL-2 spontaneous in vivo production in cancer patients until recently.[7-9] The development of sensitive ELISA method enabled us to investigate serum IL-2 concentrations in patients with malignant melanoma.

In view of the previously mentioned facts, serum levels of IL-2 were investigated in patients with malignant melanoma in comparison with healthy persons.

Patients and methods

The study included 30 patients with malignant melanoma. There were 12 male and 13 female patients with the mean age 51.4 years (range 31–76 years). Thirty healthy persons (blood donors) served as a control group; there were 15 males and 15 females with the mean age 41 years (range 25–57 years).

In all patients the tumor was histologically proven after surgical removal, and none of them was treated with exogenous recombinant interleukin-2. Disease activity was determined at the time when blood samples were collected. Eleven patients with malignant melanoma were treated intramuscularly with human leukocyte intereferon (2×10^6 U weekly for 30 weeks). The blood samples from those patients were collected throughout the duration of treatment.

Serum interleukin-2 (serum IL-2) levels were measured by means of Inter Test 2 Human IL-2 ELISA test kit (Genzyme-Corporation, Boston, Massachusetts, USA) which is a solid phase enzyme immunoassay employing the multiple antibody sandwich principle.

Results

The mean value (±SE) of serum IL-2 in healthy donors was 269 U/ml (269±66, range 6.9 - 1881 U/ml). In males the mean serum IL-2 value was 259 U/ml and in females 279 U/ml (Table 1). There was no significant sex- and age-related difference in the group of healthy persons. However, since there was a difference between mean age of healthy donors and melanoma patients, the control group was divided into two groups, i.e. persons over 40 and those under 40 years of age. The mean value in healthy donors over 40 years of age was 292 U/ml, while in younger donors it was 249 U/ml; there was no significant impact of age on serum IL-2 levels.

Table 1. Serum interleukin-2 levels in healthy donors according to the sex and age distribution.

Group	No	serum IL-2 Levels AM* ± SE**	Range
Males	15	259	13.8 – 536.2
Females	15	279	11.5 – 1881
Age:			
>40	14	292	13.8 – 1881
<40	16	249	11.5 – 891
All	30	269 ± 66	6.9 – 1881

* AM – arithmetic mean
** SE – standard error

In patients with malignant melanoma the mean value of serum IL-2 was 27±9 U/ml (Table 2); in female patients 28 U/ml and in male patients 26 U/ml. There were no sex-related differences noted in melanoma patients. In older patients (over 51 years) mean serum IL-2 level was 27 U/ml, and in younger patients (under 51 years) this value was nearly the same - 27 U/ml.

Table 2. Serum IL-2 levels in patients with malignant melanoma according to the sex and age distribution.

Group	No	serum IL-2 Levels AM* ± SE**	Range
Males	12	26.1	2 – 127
Females	13	28.4	2 – 200
Age:			
>51	11	27.3	2 – 127
<51	14	27.2	2 – 200
All	25	27.3 ± 9.3	2 – 200

* AM – arithmetic mean
** SE – standard error

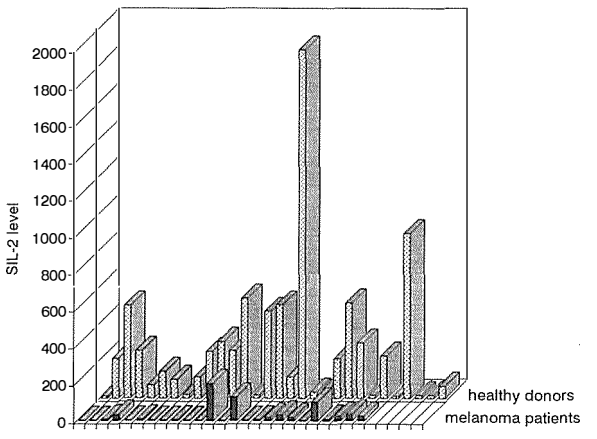

Figure 1. Serum IL-2 levels in healthy donors and melanoma patients.

Serum IL-2 levels in melanoma patients and healthy donors are presented in Figure 1. The difference between mean values of serum IL-2 in melanoma patients and in healthy donors was significant (27±9 U/ml versus 269±66 U/ml, $p<0.05$). In 5 patients the disease was found to have recurred by the time of sample taking. In these patients with recurrence, the mean value was decreased (6.8 U/ml). Despite of a low number of cases, it seems that the extent of disease (or disease activity) influenced the serum IL-2 levels.

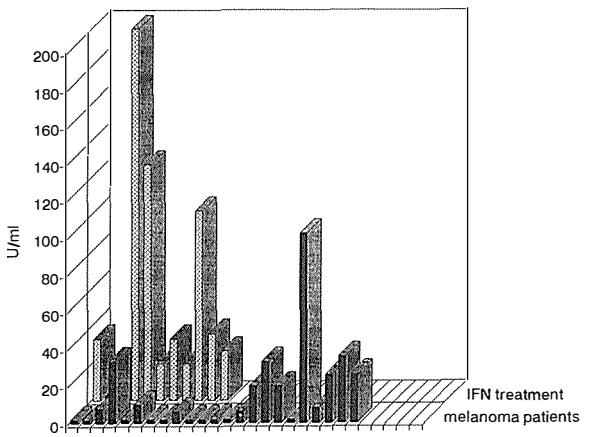

Figure 2. Serum levels in all melanoma patients and 11 patients that were on adjuvant treatment with human leukocyte interferon (2×10^6 units weekly, intramuscular application) after surgical removal of the primary tumor.

At the time of sample taking 11 patients were on adjuvant treatment with human leukocyte interferon (2 × 106 units weekly, intramuscular application) after surgical removal of the primary tumor. In these patients the mean value of serum IL-2 was 57 U/ml (Figure 2), which was higher when compared with otherwise decreased levels of serum IL-2 in melanoma patients. Although, the difference in serum IL-2 levels between all patients and patients treated with human leukocyte interferon was not significant. Also, the serum IL-2 levels were not in the range of the levels of controls employed in this study (i.e. 269 + 66 U/ml).

However, according to our findings it can be postulated that the extent of the disease could influence the serum IL-2 levels since a decrease in serum IL-2 was found in the group of patients with recurrence when compared with the mean serum IL-2 value in all patients (6.8 U/ml versus 27 U/ml).

Discussion

The aim of this study was to assess the potential role of serum IL-2 in the diagnosis and prediction of recurrence in malignant melanoma patients.

Biological significance of altered levels of Il-2 is still not clear; in particular, it is not yet clear whether the increased levels in the blood reflect possible activation of immune cells, or should be ascribed to immune dysfunction.

Although the lymphoproliferative response to mitogens or antigens is frequently depressed in cancer patients, their ability to produce IL-2 by lymphocyte stimulation with PHA appears relatively normal, as reported in literature. However, subgroups with advanced disease did have depressed IL-2 production.[1, 8] Furthermore, in some studies the presence of soluble form of IL-2 receptors was evaluated; increased values were found in patients with small-cell lung carcinoma . Additionally, in breast cancer patients serum IL-2R levels after surgery were significantly higher than those before surgery.[10] The values were found to correlate with the extent of disease.[11]

Decreased serum IL-2 levels in our study are consistent with some other reports, and so is also the finding that further decrease in serum IL-2 levels in our melanoma patients was associated with recurrence of the disease. The influence of human leukocyte interferon could be ascribed to various reasons though it is possible that, with respect to a small number of cases studied, interferon could influence the production of IL-2 in vivo. The increased serum IL-2 levels in patients treated with human leukocyte interferon could also be ascribed to crude extract of interferon containing minor quantities of IL-2.

Acknowledgements

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Natural porcine interferon gamma (PoIFN gamma)

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The natural porcine mitogen induced gamma interferon (PoIFN gamma) was studied and compared with the human interferons gamma (HuIFN gamma) and alpha (HuIFN alpha). A comparison was performed by the following criteria : pH 2.0 and 56°C stability, molecular weight, dot-blot reactions and cross reactivity (neutralisation index). The biological activity of porcine and human interferons in vitro (antiviral and antiproliferative) was correlated on human nontransformed (HEF) and transformed cells (FL).

Key words: interferon type II-analysis; cells; cultured-drug effects

Introduction

Interferons (IFNs) are defined as proteins/glycoproteins having an ability to protect the infected cells (causing the antiviral state) or to reduce and/or inhibit the growth of transformed cells *in vitro* and tumors *in vivo*.[1, 2] They are divided into at least two classes (Type I - alpha or beta and Type II - gamma) according to their mode of induction (Type I - with viruses or pI:C, Type II - with T cell mitogens or heterologous cells).[3, 4]

Because of clinical use, the main attention was focused on human interferon alpha (HuIFN alpha)[5] and much later on the human interferon gamma (HuIFN gamma).[6] For the IFN gamma production it was thought that a pure population of T lymphocytes is necessary. Latter on, it was found that partially purified spleenocytes can be used as well. The highest titers (IFN units/ml) were obtained when a mixture of peripheral blood lymphocytes and spleenocytes was used for the IFN gamma production, with specific T cell mitogens as inducers.

A comparison between HuIFN alpha and HuIFN gamma shows that they are antigenically completely different: with anti-HuIFN alpha antisera it is impossible to neutralise HuIFN gamma and vice versa.

At the beginning of the clinical use of human IFNs in natural form, the main problem was how to produce enough IFN Concomitantly, the optional solutions arose: to use human-like IFNs. In this respect, many studies were carried out to find the antigenic similarity between human and human-like interferons. Surprisingly, the highest degree of homology was found between murine and human IFNs.[7]

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Similar interferon system as in humans can be found in other animal species, such as rat, horse, cattle, pig, monkey, etc. Porcine and bovine IFNs[8–11] became of interest because of their relatively high antigenic similarity with human one. Such a similarity between Hu and PoIFN is about 78.5 % (at nucleotide level)[12] in the case of IFN alpha. When a recombinant gamma interferons (gamma IFNs) were compared (rPoIFN gamma/rHuIFN gamma) a homology was estimated in 59 % (at nucleotide level).[13] Correlation of non-recombinant forms of porcine alpha and human alpha IFNs shows even higher levels of homology (86.7 % at nucleotide level) between them.[14–15] It seems to be unusual that PoIFN gamma is cross reactive with HuIFN alpha and not with PoIFN gamma. Experiments presented herein are aimed to compare the natural porcine interferon gamma (PoIFN gamma) with the human interferon alpha (HuIFN alpha) by the following criteria: pH 2.0 and 56 °C stability, molecular weight, dot-blot analysis and neutralisation on cell culture *in vitro*.

Material and methods

Blood collection, buffy-coat preparation and interferon induction

Porcine blood was collected aseptically into sterile flasks containing 3.8 % sodium citrate to prevent coagulation. The porcine buffy-coat was separated by introducing 30 % sacha rose.[16-18] The sedimented buffy-coat was washed twice with saline and resuspended in modified Eagle's medium[19] containing 4 % of porcine plasma and antibiotics (Penicillin 5000 U/ml, Streptomycin 5 mg/ml, Gentamycin 10 mg/ml, Neomycin 10 mg/ml). The induction was performed by LCL (Lens culinaris lectin) at a concentration of 25 μg/ml. Cell concentration was adjusted to 10^7-10^8 cells/ml. After three days of cultivation on a spinner (37°C/140 RPM) the cells were sedimented by centrifugation (2000 RPM/20 minutes) and the supernatant representing the crude IFN was harvested.

Partial purification of PoIFN gamma

Interferon was isolated as follows: to 100 ml of IFN containing supernatant, 3 g of avtoclaved SiO_2 (water glass) were added and incubated overnight at + 4°C. On the next day the suspension was centrifuged at 2500 RPM for 30 minutes to sediment the water glass. The sedimented water glass was resuspended in the 1/8 of the original mixture volume of 50 % ethylene glycol monoethyl ether in 1.4 mM NaCl to elute the interferon. After 2 hours the water glass was sedimented by centrifugation (2500 RPM for 30 minutes). The elution procedure was repeated in 1/16 of the original volume. Both eluates were mixed with 0.1 % of FCS (Fetal calf serum, Flow) and dialysed against distilled water.

Measurement of antiviral and antiproliferative activity

IFNs were tested for antiviral (AV) activity by 50 % cytopathogenic inhibition assay against HSV1 (Herpes simplex virus type 1) as a challenge virus.[20] The antiproliferative activity (AP) was determined by 50 % growth inhibition assay on HEF (human embryonal fibroblasts) and FL (human amniotic cell line) cells.[21] Natural interferons alpha (Institute of Immunology, Zagreb, Croatia; EGIS Budapest, Hungary) at a concentration of 1000 AV (antiviral units/ml) were used as the control standards.

Stability tests

To determine the type of IFNs the samples were exposed to pH 2.0 and heating at 56°C for 30 minutes. Antiviral (AV) and antiproliferative (AP) units were determined before and after such treatment.

Protein content

In each sample, the quantity of proteins was determined by the modified Lowry method.[21]

Serological analyses

To determine the serological similarity and/or differences between porcine and human IFNs (gamma and alpha) the "constant method" according to LaBonnardiere et al.[14] was used. In summary, constant (twofold) dilutions of anti-IFN antisera were added to the FL cells in microtiter plates (NUNC). In the next step, three-fold dilutions of IFNs with the virus (HSV 1) were added. Simultaneously, IFN titration was performed without antisera. The neutralisation index (NI) was calculated as follows: NI = $\log_3$ (antiserum + interferon) - $\log_3$ (interferon).

Antigenic properties

Immunoblot tests were performed according to Pretnar et al.[22] In summary, the antigens HuIFN alpha (Institute for Immunology, Zagreb, Croatia; EGIS, Budapest, Hungary), HuIFN gamma (Sigma, St. Luis, USA), PoIFN gamma (Institute for Microbiology, Medical Faculty, 61000 Ljubljana, Slovenia) in the volume of 10 µl (the concentration of proteins in the samples was 0.9 mg/ml) were spotted on nylon films (Hybond, Amersham) and air dried. The nylon films with the bound antigens were incubated for 60 minutes with the primary antibodies (polyclonal) as follows: Anti-HuAlpha (Boehring, Manheim, FRG; neutralisation titer 10.000 U/mg), Anti-HuGamma (Boehringer, Manheim, FRG; neutralisation titer 10.000 U/mg) and Anti-PoGamma (INRA, Youi-en-Josas, France; neutralisation titer 10.000 U/mg), Anti-HuAlpha1 (Boehring, Manheim, FRG; neutralisation titer 10.000 U/mg), Anti-HuAlpha 2 (Institute of Virology, Bratislava, Slovakia; neutralisation titer 4000 U/mg) and Anti-Acidolabile (Institute of Virology, Bratislava, Slovakia; neutralisation titer 10.000 U/mg). After washing with TTBS (10 mM Tris + HCl, pH 8.0, 150 mM NaCl + 0.05 % Tween 80), the films were incubated in anti-rabbit IgG (Boehring, Manheim, FRG) conjugated with peroxidase. Following three washes in TTBS, the reactions were developed using DAB (diaminobenzidine, Sigma, St.Luis, USA) in the concentration of 50 mg/100 ml TBS and 30 fl of hydrogen peroxide.

Results

Stability tests

Table 1 shows the differences between porcine (PoIFNs) and human interferons (HuIFNs, alpha, gamma) as follows: PoIFN gamma is pH 2.0 stable in contrast to HuIFN gamma which is not. On the other side, PoIFN alpha is pH 2.0 labile, i.e. different from HuIFN alpha which is not. Similar data were found when the resistance to heating to 56°C were compared. It seems that PoIFN gamma is more similar to HuIFN alpha than to its human counterpart.

Table 1. Stability tests for PoIFNs and HuIFNs.

IFN type	pH 2.0	56°C	M.W.[1] (Daltons)
PoIFN gamma	Stable	Stable	21000
PoIFN alpha	Labile	Labile	20000
HuIFN gamma	Labile	Labile	19000
HuIFN alpha	Stable	Stable	19500

[1] Molecular weight was determined by PAG-SDS electrophoresis

Dot-blot analysis

Using dot-blot tests, the antigenic properties are described (Table 2) in terms of positive or negative reactions. PoIFN gamma shows a positive reaction with antisera against PoIFN gamma, HuIFN alpha and HuIFN alpha 2. HuIFN gamma reacts only with the antiserum against HuIFN gamma, and not with PoIFN gamma.

Table 2. Dot-blot analysis of Po and Hu IFNs.

IFN type	Reaction with Anti[1]				
	Po gamma	Hu gamma	Hu alpha	Hu alpha 1	Hu alpha 2
PoIFN gamma	+	–	+	–	+
PoIFN alpha	–	–	+	–	+
HuIFN gamma	–	+	–	–	+
HuIFN alpha	+	–	+	+	+

[1] Dot-blot reaction: "+" = gives positive reaction
"–" = gives negative reaction

In the case of alpha IFNs, PoIFN reacts with the antiserum against HuIFN alpha and HuIFN alpha 2. HuIFN alpha gave a positive reaction with the antisera against PoIFN gamma, HuIFN alpha, HuIFN alpha 1 and HuIFN alpha 2.

Serological tests

By serological tests in vitro (on FL cells) (Table 3) the level of the neutralisation of IFN's antiviral activity with anti-IFN antisera was quantified by the Neutralisation index (NI). When PoIFN gamma was tested, the following NIs

Table 3. Neutralisation indexes for Po and Hu IFNs.

IFN type	Neutralisation with Anti[1]				
	Po gamma	Hu gamma	Hu alpha	Hu alpha 1	Hu alpha 2
PoIFN gamma	–1.15	0	–1.94	0	–0.29
PoIFN alpha	0	0	–2.43	0	–0.53
HuIFN gamma	0	–0.98	0	0	0
HuIFN alpha	–0.92	0	–2.85	–2.00	–1.60

[1] NI = $\log_3$ (antiserum + IFN) – $\log_3$ (IFN)

were found: With anti-PoIFN gamma – 1.15, with anti-HuIFN alpha – 1.94 and with anti-HuIFN alpha 2, –0.29. PoIFN alpha shows much higher NI for anti-HuIFN alpha (NI = -2.43) as well as for anti-Hu alpha 2 (–0.53). HuIFN alpha shows the following values for NIs: Anti-PoIFN gamma + 0.92, anti-HuIFN alpha –2.85, anti-HuIFN alpha 1, –2.00, and anti-HuIFN alpha2, –1.60

Discussion

PoIFN alpha and HuIFN alpha were found to be antigenically similar, though the differences between them were found when some of the physico-chemical characteristics were tested (temperature stability, acid-resistance).[9-11] Contrarily, natural PoIFN gamma is different from its natural counterpart in humans. Its pH and temperature stability are more similar to that of HuIFN alpha. PoIFN gamma also shows cross reactivity in vitro with HuIFN alpha (complete) and its natural subtype HuIFN alpha 2 When these data were correlated with those obtained by comparison of recombinant IFNs (human, porcine) in the case of gamma interferons, homology was established in 59% (at nucleotide level). It seems possible that in the case of recombinant forms only selected molecules, in contrast to the natural ones (partly purified or purified) when complete molecules composed from different numbers of natural subtypes, were compared.

In this respect it should be mentioned that the comparison by NI (Neutralisation index) in vitro gives a picture of biological activity.

Future experiments with pure porcine interferons (alpha and gamma) are expected to disclose the real level of similarity/differences with human interferons (alpha, gamma), and thus enable clinical use of porcine IFNs in veterinary and human medicine.

Acknowledgment

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Biological activity of rat fibroblast interferon beta

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The biological activity of rat fibroblast beta interferon was studied. Rat embryonal fibroblasts (Wistar strain-WiREF) were used as a source of interferon. Cells were grown in suspension and on the microcarriers (Cytodex 3). In the exponential phase of cell growth the induction was performed. The biological activity of the obtained interferon was tested on the homologous cells. Interferon's influence on cell growth, morphology and intracellular level of some hydrolases was measured.

Key words: fibroblasts-drug effects; interferon- beta; rats

Introduction

Generally, interferons (IFNs) are very small glycoproteins/proteins produced by eukaryotic cells in response to induction by certain chemicals or infection with various viruses.[1] They are divided into two groups: Type I with three distinct families: alpha, beta and omega, and Type II with only one family: gamma.[2, 3]

Beta IFN can be obtained from various types of mammalian fibroblasts, such as human, bovine and murine[4, 5] by viral or pI:C induction. An interferon molecule has a molecular weight of around 22.000, although some smaller components with molecular weights between 17.000 and 18.000 can be found. It is less stable than alpha interferon. Schiff's staining revealed that beta interferon is a glycoprotein. Aminosugar and aminoacid analyses confirmed its hydrophobic characteristics.

Among other biological activities of beta IFNs, the antiproliferative activity was described.[6] This activity became important because of possible use of IFN as an antineoplastic agent. Mostly, the attention has been focused on the human interferons.

In our previous experiments[7, 8] we have found that spontaneously transformed rat embryonal fibroblasts of Wistar strain (WiREF) can produce relatively large amounts of beta interferon. In search of the possible mechanisms of the anticellular activity of IFN the growth inhibition was found to be parallel to the intracellular enzymatic changes in various model systems.[9, 10] Different hypotheses were postulated on the anticellular effects of IFN, though little is known about the distinct changes of the normal or transformed cells after a short-term treatment. It is generally accepted that at least 18-hour treatment is needed to produce an inhibition of cell growth in vitro.

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The experiments presented are aimed to show the distinct changes (morphological, growth characteristics, nuclear blebs, alkaline phosphatase) after the treatment of nontransformed and transformed WiREF cells with IFN for a short time (15, 30, 45, 60, 90 and 180 minutes).

Materials and methods

Cells

In these experiments we used rat embryonal fibroblasts (WiREF) in their nontransformed (Phase A) and transformed (Phase E) form.[7] Cells were grown in Eagle's medium supplemented with 10% of FCS (foetal calf serum).

Interferon

To obtain rat fibroblast (beta) interferon, the transformed WiREF cells were grown in up to two liters of suspension (10^7-10^8 cells/ml). IFN was induced using pI:C (polyionosinic: polycitidilic acid) at a concentration of 25 μg/ml. After 8 hours Actinomycin (10 μg/ml) was added. The cells were then sedimented (1200 RPM/20 minutes) and resuspended in fresh medium. The cultivation was carried on for the next 24 hours. Thereafter, the cells were pelleted (1200 RPM/ 20 minutes) and the supernatant was tested for the IFN content (antiviral units/ml). The IFN was further purified using the selective precipitation method.[8] The IFN used in the experiments has a specific activity of 10^8 antiviral unit/mg of proteins.

Analysis of cell morphology

In order to establish the differences in cell morphology, the cells were cultivated by two methods: (i) on small glass slides for four days, and (ii) on small glass slides in test tubes for 15, 30, 45, 60, 90 and 180 minutes after adsorbtion (control) and treatment with 1000 units of IFN/ml or mock IFN for 15, 30, 45, 60, 90 and 180 minutes. To analyse morphological changes, the cells were fixed with 4% paraformaldehyde, washed with PBS (phosphate buffer saline pH 7.2) and stained with Giemsa. In the parallel experiments, the cells were fixed and washed as in the case of staining with Giemsa, but afterwards stained with acetoorcein for two minutes. The following parameters were analysed: the diameter of the nucleus (>1/2 of the cell), presence or absence of phylopodia, presence or absence of binuclear cells.

Analysis of nuclear blebs

Throughout the experiments the number of nuclear blebs was determined using the method described by Fraccaro et al.[11] In summary, cells were adsorbed for 45 minutes and treated with 1000 units/ml of IFN (mock in control) for 15, 30, 45, 60, 90 and 180 minutes. The cells were then detached using trypsin (0.25%) and resuspended in 5 ml of 0.83 mM of Ammonium chloride for 45 minutes. The nuclei were sedimented by centrifugation for 10 minutes at 1200 RPM, and resuspended in PBS and put onto glass slides, air dried and fixed with methanol. Thereafter, they were stained with Giemsa and analysed by dark field microscopy.

Growth characteristics

The following growth characteristics were analysed: growth index (GI), cumulative population density (CPD) and calcium dependence growth assay.[7]

Growth index (GI): Cells were seeded into 5 cm Petri dishes for 45 minuts to allow the adsorbtion. After the treatment with 1000 units of IFN/ml (mock in control) for 15, 30, 45, 60, 90 and 180 minutes, the medium was replaced with medium containing 1% and/or 10% of FCS. The incubation was continued for four days. Cells were detached using trypsin (0.25%) and counted by means of a hematocytometer.

Cumulative population density (CPD): The values were obtained from the data (number of cells/ml) of growth experiments.

Calcium dependence growth assay: To obtain the values for calcium dependence growth in

vitro, the cells were seeded into 5 cm Petri dishes for 45 minutes and treated with 1000 units of IFN/ml (mock in control) for 15, 30, 45, 60, 90 and 180 minutes. Afterwards, the medium was replaced with medium containing 10% FCS, and incubated for 18 hours. On the next day, the medium was replaced with another one containing 0.01 mM Ca or 1.00 mM Ca with 10% of dialysed FCS. The number of cells was determined after four days of incubation by means of a hematocytometer.

Alkaline phosphatase

The enzyme level was measured according to the method described by Chou.[12] In summary, 1 ml of distilled water was added to 50 µl of substrate (p-nitrophenyl phosphate in 0.05 M Tris-HCl, pH 8.6). After 5 minutes of preincubation, cell supernatant (100 µl) was added. Thereafter, the incubation was continued for 20 minutes, in the presence of PBS pH 9.0. The extinction was measured at 550 nm using a spectrophotometer.

Protein content

In each sample the protein content was determined by modified Lowry method.[13]

Results

Cell morphology

Even though a significant differences can be seen between nontransformed and transformed WiREF cells, we were interested to find out whether a short time IFN treatment could change the following parameters: diameter of the nuclei, number of binuclear cells and the number of cells without philopodia. The results obtained (Figure 1) show a relatively fast increase in the number of cells with nuclei bigger than 1/2 of the cell. In contrast to this, in transformed cells these changes were much slower.

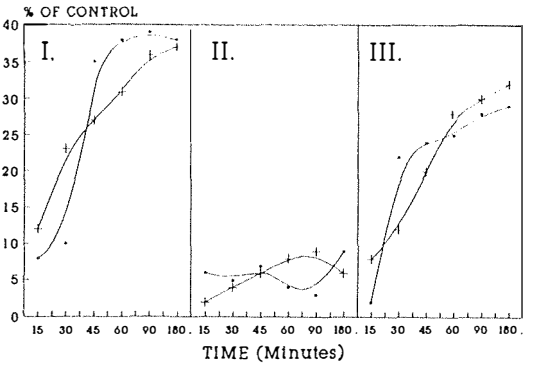

Figure 1. Morphological changes after interferon treatment of nontransformed (•) and transformed (+) WiREF cells. I. % of big nuclei, II. % of fused nuclei, III. % of cells without phylopodia.

The analysis of binuclear cells showed no differences between nontransformed and transformed cells at a higher rate, but it can be seen, that the percentage of binuclear cells begins to increase at a higher rate in nontransformed than in transformed cells.

When the phylopodia were analysed, the following results were obtained: in nontransformed cells the increase of their number began after 30 minutes, whereas in transformed cells this began after 15 minutes of treatment.

Growth characteristics

When the effect of IFN on the growth characteristics (Table 1, Figure 2) was analysed, in general the expected data were observed, i.e. a higher sensitivity of transformed versus nontransformed cells. A completly reverse situation, however, was observed in the case of CPD. The reduction rate was higher in nontransformed cells.

Alkaline phosphatase

In the case of alkaline phosphatase (Figure 3), the kinetics of enzyme levels seemd similar in both nontreated and IFN- trated transformed cells, but quantitative differences could be seen. In nontransformed cells, the enzyme level decreased in untreated cells, whereas in IFN-trea-

Table 1. Growth characteristics of nontransformed and transformed rat embryonal fibroblasts (Wistar strain) (WiREF).

Cells	Growth index[1]		Cumulative[2] population doublings:		Ca Dependence[3]
	(1%)[x]	(10%)[x]	(1%)[x]	(10%)[x]	
Nontransformed (Phase A)	2.19	3.19	1.29	1.67	0.42
Transformed (Phase E)	3.11	4.87	2.09	2.29	0.96

1) Values obtained by equation: $\frac{\text{No. of cells after 4 days}}{\text{No. of cells on day 0}}$

2) Values obtained by equation: $\frac{(\text{Log N1} - \text{Log N0})}{\text{Log 2}}$

N0 = Number of cells on day 0
N1 = Number of cells after four days

1) Values obtained by equation: $\frac{\text{No. of cells at. 0.01 mM Ca}}{\text{No. of cells at 1.00 mM Ca}}$

x) Denotes the presence of 1% or 10% of FCS in Eagle's medium

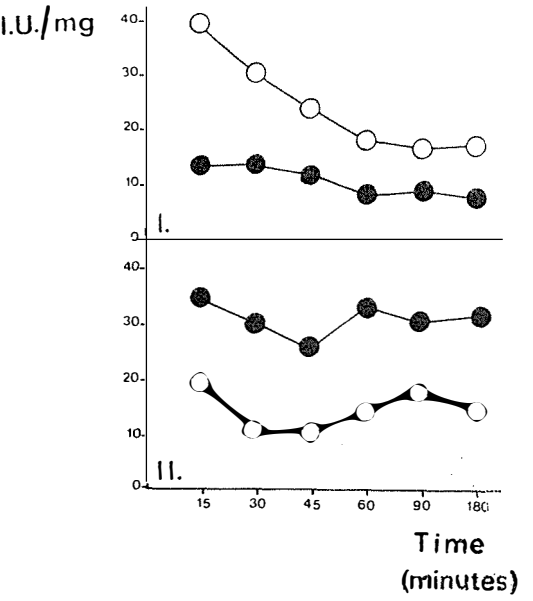

Figure 3. Effect of a short term rat Beta IFN on the intracellular level of alkaline phosphatase (specific activity). I. Nontransformed WiREF, II. Transformed WiREF. (o) Nontreated cells, (•) Treated cells.

ted ones a time independent decrease was seen throughout.

Discussion

The experiments were conducted on spontaneously transformed rat embryonal fibroblasts (WiREF) cell line and on its nontransformed counterpart. We have been interested to find out whether homologous rat (beta) interferon exerts an effect on cell morphology, growth characteristics and enzyme level. The experiments showed differences in the sensitivity of nontransformed cells. As to the morphology, in general there was no difference, but the effects on transformed cells were observed earlier than on nontransformed cells. The first morphological change in the diameter of cell nucleus can be found after a 15 minute treatment. It is interesting to note that immunochemical methods have shown that interferon can be detected around the cell nucleus even after 30 minutes.[14] These results can be correlated with the changes in the number of nuclear blebs and calcium dependent cell growth *in vitro*. In contrast to this, in our study of IFN effect on the actin organisation,[15] the nontransformed cells were found to be more sensitive than transformed ones. Having all this in mind, we attempted to find out whether the effects of IFN listed above (morphology, growth characteristics, nuclear blebs, actin organisation) were

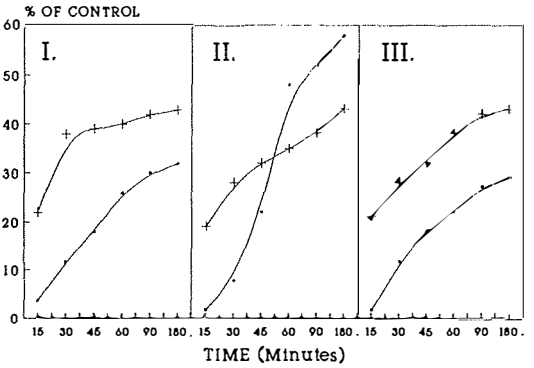

Figure 2. Growth characteristics of nontransformed (•) and transformed (+) WiREF cells after treatment with 1000 units of IFN/ml for 15, 30, 45, 60, 90 and 180 minutes. The values are expressed as the percentages of the control (Table 1). I. Growth index, II. Cumulative population doubling, III. Ca dependence growth *in vitro*.

parallelled by changes in enzyme levels (alkaline phosphatase). In notransformed cells the decrease of alkaline phosphatase was time independent, in contrast to transformed cells, where time-dependent changes were observed (alkaline phosphatase reached a maximum after 180 minutes).

It can be concluded that after the binding to cell surface, interferon acts relatively fast, and that the intracellular changes (enzyme level, morphology) are probably different depending on the phase of the cell cycle as well as on the phase of transformation. But it seems that the majority of the changes connected with the anticellular activity are triggered during the first 60–180 minutes. Concomitantly, the transformed cells show a trend toward a time dependent sensitivity of IFN action.[16, 17] The mechanism of a relatively greater sensitivity of transformed cells to IFN remains unknown, even though it seems possible that the regulatorly role of different intracellular hydrolases and consequently also the role of proteases and their inhibitors should be taken into account.

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Our experience with alpha 2-b interferon in the treatment of chronic active hepatitis B

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Clinical investigations of alpha-2 b interferon (Intron A - Schering-Plough) and other interferons have led to the registration of this substances for the treatment of chronic active hepatitis (CAH) B. In the present study patients were administered a dose of 3 million units (or 6 million units) of Intron A three times a week for three months (treatment failure was observed in one woman only; in this patient the dose was increased to 6 million units of Intron A and the treatment continued for another three months). As regards the therapeutical scheme, optimal instructions, as recommended by other European authors, were followed. Before therapy was introduced, all objective factors influencing the outcome of treatment were thoroughly examined. Liver histology, repeated comparative tests of the virus replication markers (HBV-DNA, HBeAg), and biochemical liver examinations (bilirubin, ALT, AST) were good indicators of the efficacy of the therapy. All the above laboratory tests were performed monthly. Therapy was stopped as soon as the laboratory tests and histological liver findings showed satisfying results. A prolongation of therapy was indicated only in one patient (non-responder) in whom therapy was prolonged until an improvement of the laboratory and histological liver test was achieved.

Key words: hepatitis B-drug therapy; hepatitis, chronic active; interferon-alpha-2B

Introduction

Chronic active hepatitis (CAH) B still represents a therapeutic problem. Corticosteroids, which had been widely used for the treatment of CAH B in the past, proved unsuccessful because they were shown to even induce HBV replication without reducing the clinical signs of the disease.[1,2] At present, interferon alpha is considered to be the most promising antiviral agent in the treatment of chronic HBV infection.[3-6] It exerts an additional effect on clearing HBeAg and HBV DNA from serum with or without the disappearance of HBs Ag. By this action interferon alpha is believed to cause an interruption of the viral replication phase (proved by HBeAg seroconversion) resulting in an improvement of the clinical, biochemical and histological changes of the liver.[7] It has been demonstrated that HBeAg seroconversion occurs in about 30–40 % of patients treated with alpha-interferon (IFN).[6-8] In the remaining over 50 % of patients the treatment is unsuccessful

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leading to further development of the inflammatory activity of the HBV infection.

A better therapeutical approach has not been found yet.

Patients and methods

In the period from June 1988 till December 1992 6 patients underwent treatment.

Table 1. Patients characteristics prior to interferon therapy.

Men/women	4/2
Age/women	25 and 61 years
Age/men	18, 20, 26, and 32 years
History of acute hepatitis	M 3/W 1
Duration of infection (months)	beyond 6 moths or up to several years
Time preceding the IFN therapy	6 months up to several years
CPH/CAH	0/6
HBV DNA (pg/ml)	0-210
AST (μkat/L)	0-5.3
anti-HIV	negative

Prior to the initiation of therapy, chronic active hepatitis B following HBV infection was histologically confirmed in all 6 patients under therapy. All patients were HBsAg positive, anti HBs negative, anti HBc positive (IgM negative), and HBeAg positive. Markers of active viral replication (positive HBeAg and HBV DNA) were detected by serum testing. Hepatitis D infection was excluded. All patients had signs of HBV infection (positive HBsAg) for at least 6 months or more. To 3 out of 4 men Intron A was administered at a dose of 6 million units three times a week for three months. Only one male patient received a dose of 3 million units during an equal treatment period.

In 2 women the therapeutic dose was 3 million units of Intron A given three times a week for 3 months. Since the older female patient (61 years) did not respond to the above treatment regimen therapy was prolonged for another 3 months during which time she was given a dose of 6 million units three times a week. During the course of therapy laboratory liver function tests (bilirubin, AST, ALT, prothrombin, proteinogramme) and tests for hepatitis B and HBV DNA serum markers were carried out regularly. Tests were performed every fortnight during the first month of treatment, later monthly, and every 3 months during the follow-up period. After three months of treatment all patients underwent liver biopsy which was used to evaluate the efficacy of interferon therapy. In the older woman (aged 61) biopsy was repeated at the end of the prolonged therapy. Blood count values were expressed in SI units, AST, ALT in μkat/L; HBsAg, and anti HBc were determined according to the RIA method with reagents obtained from Abbott, and were expressed in mmol/L, while anti HBs was determined according to the enzymatic method developed by Berhringwerke and was expressed in IU/L. HBV DNA was expressed in pg/ml and was determined according to the RIA method with reagents obtained from Abbott.

Results

After therapy with Intron A the histologic picture of the liver showed that chronic persistent hepatitis B (CPH) was present in 4 men. In 1 (32 years) of the 2 women, to whom Intron A was administered at a dose of 3 million units three times a week for 3 months, repeated liver biopsy showed chronic CPH B, while in the other patient (61 years) the therapy was ineffective. In this non-responding patient, the dose of Intron A was increased to 6 million units given three times a week and the treatment prolonged for another 3 months. The histological finding obtained after this prolonged administration confirmed the presence of chronic persistent hepatitis (CPH) B.

Four men and one woman (5 patients) remained HBsAg positive throughout the whole treatment and follow-up period. In the 61 year old woman in whom therapy was prolonged for another 3 months, HBsAg disappeared from serum and has remained negative up to the present. In all patients converted to HBeAg

negative, seroconversion of anti HBe was observed after the cessation of therapy.

At the end of therapy HBV DNA was normal in all patients. In one woman (32 years) an increase reappeared after therapy had been stopped, but disappeared spontaneously after some time.

Aminotransferases returned to normal values in all patients at the end of treatment.

As regards the side effects, a flue-like syndrome was noticed which was most frequent after the first application of the drug and disappeared spontaneously in the majority of patients during the continuation of therapy. Only in the 61 year old woman who was under prolonged interferon treatment, an increase in body temperature occurred (lasting for several hours after application) associated with nausea, loss of appetite, and reduced body weight. After repeated application these side effects diminished and totally disappeared by the end of therapy.

In all patients leukopenia associated with a relative lymphocytosis and mild thrombocytopenia occurred after application, so that therapy could be continued and concluded in the whole group of patients.

The patients are followed up every three months.

Discussion

The aim of alpha 2b interferon therapy (Intron A) was to prevent further development of HBV infection. The sole disappearance of HBeAg and HBV DNA from serum cannot be seen as a sign of complete eradication of HBV since many investigators have found that in patients with chronic liver diseases HBV DNA division in liver cells occurs even after complete disappearance of HBeAg and occurrence of anti HBe antibodies.[9,10] In patients under immunosupressive therapy (and also spontaneously) clinical activation of hepatitis and reactivation of HBV replication developed despite the occurrence of anti HBe antibodies.[11,12]

In all 6 patients under trial interferon therapy led to disappearance of HBeAg and HBV DNA from serum. In our patient group the occurrence of anti HBe in serum was, except for one patient, not followed by reappearance of increased HBV DNA values. Later on these values were not detectable. The HBV DNA value before treatment is another important parameter. Some authors have reported that lower serum HBV DNA was associated with a better response to interferon therapy.[13,14]

In our patient group the loss of HBeAg from serum was associated with normalization of aminotransferases and an improvement of the hepatic histology (histologically, in all patients CAH B turned to CPH before the beginning of therapy).

It is known that in about 5-15 % of patients with chronic liver disease seroconversion from HBeAg can occur also spontaneously.[15-17]

Disappearance of HBsAg from serum (lasting up to the present) occurred only in one patient under prolonged therapy. This finding is in accordance with the study results obtained by other authors,[8,18,19] who reported that after therapy with alpha-interferon disappearance of HBsAg from serum occurred in 0–20 % of patients under therapy.

Due to a small number of patients involved in the trial, the evaluation of the obtained results lacks to a certain degree the required objectivity. However, the frequency of the disappearance of HBs from serum will probably increase with prolonged therapy and follow-up period, depending, of course, on the dose of interferon. For that very reason my work has been directed towards further investigation of the efficacy of this treatment regimen already from the beginning.

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Alpha interferon in the treatment of chronic hepatitis C

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To determine the effectiveness of treatment with recombinant interferon alpha (rIFN-α) we treated 100 patients with chronic hepatitis C. rIFN-α 2a 3MU thrice weekly were administered to 35 patients with chronic persistent hepatitis C (CPH-C), 60 with chronic active hepatitis C (CAH-C) and five with combined CAH-C and cirrhosis. After two months of treatment 36% of patients were good responders (CPH-C: 10, CAH-C: 26), 34% partial responders (CPH-C: 14, CAH-C: 19, CAH-C + Cirrhosis: 1) and 30% non-responders (CPH-C: 11, CAH-C: 15, CAH-C + Cirrhosis: 4). The treatment was performed during 12 months in 40 patients (CPH-C: 13, CAH-C: 26, CAH-C + Cirrhosis: 1). Good response was noticed in 40% (CPH-C: 6, CAH-C: 10), partial response in 38% (CPH-C: 5, CAH-C: 9, CAH-C + Cirrhosis: 1) and no response in 22% (CPH-C: 2, CAH-C: 7). Among 11 patients with good response to IFN-α treatment in 12-month period after the cessation of therapy relapses occurred in three of them (27%). The data suggest that long-term treatment with 3 MU rIFN-α trice weekly could be benefit for about one half of patients with chronic hepatitis C.

Key words: hepatitis C-drug therapy; interferon-alpha

Introduction

Hepatitis C virus (HCV) is a lipid-enveloped single-stranded RNA virus. The physical structure of HCV is unknown and only putative HCV particles have been identified by electron microscopy.[1] HCV causes chronic infection in 40-60% of infected individuals. A wide spectrum of liver diseases, ranging from normal findings to chronic persistent and active hepatitis, cirrhosis of the liver in about 20 percent of cases, and finally hepatocellular carcinoma, could be a consequence of genetic heterogeneity of HCV.[2,3] Diagnosis of HCV infection relies on anti-HCV and HCV RNA detection. Besides the first generation tests (ELISA, RIBA) which employed the non-structural polypeptide (C100-3), second-generation tests employing both structural and non-structural polypeptides increased diagnostic sensitivity to about 95%. Unfortunately, the detection of anti-HCV does not distinguish past from recent infection, except in patients with anti-HCV seroconversion. HCV RNA is the most reliable marker of HCV infection, especially in anti-HCV negative infections and cases of early acute hepatitis.[4,5] HCV RNA detection by polymerase chain reaction (RT-PCR) in serum, liver and peripheral blood

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mononuclear cells (PBMC) represents an important diagnostic procedure in all stages of HCV infection, as well as a valid tool to monitor response in patients undergoing interferon therapy.[6, 7] Interferon (IFN) therapy of chronic hepatitis C has proved useful in recent years, but many open questions about optimal dose, duration of treatment, prediction of responders or relapses are unresolved.[8, 9] The aim of our study was to investigate the efficacy of 12-month course of recombinant IFN-α 2a 3 MU 3-times weekly in patients with chronic hepatitis C.

Materials and methods

We treated 100 patients with chronic hepatitis C by the use of recombinant interferon-alpha 2a (rIFN-α) 3 MU thrice weekly during two months. Every patient was biopsied before starting the therapy. Before treatment, hepatitis A, acute and chronic hepatitis B, alcoholic liver disease, drug-induced liver injury, as well as Epstein-Barr virus (EBV) and cytomegalovirus (CMV) hepatitis were excluded. The values of alanine aminotransferase (ALT normal range under 35 IU/L) as markers of disease activity, were measured before therapy, and once monthly during the course of treatment. In all patients, antibody to HCV was examined before initial therapy, and thereafter once monthly too, by means of second-generation test (LIA-HCV, SORIN, Italy). Anti-HCV negative patients were excluded from treatment. In all treated patients complete blood count was examined once a week.

After two months of treatment, the patients were classified as good responders (normal ALT levels), partial responders (lower but still not normal ALT levels), and non-responders (equal or higher ALT levels).

The treatment lasted 12 months in 40 patients only. These patients were classified as good responders, partial responders and non-responders at the end of therapy.

The influence of patohistological diagnosis (CPH, CAH or CAH with cirrhosis) on the results of treatment was especially examined and calculated (Chi-squares with Yates corrected, Fisher's exact: 2-tailed). The percentage of relapses in 11 good-responders receiving 12-month treatment one year after completed therapy was calculated separately. The main side-effects of one year rIFN-α treatment are described. The influence of age and sex on treatment results was not calculated separately, but some observations are mentioned.

Results

Inclusion and exclusion criteria in 100 patients with chronic hepatitis C, treated with rIFN-α are summarised (Table 1). All patients of both sexes were between 18 and 60 years old, and had active liver disease with serum levels at least two times the upper limit of the normal range one year before the treatment. All patients were tested with second-generation assay for anti-HCV, and only positive persons were treated. All patients underwent liver biopsy, which was performed percutaneously with Meneghini needle. Patients with severe cirrhosis of the liver (Grade B and C), as well as patients with other liver diseases, were excluded from the treatment.

Table 1. Inclusion and exclusion criteria in patients with chronic hepatitis C.

Inclusion criteria
Age between 18 and 60 years
Either sex
Increased ALT value (> ×2) for at least 12 months
Anti-HCV positivity
Chronic hepatitis with or without cirrhosis on liver biopsy
Exclusion of other etiologic factors for chronic hepatitis
Exclusion criteria
Previous treatment with interferons or steroids
Severe cirrhosis (Grade B and C)
Drug addiction
Alcohol abuse
Anti-HIV positivity
Malignancy
Autoimmune markers or disease
Immunosupression (drugs, severe chronic diseases)

Table 2. Characteristics of patients on two month treatment with rIFN-α.

	two month treatment
No. of patients	100
Mean age (years)	45.2 ± 14.0
Sex (M/F ratio)	3.2
CPH-C	35
CAH-C	60
CAH-C and cirrhosis	5

Baseline demographic and histologic features of treated patients are shown in Table 2. We treated 100 patients of both sexes (69 males and 31 females, M/F ratio 2.2). The mean age of all patients was 45.2±14 yrs.

Liver sections stained by conventional procedures revealed features of chronic persistent hepatitis (CPH-C) in 35 cases, chronic active hepatitis (CAH-C) in 60, and chronic active hepatitis with cirrhosis (Grade A) in 5.

The characteristics of response to 2-month rIFN-α treatment are presented (Table 3). After two months of therapy 10 of 35 patients with CPH-C were good responders (28.6%), 14 were partial responders (40%), and 11 were non-responders (31.4%). Among 60 patients with CAH-C, 26 (43.3%) presented with good response, 19 with partial (31.7%) and 15 with unfavourable response (25%). None of the five patients with combined CAH-C and cirrhosis was a good responder; only one (20%) had partial response to rIFN-α treatment, whereas the remaining four (80%) were non responders. In the group with CAH-C a higher percentage of good responders than among patients with CPH-C is evident (43.3% vs. 28.6%, $p = 0.22$). The treatment was performed during 12 month

Table 3. Response to rIFN-α after two month treatment.

	Good responder (%)	Partial responder (%)	Non-responder (%)	Total
Initial histology				
CPH-C	10 (28.6%)	14 (40%)	11 (31.4%)	35
CAH-C	26 (43.3%)	19 (31.7%)	15 (25%)	60
CAH-C and cirrhosis	-	1 (20%)	4 (80%)	5
Total(%)	36 (36)	34 (34)	30 (30)	100

Table 4. Response to rIFN-α after 12 month treatment.

	Good responder (%)	Partial responder (%)	Non-responder (%)	Total
Initial histology				
CPH-C	6 (46.1%)	5 (38.5%)	2 (15.47)	13
CAH-C	10 (38.5%)	9 (34.6%)	7 (26.9%)	26
CAH-C and cirrhosis	-	1		1
Total (%)	16 (40)	15 (37.5)	9 (22.5)	40

period in 36 good responders and four partial responders (Table 4).

After one year of therapy 6 of 13 patients with CPH-C were good responders (46.1%), nine were partial responders (38.5%) and two were non-responders (15.4%). In 10 of 26 patients with CAH-C good response occurred (38.5%), nine had partial response (34.6%) and seven were non-responders (26.9%). One patient with combined CAH-C and cirrhosis, who previously had partial response to two month rIFN-α treatment, was non-responder after full course of 12 months therapy. Although the difference among patients with CAH-C and CPH-C is now negligible, it seems surprising that the rate of good responders among patients with CPH-C is obviously increasing (46.1% vs. 28.6%, $p = 0.31$).

Long-term follow-up in the duration of one year after cessation of therapy was feasible in 11 good responders (CPH-C : 3, CAH-C : 8) after 12 months of rIFN-α treatment. In three of them (27%) ALT values flared up; in one of them CPH- C and in two others CAH-C was found on initial liver biopsy; all of them were men.

The major side-effects during one-year rIFN-α treatment were flu-like syndrome, especially with initial doses, nervousness, depression, loss of hair, leucopenia and lymphocytosis. Serious side effects were not noted.

Discussion

Multiple randomised controlled trials have documented that alpha-interferon suppresses di-

sease activity and induces remissions in a high proportion of patients with chronic hepatitis C. Biochemical response (ALT levels) has been associated with doses of 3-5 million units (MU) in 30-70 % of patients with compensated chronic hepatitis C if ALT levels are at least 1.3 times the upper limit of normal and disease activity is demonstrated by liver biopsy. A long-term, sustained improvement expressed by normal serum ALT occurred in 10-25 % of patients.[1, 10, 11, 12]

Although, our study is still ongoing, a preliminary evaluation of 12 month treatment with 3 MU rIFN-α three times weekly is eligible. Normalisation or improvement in ALT values occurred in 70 % of patients after two months of treatment. Although the percentage of good responders is significantly higher among patients with CAH-C than among those with CPH-C, interestingly, this difference diminished after 12 months of treatment. On the other hand, regardless of the small number of patients a significantly lower rate of response was noted in patients with CAH-C and cirrhosis on initial biopsy. By the end of one-year rIFN-α therapy, the percentage of good responders among patients with CPH-C significantly increased, whereas in patients with CAH-C this rate gradually decreased, but altogether, almost equal rate of good response in both groups was noted after two months of treatment. Thus, in good responders after two months of treatment with rIFN-α 3 MU three times weekly, regardless of initial histological diagnosis of CPH-C or CAH-C further continuation of IFN therapy seems to be warranted.

Although the influence of age and sex on our results of one year treatment were not estimated separately, it seems that women were better responders, and that in younger persons (<45 years) the efficacy of treatment was significantly better.

In general, the results reported by some other authors commonly confirm a positive influence of younger age on the probability of response to IFN therapy. On the other hand, the significance of sex remains to be elucidated.[1, 8, 9, 13]

The percentage of relapse (27 %) one year after the cessation of therapy, which we have noted among good responders, is similar to some previously described observations.[1, 8, 9, 14, 15] Our data suggest that long-term treatment with 3 MU rIFN-α thrice weekly could be beneficial for about one half of patients with chronic hepatitis C.

There are many unresolved questions associated with the use of rIFN-α in the treatment of chronic hepatitis C, such as increasing of response rate, finding of optimal dose and duration of therapy, treating of patients with severe disease or immunosupression, predicting of response or relapse etc. Although interferon-alpha is now considered standard therapy for patients with active HCV-induced liver disease, further research of antiviral therapy is needed in order to provide safe and highly effective therapy for all patients with HCV-induced liver injury.

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Radiol Oncol 1993; 27: 321-5.

Treatment of cervical intraepithelial neoplasia associated with human pappilomavirus by interferon vaginalettes

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The randomized prospective study of the manifested 176 cases of cervical intraepithelial lesions (CIN) associated with human papillomavirus infections during 24 to 36 months was performed. The control cases were 240 patients which have had the same findings but were not treated with human leukocyte interferon (n-IFN-α). For the diagnosis and follow up, cytology, histology, colposcopy and dot-blot HPV typing were used. Patients were treated with n-IFN-α vaginalettes containing 1×10^6 I.U. of the natural interferon alpha and Excip. Macrogoli as a daily dose. In the course of two menstrual cycles 42 vaginalettes were given to each patient. Vaginalettes may be given as a monotherapy, or in combination with other remedies, depending on the micro biological findings or persistence. For statistical evaluation Chi-square, Fisher exact probability and Kolmogarov-Smirnow goodness of fit test were used. After 24 to 36 months follow up significant improvement in the treated group was found, and progressions of carcinogenesis was not registered. At the same time significant persistence of CIN was found in the control cases as well as twenty five or 42 % controls progressed into higher grade lesions. Better results of treatment were obtained with dot-blot hybridization negatives and with low-grade cases of cervical intraepithelial lesions. Our conclusion is that especially in young women, for the preservation of fertility and in pregnancy, n-IFN-α vaginalettes may be a remedy of choice.

Key words: cervix neoplasms-drug therapy; papillomaviruses; natural interferon-alpha; administration, intravaginal

Introduction

Human papillomaviruses (HPV) are clearly related to genital squamous precursor lesions and invasive carcinomas.[1-5]

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Prospective, randomized, double blind study on the natural human leukocyte interferon treatment have been performed and published previously.[6, 7] Preliminary results of treatment with n-IFN-α vaginalettes resulted in significant improvement in the treated group.[8]

The aim of our work is to eliminate the cervical intraepithelial neoplasia (CIN) as a potential cancer precursor.

Materials and methods

Out of 250 randomized[9] cases 176 were treated with vaginalettes. The remaining 74 cases were without n-IFN-α treatment for different reasons, or were lost from evidence. Each vaginalette, as a daily dose, contained 1×10^6 I.U. of curde interferon preparation and Excip. Macrogoli. A total dose of 42 vaginalettes was given to each patient. For diagnosis and follow up cytology,[10] colposcopy,[11] histology and dot-blot hybridization of the HPV deoxyribonucleic acid (DNA)were used.[9]

Before treatment, Chlamydia, Gardnerella, fungi or other microbiological causes of inflammation were treated. Prospective study was performed after three month interval using cytology/colposcopy investigations throughout 24 to 36 months. The treated group was compared with randomized cooperative 240 controls.

For statistical evaluation Chi square, Fisher exact probability and Kolmogarov-Smirnow goodness of fit test were used.

Results

Table 1 shows randomized groups and treatment results. Table 2 shows significant improvement ($P < 0,01$) in the treated group. Important differences may be seen in the eradication of the disease in treated group and progression of the disease in the controls. Table 3 presents the HPV DNA analysis; high-risk types 16/18 were found in more than 50% of treated cases. Table 4 and 5 show that better results have been obtained with dot blot negatives ($P < 0,05$) and with low-grade cases of cervical intraepithelial lesions ($P < 0,05$).

Discussion

Double blind study was not performed in this investigation for two reasons. Firstly double blind study was done in our previous investigations.[6, 7] Secondly a double blind is not always feasible because of the medico legal reasons.

Table 1. Results of treatment with interferon vaginalettes.

Results	N	%	Remarks
Eradication	73	41.48	Two six-month interval negative findings
Regression	55	31.25	"Borderline" cases
Persistency	48	27.27	Permanently the same findings
Total treated	176	100.00	
Excluded	74		Without treatment or lost to follow up
			Random numbers used
All randomized	250		

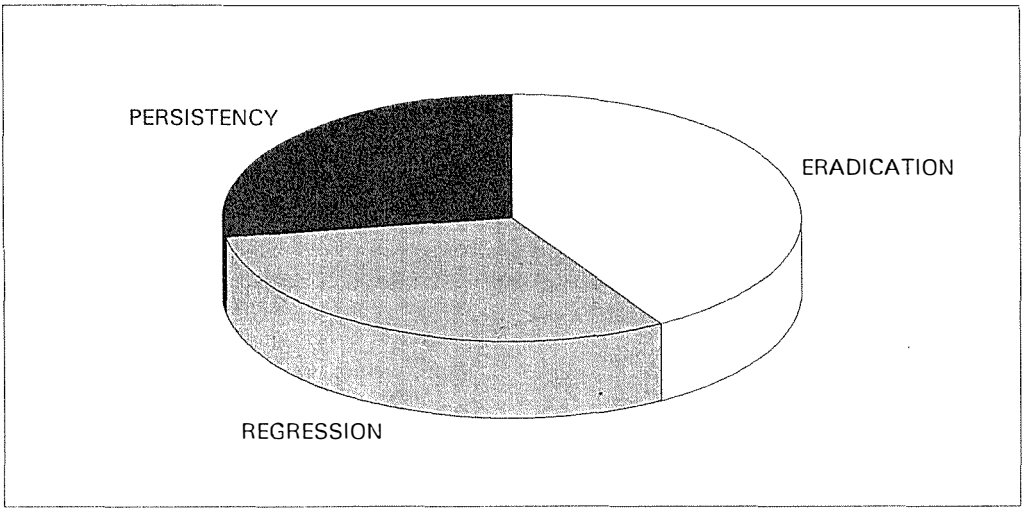
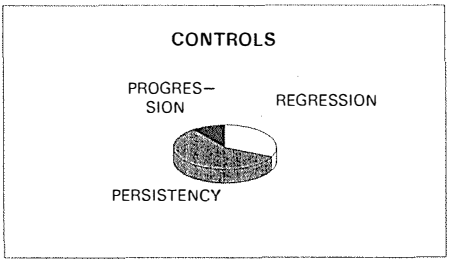


Table 2. Correlation of treated cases with controls.

Results	Controls N	%	Results	Treated N	%
Eradication	–		Eradication	73	41.48
Regression	77	32.08	Regression	55	31.25
Persistency	138	57.50	Persistency	48	27.27
Progression	25	10.42	Progression	–	–
Total treated	240	100.00	Total	176	100.00



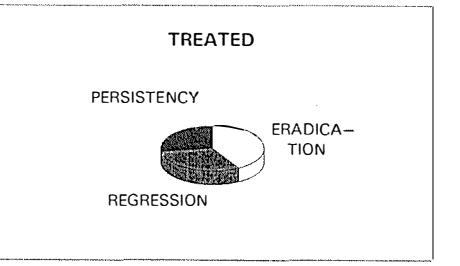
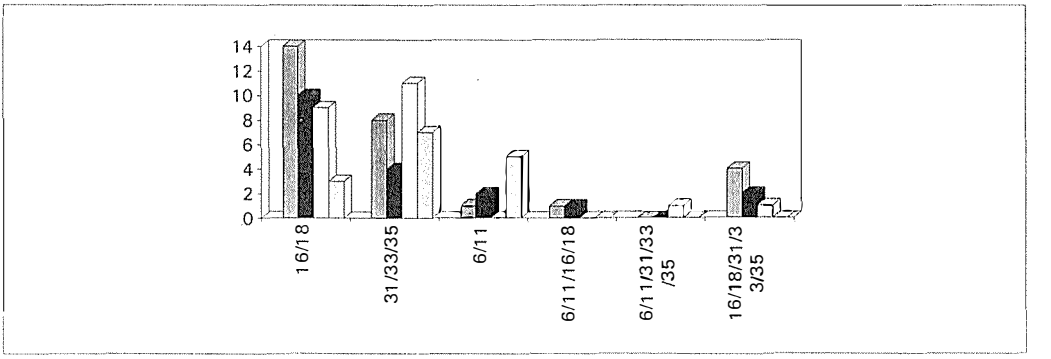


Table 3. Results of treatment with interferon vaginalettes regarding viral types.

	Results	N	16/18	31/33/35	6/11	6/11/16/18	6/11/31/33/35	16/18/31/33/35	N
1	Eradication	73	14	8	1	1	–	4	28
2	Regression	55	10	4	2	1	–	2	19
3	Persistency	48	9	11	–	–	1	1	22
4	Excluded	74	3	7	5	–	–	–	15
	Total	250	36	30	8	2	1	7	84

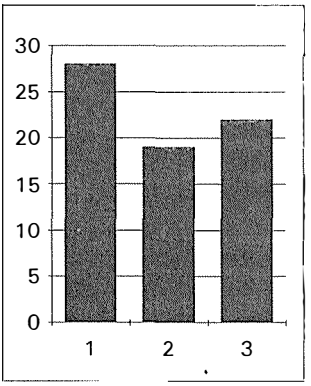


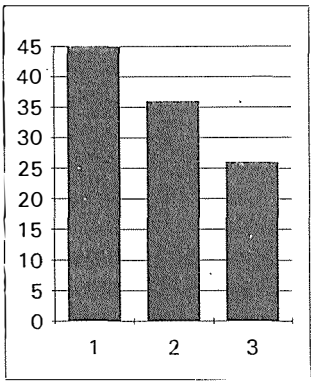
The same considerations have been stated also by other authors.[12]

Characteristic of our strategy was that interferon vaginalettes were used topically, also in pregnancy (10% of treated group), and did not interfere with the human reproduction. Surgical treatments were performed (cold-knife conization) in persistent cases, over 30 years of age, if the condition lasted for more than 30 months, and in lower grades of CIN with intent of a final eradication in younger women (CO2 laser). The vaginalettes were mostly used as monotherapy, but also in combination with other modes of treatments, depending on the case. In order to prevent HPV reinfections, the specific treatment of partners should be taken in consideration.

Table 4. Results of treatment with interferon vaginalettes and blot hybridization findings.

Results	Positive		Negative		Total	
	N	%	N	%	N	%
1 Eradication	28	40.58	45	42.06	73	
2 Regression	19	27.54	36	33.65	55	
3 Persistency	22	31.88	26	24.99	48	
Total Treated	69	100.00	107	100.00	176	70.40
Exluded	15		59		74	29.60
Total randomized	84	33.12	166	66.88	250	100.00





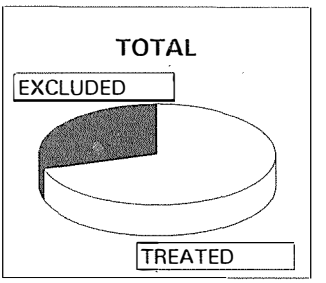
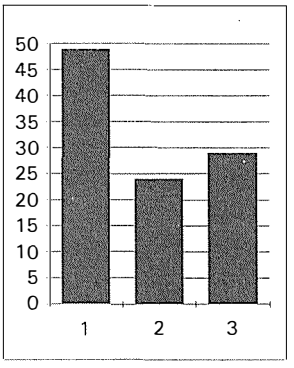
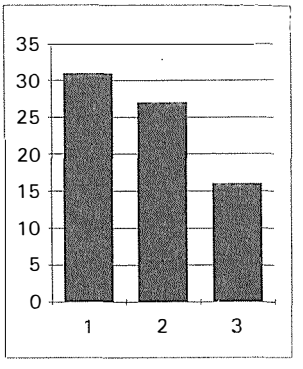
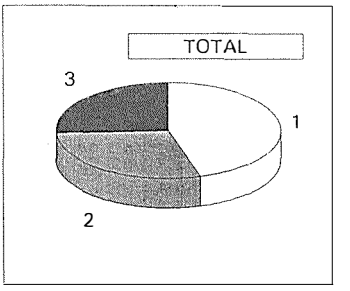


Table 5. Results of treatment with interferon vaginalettes and Pap test in Squamous Intraepithelilal Lesions

Results	Low-grade		High-grade		Total	
	N	%176	N	%176	N	%
1 Eradication	49	27.84	31	17.61	80	45.45
2 Regression	24	13.64	27	15.34	51	28.98
3 Persistency	29	16.48	16	9.09	45	25.57
Total	102	57.96	74	42.04	176	100.00







Morphogenetic grades of CIN and HPV DNA types appear to be of value as prognostic indices.[13] The low or high-grade of CIN and data obtained by colposcopy and HPV typing, as well as by follow up of the lesions, were analyzed in both groups, but from the patient's point of view it is essential that clinical terminology does not result in over treatment.[14] The

results of interferon treatment were also found to be in close correlation with CIN and Pap grade classification.[15] In our opinion, patients carrying high-risk HPV types should have regular checks at shorter intervals.[16] Some findings indicate long (up to 10 years) persistence of HPV infection in the natural course of progression to the carcinoma of the uterine cervix.[17] Therefore, follow up and further investigations are under way.

In conclusion, we want to point out that especially in young women, for preservation of fertility and in pregnancy, the natural crude interferon alpha vaginalettes may be the treatment of choice.

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Radiol Oncol 1993; 27: 326-31.

Natural IFN-α for non-small-cell lung cancer with pleural carcinosis

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The survival of patients with pleural effusion from bronchial carcinoma is short. Ten patients with ipsilateral pleural effusion from non-small cell cancer of the lung, localized to the thoracic cavity, were treated with intrapleural application of IFN-α and radiation therapy. All had malignant pleural effusion confirmed by cytology. Radiation therapy was given to the hemithorax with boost to the area of local tumor and mediastinal lymph node metastases. The dose to the hemithorax was 20-25 Gy, whereas the total dose to the tumor bed and mediastinum was 45 Gy. IFN-α 2 × 10^6 IU diluted in 20 ml of distilled water was injected intrapleurally once weekly. The treatment, as a rule, is suitable for palliation only. The effect of IFN-α was evaluated according to the cellular morphology of the pleural fluid and the patients' survival. The median survival of IFN-α treated patients was 17 months. The median survival of the matched control pts treated only for palliation with pleurodesis or radiation therapy was 7 months. The patients in the experimental group had a slightly better chance of prolonged survival. A randomized clinical trial seems to be indicated.

Key words: carcinoma, non-small cell lung-drug therapy; interferon-alpha

Introduction

Non small cell lung cancer (NSCLC) represents 75 % of lung cancer, the most common cancer in males. The diagnosis is late in the great majority of cases (70-75 %) and the overall survival of NSCLC patients is poor. The 5-year survival of operable (Stage I and Stage II) patients treated by surgery is 30-40 %. The survival of patients with Stage III is less than 5 % whereas the survival of those with malignant pleural effusion and those with Stage IV is practically nil.[1, 2] Neither chemotherapy nor radiation have contributed much to the survival of these patients. Surgery has been attempted for cure in patients with stage III,[3-8] but was not succesfull, especially not in those with pleural effusion. To palliate symptoms and prolong the survival was the aim of several trials; recently, there have been reports with encouraging results of treatment with intrapleural applica-

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tions of chemotherapy and biologic response modifiers.[9-12]

IFN-α has proved to be locally effective in several solid malignant tumors,[13] and systemically effective in several haematological malignancies.[14, 15] As a single therapeutic agent for adenocarcinoma and other solid tumors it has shown only modest results.[16] Enhancement of chemo- and radiation therapy with IFN-α has been shown in vivo[17-20] and in vitro studies.[21] Also, some enhancing effect of IFN-α on chemotherapy and radiation has been established.[22]

Earlier, we have reported on 14 patients with NSCLC and pleural effusion treated with intrapleural application of IFN-α; it was found that IFN-α could clear effusion from cancer cells and haemorrhagic admixture with minimal side effects. The treatment also prolonged the survival of patients.[23]

In the presented series, 10 patients with NSCLC and pleural effusion, without distant metastases (Stage IIIb), were treated by radiation and intrapleural applications of IFN-α, with the aim of permanent local control and prolonged survival.

Materials and methods

Experimental group

Ten patients admitted to the Institute of Oncology between december 1988 through november 1991, were treated for pulmonary cancer and pleural carcinosis. They had malignant cells in the pleural effusion proved by cytology; the primary tumor was confirmed to be adenocarcinoma by bronchoscopy and biopsy in all cases. The extent of the disease was further defined on plain chest radiograms, by CT of the chest and the brain, abdominal echogram, 99mTc bone scan in addition to the conventional biochemical and haematological laboratory tests. Their clinical data are presented in Table 1. The disease was confined to the chest in all patients, those with metastases outside of the chest were not included.

All the patients had radiation therapy 20–24 Gy to the whole hemithorax with a boost to the primary tumor and mediastinal metastases to a total dose of 40-45 Gy (Table 1). IFN-α was given by intrapleural application weekly as long as pleural effusion was present. After that it was given intramuscularly, twice weekly. In one patient (No. 4) it was only given i.m. because of a high risk for bleeding.

2×10^6 units of natural IFN-α were diluted in distilled water and after thoracocenthesis, with removal of as much fluid as possible, injected into the pleural cavity.

Control group

During the same period the great majority of patients with lung cancer and pleural carcinosis were treated for paliation by other methods both, at the Institute of Oncology as well as at the Institute for Lung Diseases, Golnik, the choice being made by the referring physician. Among these, 10 were chosen who matched the patients in the experimental group in terms of age, sex, and extent of disease. There were, however, 3 patients with squamous cell carcinoma in the control group. None of the patients had radical surgery performed previously, two had pleurodesis with Achromycin, 3 received palliative radiation and 5 analgetics only. The mean age of this group of patients was 56 years as compared with the mean age of 54 in the experimental group.

The level of IFN-α was measured in the pleural fluid and in the blood serum before and after IFN-α treatment in 2 patients, one from the experimental group and one from the control group.

In our experiments we used WISH (epithelial cells; European Collection for Animal Cell Cultures, ECACC, England) and MDBKK (bovine kidney, epithelial; American Tissue Type Collection, ATCC). As cell- virus combination using MDBK + VSV is not suitable for the detection of IFN-α, recently we have neglected MDBK cells, esspecially after we have got enough monoclonal antibodies to identify the type of IFN-αin samples of unknown IFN constitution.[24]

Table 1. Clinical data of patients in the experimental group.

Pat. No.	Age	Sex	Site (lobe)	Other metastases	Chemotherapy	RT dose/volume		IFN-α treatment dose effect			Spread	Survival (mos)
								10^6 IU	effusion	complications		
1	60	M	RLL	mediastinum	–	R thorax tumor bed	2100 1500 3600	8 × i.p.	–	fibrosis	brain, L lung, liver, peritoneum	18 DOD
2	26	M	LLL	–	5-Fu, Cis-P VP 16 } 5×	L thorax tumor bed	1500 3000 4500	5 × i.p.	–	fever lymphadenopathy	brain	25 DOD
3	51	F	LLL	–	–	L thorax tumor bed	2000 1750 3750	3 × i.p. 12 × i.m.	–	–	brain	9 DOD
4	50	F	LLL	subclavian ven. thrombosis	–	L thorax tumor bed	1500 2800 4300	8 × i.m. 1 × i.p.	residual	coagulopathy	–	1 tumor on autopsy not proven
5+	38	M	RLL	–	–	R thorax	4000	15 × i.m.	–	fever	L lung, R thoracic wall	46 AWD
6	56	M	RLL	bil. lymph-angiocarcinosis	–	R thorax tumor bed	1500 2000 3500	9 × i.p.	residual	–	lymphangio-carcinosis	5 DOD
7	72	F	LLL	–	–	L thorax tumor bed	1500 2000 3500	9 × i.p.	residual	–	lymphangio-carcinosis	18 DOD
8	72	F	LLL	mediastinum	–	L thorax tumor bed	1650 1800 3450	9 × i.p.	residual	–	–	8 DOD
9	52	M	LUL	mediastinum	Thiotepa i. p. 1×	L thorax	3000	13 × i.m.	residual	fever	liver, peritoneum	23 DOD
10	66	F	RUL	mediastinum	–	R thorax tumor bed	2500 2000 4500	2 × i.p. 11 × i. m.	–	–	L lung	30 DOD

DOD = dead of disease
AWD = alive with disease
+ = pleuropneumectomy

R = right
L = left

U = upper
L = lower

i. p. = intrapleural
i. m. = intramuscular

All patients in the experimental group have been regularly followed by clinical examination, laboratory and blood tests, chest X-ray and CT of the brain. The follow up of the patients in the control group was by the referring physician, who has treated them symptomatically. Therefore, only the date of death is reported for these patients and no details about the

The survival was calculated by the Kaplan-Meier method from the date of diagnosis until death or the date of the last follow up.[25]

The difference in the survival of the two groups was calculated with the log-rank test.

Results

At the end of the study in July 1993, 3 patients were still alive one patient from the experimental group more than 4 years, and 2 from the control group 17 and 15 months respectively (both had squamous cell carcinoma), all with residual disease. The survival is shown in Figure 1. The median survival of the patients in the experimental group was 17 months as compared to 7 months in control patients.

Malignant cells have disappeared from the pleural fluid after treatment with IFN-α in all patients, in the majority the fluid was still present.

Cytology was possible in 9 out of 10 patients, in 2 of them without the influence of radiation therapy. In all patients it showed essentially the same findings as in a previous[26] study of IFN-α in pleural effusions from breast cancer, i.e.:

a) increase in the number of transported lymphocytes and histiocytes in the sediment of the exudate,

b) marked decrease in the number of malignant cells, and

c) marked degenerative changes in the remaining malignant cells.

The levels of IFN-α in the pleural fluid and serum are presented in Figure 2 for patient No. 1 of the experimental group, and in Figure 3 for a patient in the control group. Only a minimal rise was observed in the serum in either of the two patients.

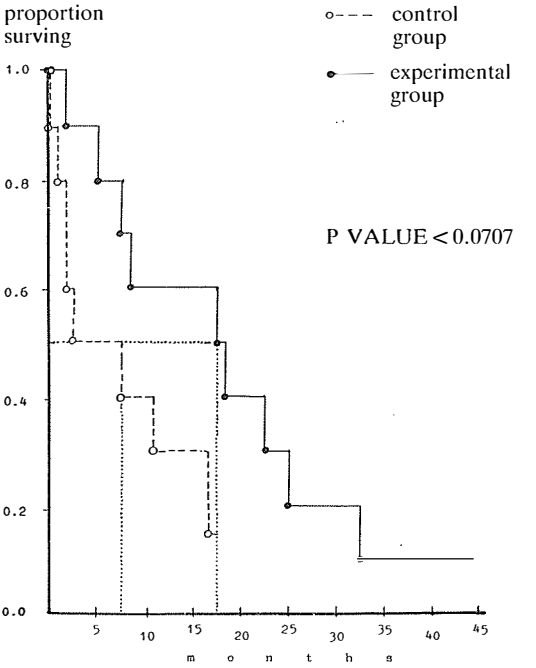

Figure 1. Survival of patients with non-small-cell lung cancer and pleural carcinosis.

Discussion

In a previous study of patients with pulmonary cancer and pleural effusion it was observed that IFN-α treatment may clear the effusion of cancer cells and haemorrhagic admixture and arrest fluid accumulation with minimal side effects.[23]

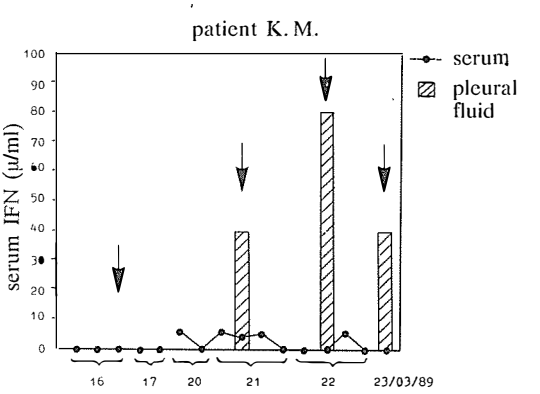

Figure 2. Pharmacokinetics of serum IFN during therapy. Bars represent IFN in pleural fluid. IFN application is indicated by arrows. Curve(s) are daily serum IFN levels.

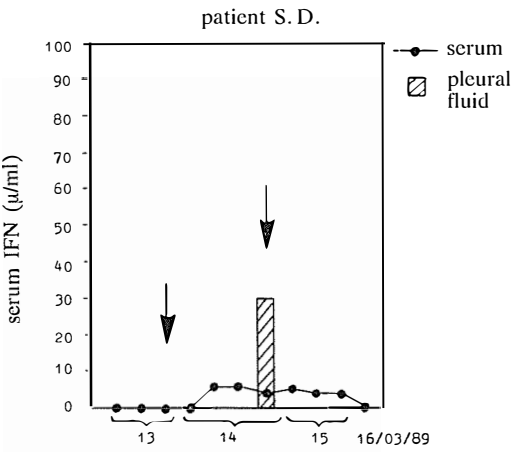

Figure 3. Pharmacokinetics of serum IFN during therapy. Bars represent IFN in pleural fluid. IFN application is indicated by arrows. Curve(s) are daily serum IFN levels.

Improvement in the survival was also noted. Even in this series of patients treated with radiation therapy and IFN-α, the treatment was tolerated well; an increased temperature and local pain within the 24 hours after application and reactive lymphadenopathy (Pat.No. 2) were the only complications. Coagulopathy (Pat. No. 4) was more likely a complication of the treatment for thrombosis than of IFN-α application.

The level of IFN-α in 2 patients under investigation showed only a minimal rise of the serum levels, an observation different from the one in a recent series of patients with pleural effusion due to breast cancer treated in a similar way.[24] As this observation is based only on 2 patients, a larger group studied, though not included in this series, will be part of a separate report.

There are still many uncertainties regarding the treatment of lung cancer with IFN-α, the dosage and the timing of treatment being the most obvious. While in our series a tendency to better survival is shown for the patients treated with IFN-α, the difference in survival is not statistically significant (possibly due to the small series), and on the other hand, it could be due to radiation therapy alone. Radiation therapy has not been shown to affect the survival of patients with inoperable lung cancer. It has, however, not been tried in patients with pleural carcinosis.[27] Because of a trend towards improved survival in patients treated with IFN-α and the good tolerance for combined treatment with radiation and IFN-α we have started a randomized trial.

The patients with lung cancer and pleural carcinosis will be treated either with radiation alone or radiation and IFN-α. We will also continue to study the serum levels after intrapleural applications of IFN-α.

Acknowledgement

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Adjuvant treatment of malignant melanoma with human leukocyte interferon after radical surgery: I. general analysis

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In our randomized prospective study, patients with malignant melanoma were treated with human leukocyte interferon (HLI) after surgical removal of primary tumor (Clark level of invasion IV, V and/or thickness exceeding 1.5 mm). They were randomized in two groups: (1) those treated with HLI and (2) a control group with no immediate treatment. HLI was applied through 30 weeks in cummulative dose 6×10^7 U ,and 2×10^6 U weekly. Both arms of the study included altogether 321 patients. The results of 5-year analysis showed significant differences in disease-free interval as well as in survival between both groups in favour of HLI treated patients ($p < 0.005$). In the treated group the rate of NED patients was significantly higher than in the control group. According to the stratification by sex, the difference was significant also between female as well as male patients of both groups ($p < 0.005$). In a majority of patients HLI application caused a flu-like syndrome, whereas adverse effects on blood count and chemistry could not be established. The treatment (given in the reported dose) was not toxic and could be applied on an out-patients basis.

Key words: melanoma-therapy; surgery, operative; interferons

Introduction

In the world, patients with malignant melanoma of the skin represent approximately 1 % of all cancer patients. The incidence of melanoma has been rapidly increasing, reaching its double value every 6-10 years, and likewise, also melanoma-related mortality has been exhibiting a trend of constant increase. Also in Slovenia, the yearly incidence of cutaneous melanoma by sex shows tendency of increase.[1] In the survival analysis study[2] of malignant melanoma in Slovenia, overall 5-year survival was 57.5 %, and median survival 108 months; 5-year survival by sex was 66.4 % for females and 38.5 % for males. Using univariate analysis of the sex and other clinical and pathohistological variables on the survival a statistical significant difference was established so for sex as well as for the extent of the invasion by Clark levels. Irrespective of the sex, a statistically significant better survival was found in the group of patients with thinner melanoma.

Considering the high mortality rates observed in patients with malignant melanoma (with deep level of invasion) as well as ineffective treat-

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ment of advanced disease, many studies have been investigating the potential of various treatment modalities.

Since these results of malignant melanoma treatment are still unsatisfactory, especially in advanced stages of disease, an effort should be directed to earlier treatment. Unfortunately, the results of adjuvant treatment in the early stage of the disease with chemotherapy[3] have also not confirmed the effectiveness of treatment so far.

During the last decade a number of clinical studies have been performed to investigate the therapeutic potential of interferons in the treatment of various malignant diseases.[4] Although partial and occasional complete regressions have been observed in some cancer patients[5] the overall results of single-agent interferon treatment point out the need for further clinical and laboratory research in order to establish the role of interferon in cancer treatment, particularly in solid tumors. Besides exerting a direct antiproliferative effect on mammalian cells, interferons have proved to be potent activators of natural killer cells and macrophages.[6] These cells have also been involved as effectors in host resistance to tumor development and in tumor control processes.[7, 8]

In view of the previously mentioned facts, we decided to established the role of interferon as an adjunct to surgical treatment of primary malignant melanoma. A prospective randomized trial[9] was commenced in 1988 in patients with malignant melanoma stage IIA and B according to the AJCC classification.[10]

Patients and methods

Three-hundred and twenty-one patient with malignant melanoma entered the study. In the protocol only patients with histologically proven primary tumor after radical surgery were included. As mentioned previously, all the patients were in Stage IIA and IIB of the disease which means that the primary tumors were classified as Clark IV,V level of invasion and/or tumor thickness exceeding 1.5 mm. The patients were randomized in two protocol arms – a group treated with human leukocyte interferon (HHLI) and control group with no immediate treatment after radical surgery (HCON) as shown in the protocol summary (Figure 1).

All patients in both groups were on regular clinical follow-up. Complete blood counts, blood chemistry, renal and liver function tests were taken each check; these were performed monthly in the first 2 years, and later on in 2 month intervals. Complete evaluation of patients was done before and after the treatment. Patients with relapse (in both groups) were further treated as necessary (with surgery, radiotherapy, chemotherapy) and were afterward also on regular follow-up.

Treatment

Treatment consisted of i/m application of crude human leukocyte interferon (Imunološki zavod, Zagreb, Croatia) and started within the first month after surgical excision. Interferon was applied for 30 weeks in cumulative dose of 6×10^7 units. Each patient received 2×10^6 units of interferon weekly. Since at the start of the study only human leukocyte interferon was available, this analysis refers only to the application of this agent, while later in the study the additional group of patients treated with recombinant interferon alpha was introduced and the results will be published separately.

HLI group

A total of 160 patients, 70 males and 90 females, have been entered in the HHLI group. The mean age of patients was 48 years (48 ± 14 years, range 20 – 78 years). Patients were distributed according to tthe primary tumor site as follows: head and neck region (HN) – 15 ; trunk (T) – 79; limbs (L) – 66 . Primary tumors were determined as superficial-spreading type (SSM) in 31 cases, nodular type (NM) in 127 cases and *lentigo maligna* type (LMM) in two cases. The level of invasion was Clark IV in 109 cases, and Clark V in 12 cases. In 39 cases the level of invasion was Clark III, but tumor thickness exceeded 1.5 mm, which was in accordance with protocol criteria.

CON group

The control group comprised 161 randomly selected patients (71 males and 90 females) in the mean age of 52 years (52 ± 13 years, range 21-84 years). As to the primary tumor site, lesions were located in head and neck region in 16 cases, on the limbs in 70 and on the trunk in 75 cases. In 92 patients tumors were assessed as SSM type, in 3 patients as LMM and in 66 patients as NM type. The level of invasion was Clark III in 23 cases (but thickness more than 1.5 mm), Clark IV in 101 cases, and Clark V in 12 cases.

Patient distribution by various potential prognostic factors is presented in Table 1. Our analysis showed that both protocol groups, i.e. HHLI and HCON, were similar as to their sex and age distribution. Also, there was no major difference in site and type of primary tumor, and neither in its level of invasion.

Statistical analysis

The statistical analysis was done using the Kaplan-Meier product-limit method[11, 12] which is a non-parametrical method to estimate the probability of an event occuring during a given time-interval. Statistical significance of graphed survival curves was tested using logrank program which performs a chi-square like analysis.[13, 14, 15]

Table 1. Comparison of HLI and CON group according to sex and age distribution, type and localization of primary tumor and level of invasion.

Data		H L I No.	Group Percentage	C O N No.	Group Percentage
Sex:	M	70	44 %	71	44 %
	F	90	56 %	90	56 %
Age:	<53	100	63 %	81	50 %
	>53	60	37 %	80	50 %
Type:	NM	127	79 %	66	41 %
	SSM	31	20 %	92	57 %
	LM	2	1 %	3	2 %
Local.:	Trunk	79	49 %	75	47 %
	HNeck	15	9 %	16	10 %
	Limbs	68	41 %	70	43 %
Clark	III	39	24 %	38	23 %
	IV	109	68 %	101	62 %
	V	12	8 %	12	7 %
TOTAL		160	100 %	161	100 %

Results

Survival analysis

Survival curves of patients in HHLI and HCON group are presented in Figure 2. Survival of patients treated with human leukocyte interferon (HHLI.dbf) is significantly higher than in the control (HCON.dbf) group ($p < 0.005$).

Survival curves of patients according to sex distribution in control group (HCON) are presented in Figure 3. Control female patients had significantly higher survival (HCONF.dbf curve) when compared with male controls (HCONM.dbf curve), which is consistent with previous observations about influence of sex on the prognosis. The difference between both groups is significant ($p < 0.001$). Similar is the situation in HHLI group (Figure 4), though the difference between female (HHLIF.dbf curve) and male (HHLIM.dbf curve) patients is not significant ($p = 0.07$). Primary tumor site influenced the survival of patients in the control group. The difference between group of patients with tumors on limbs (HCONL.dbf curve) and patients with tumors in trunk region (HCONT.dbf curve) is significant ($p < 0.05$) in favour of limbs site, which is presented in Figure 5. In HLI group the similar difference was not significant (HHLIL.dbf curve versus

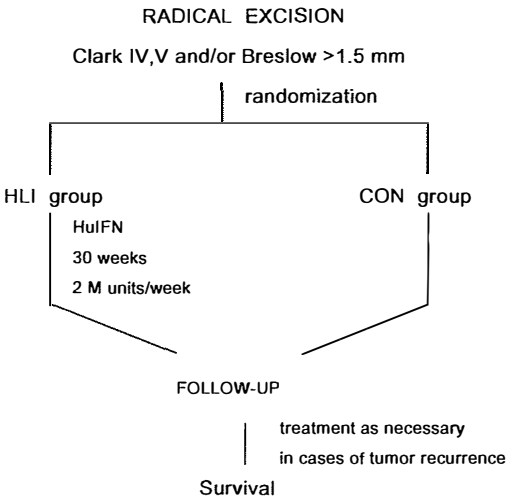

Figure 1. Protocol summary.

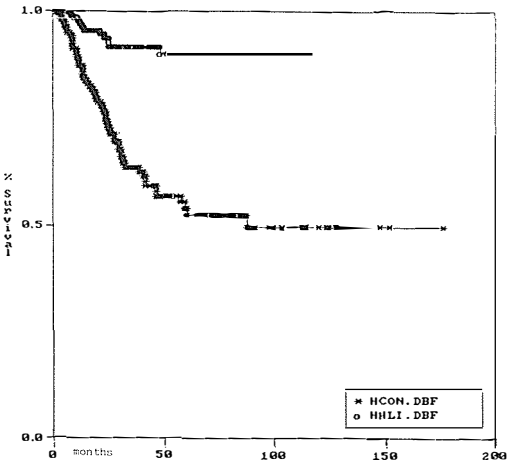

Figure 2. Survival analysis of melanoma patients in both protocol groups. (HCON.dbf – patients in control group, HHLI.dbf – patients in treated group; the difference is significant, $p < 0.001$).

HHLIT.dbf curve, $p = 0.5$). The type of primary tumor did not significantly influence the survival in both protocol groups (Figure 6), and also the impact of age of patients could not be established, as illustrated in Figure 7.

The difference between treated and control patients is significant also by sex stratification (Figure 8). Females in the treated group had better survival than female controls; likewise, male patients treated with interferon survived longer than male patients in the control group (HHLIF.dbf curve vs. HCONF.dbf curve, and HHLIM.dbf curve vs. HCONM.dbf curve; $p < 0.005$).

Interferon treatment was well tolerated by majority of patients and no patient declined it because of toxic side effects. In all patients the

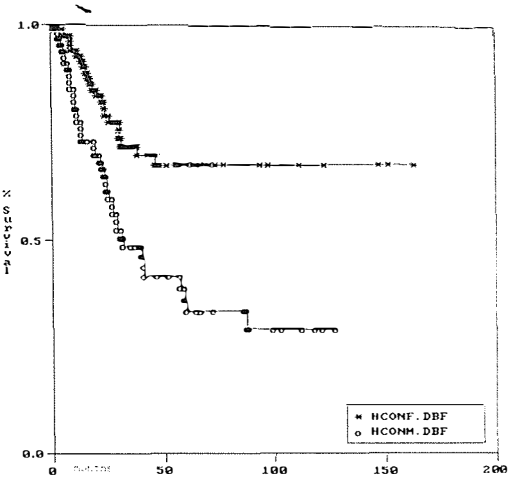

Figure 3. Survival comparison between male and female patients in control group. (HCONM.dbf - males, HCONF.dbf – females; the difference is significant, $p < 0.001$).

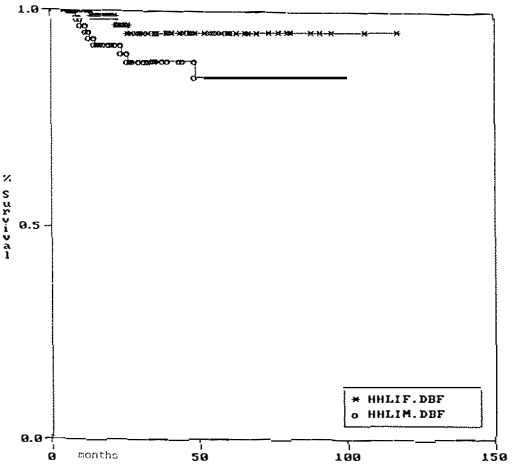

Figure 4. Survival comparison between male and female patients in treated group. (HHLIM.dbf – males, HHLIF.dbf – females; the difference is not significant, $p = 0.07$).

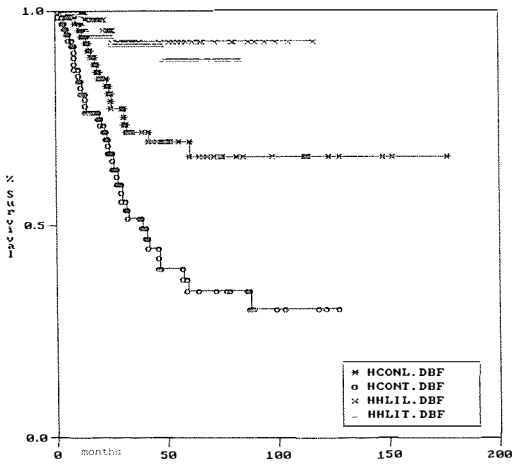

Figure 5. Survival comparison in both protocol groups according to the primary tumor site. (HCONL.dbf – control patients, primary tumor on limbs, HCONT.dbf – control patients, primary tumor in trunk, HHLIL.dbf – created patients, primary tumor on limbs, HHLIT.dbf – treated patients, primary tumor in trunk; HCONL.dbf vs. HCONT.dbf significant, $p < 0.05$; HHLIL.dbf vs HHLIT.dbf not significant, $p = 0.5$).

application of interferon was followed by mild up to moderate fever (less than 39° C) which was transient. The patients experienced also

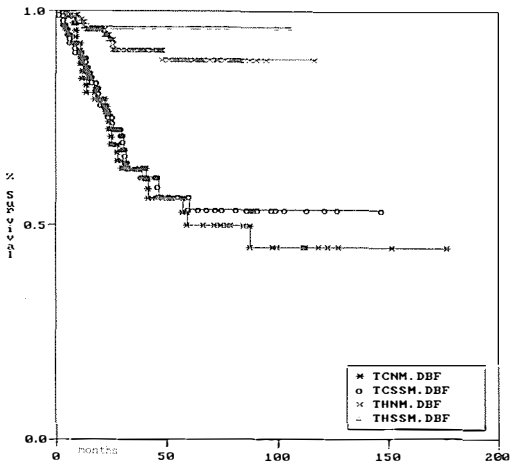

Figure 6. Survival comparison in both protocol groups according to the primary tumor type. (TCNM.dbf – control patients, nodular melanoma, TCSSM.dbf – control patients, superficial spreading melanoma, THNM.dbf – treated patients, nodular melanoma, THSSM.dbf – treated patients, superficial spreading melanoma).

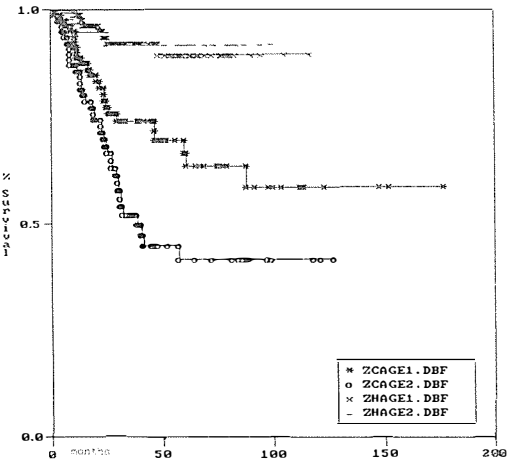

Figure 7. Survival comparison in both protocol groups according to the age of melanoma patients. (ZCAGE1.dbf – control patients, age <53 years, ZCAGE2.dbf – control patients, age >53 years, ZHAGE1.dbf – treated patients, age <53 years, ZHAGE2.dbf – treated patients, age >53 years).

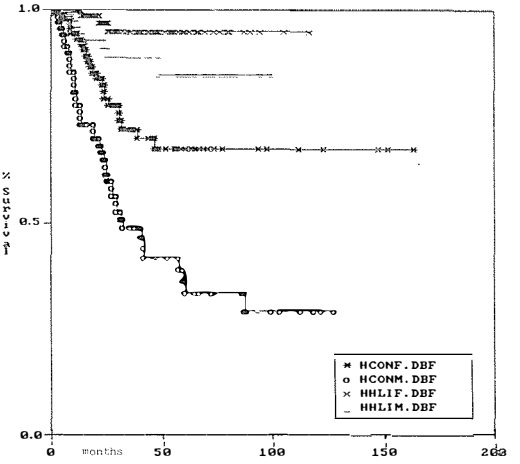

Figure 8. Comparison of survival between both protocol groups by sex stratification. (HHLIF.dbf – treated females, HHLIM.dbf – treated males, HCONF.dbf – female controls, HCONM.dbf – male controls; HHLI.dbf vs. HCONF.dbf and HHLIM.dbf vs. HCONM.dbf significant, $p < 0.005$).

flu-like syndrom, which was antecipated. The application of interferon in performed dosage exerted no effect on blood counts and chemistry. In one case, as reported previously,[16]

moderate allergic reaction manifested with urticaria followed the second course of the treatment. Since the fever and flu-like syndrom were transient, after pilot study it was decided that the regimen should be applied on out-patients basis.

Discussion

The increasing incidence of melanoma and its tendency to affect younger adults, as well as relative ineffectiveness of treatment in advanced stages, point out the need for an effective adjuvant therapy.

In our study interferon treatment was found to have influenced the survival of patients, since the difference between both protocol groups (HLI and CON) was significant. In addition, the difference between male as well as female patients of both groups was also significant. According to these results, it can be postulated that the interferon treatment prolonged the survival of patients in treated group.

Also, the treatment was not associated with significant toxic side effects. The reason for allergic reaction in one case is most probably in crude extract of human leukocyte interferon containing various potential allergens.

Possible mechanisms of interferon action have not been fully explained yet. Theoretically, interferons could exert their antitumor effects in three ways: (1) via the host immune system; (2) by altering some non-immune host/tumor cell interactions; or (3) by direct effects on the tumor cells. It is known that tumors sensitive to interferon alpha tend to be slow growing and moderately well differentiated. Moreover, in contrast to other types of cancer therapy, responses to alpha interferons are typically slow, with haematological and bone marrow improvement often taking several months in leukemia cases. Data from laboratory animals suggest that interferon act best when tumor load is low, such as was the situation in our study.[4, 9] Interferons are also an important part of lymphokine cascade. It is reasonable to conclude, that interferon could act as biological response modifier through many yet uknown mechanisms including lymphokine cascade.

According to our results, the interferon adjuvant treatment can be advised in cases with prognostically unfavourable melanoma, i.e. primary melanoma tumors with level of invasion Clark IV,V and/or thickness more than 1.5 mm.

Acknowledgement

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Breast imaging at ECR'93

Vienna, September 12–17, 1993

The 8th European Congress of Radiology held in Vienna in early autumn this year was attended by about ten thousand professionals. Technical, educational and scientific aspects of numerous topics including breast imaging were discussed. Breast imaging, now firmly rooted in early detection and management of breast cancer together with recent advances and relevant views about this rapidly evolving discipline were dealt with in a "Categorical course" offered by the organiser.

Mammography (MG) won the central role in the diagnosis of breast disease and particularly in the very sensitive field of mass-screening for asymptomatic breast cancer which, in developed countries, will soon afflict up to one third of women. Early objections against extensive use of MG because of the risk of radiation-induced cancer, have proven unfounded. The exhibition accompanying the congress showed that the industry had fully embraced the modern concepts of dedicated apparatus which limits the radiation burden of a typical investigation to mere 0.5 mGv and simultaneously enables imaging with high contrast and fine detail. This is being achieved with special anti-scatter grids, an automatic exposure-control, a focus of 0.6 mm or less, a voltage range from 25 to 35 kV and high-resolution film-screens (Friedrich M). It was stressed, however, that in order to insure adequate functioning of this equipment, regular daily, weekly, monthly and annual quality-control program should be rigorously followed and only accepted phantoms used (Thomas BA).

Equal emphasis was laid on the professional skill of image-readers, particularly by German and Dutch authors with wide experience in mass-screening programs (Hessler C; Hendriks JHCL). Intensive, repeated training courses for medical and technical personnel should replace self-education wherever possible. It was even suggested that the accreditation for MG should be time-limited and attendance in regular refreshing courses a sine qua non (Friedrich M). The performance quality of the diagnostic team should be periodically assessed by reviewing the final results including the reevaluation of false reports and interval cancer cases (10). In MG, it was said, mediocrity can hardly be tolerated (Hendriks JHCL).

MG features of breast cancer are well known and, in most cases, a skilled radiologist can deliver a correct diagnosis. For various reasons, though, false-negatives cannot be avoided, particularly in screening as proved by the incidence of interval cancers. In clinical work, false-negatives destroy the clinician's confidence and their rate should not exceed 5 %–10 % of histologically proven cases. The overall incidence of false-positive reports is hard to estimate but they seem to result from overinterpretation of microcalcifications.

Microcalcifications themselves are a rather sensitive sign of malignancy though highly unspecific. Efforts to improve the accuracy of MG images by analysing their semiology in great detail were not successful. Therefored, simpler, commonly identifiable and easily describable morphological criteria (according to "Le Gal")

were advocated, therefore, particularly to facilitate reporting, communication and teaching (Lamarque JL). High quality of the image is essential and magnification techniques may be helpful (Del Turco MR).

The radiologist's task of detecting locally recurrent cancer is becoming ever so frequent since more patients are being new treated conservatively. Swelling, scarring and haematomas after surgery and/or irradiation may utterly change the radiological appearance of the breast tissue and obscure early malignant regrowth. Nevertheless, a recurrence can be recognised during regular MG follow-up after initial treatment since its density and size increases, while post-treatment changes tend to clear up with time (Schreer I).

Benign breast lesions are very frequent, very variable and difficult to diagnose correctly. In all doubtful cases, additional diagnostic techniques should be used liberally including ductography, pneumocystography, ultrasound, clinical examination and stereotaxically guided thin-needle biopsy before surgical biopsy is resorted to. Unlike malignant lesions where a mere suspicion may suffice for further diagnostic work-up, benign diseases call for an accurate diagnosis. In order to reduce the number of unnecessary biopsies, the skill of the radiologist, again, cannot be overemphasized (di Maggio C).

Ultrasound imaging is safe and generally accepted. In the examination of the breast, however, its role remains limited in spite of the experience gathered in the last ten years with this diagnostic modality. In the screening of unsymptomatic women it has no value at all but has retained a few firm indications in clinical diagnostic work particularly in fluid detection in palpable lumps, examination of young women and visual guidance of the needle during biopsy (van Kaick G).

Magnetic resonance imaging (MRI) is a relatively new diagnostic modality. Its main advantage is: absence of any radiation risk. It enables a much better distinction between fat, cellular tissue and fluid which together with the possibility of visualisation in any desired plane, greatly facilitates recognition of the disease. The disadvantage of MRI is: high cost and very limited availability of the procedure (Heywang-Koebrunner SH).

ECR'93 was a great professional event in every respect including the splendid exhibition of radiological equipment. It is a pity that so few Slovene radiologists attended the very instructive categorical course on breast imaging.

Breda Jančar, MD
The Institute of Oncology

Radiol Oncol 1993; 27: 341.

Book review

Gastrointestinal Radiology: A concise Text. By Branko M. Plavsic, Arvin E. Robinson and R. Brooke Jeffrey Jr. New York: McGraw Hill, 1992 (557 pp)

The book, absolutely necessary at the time of modern image technique, shortly summarizes all knowledge from the conventional gastrointestinal radiology. During the past several years many descriptions of the CT, MR and ultrasound have been found in the literature. The place of the conventional barium studies of the gastrointestinal tract remains the important base in the radiological diagnostic procedures. The book presented here fills that important space in the current literature and proves the complete overwiev over the methods, techniques and radiological characteristics of the gastrointestinal radiological procedures. The modern image technique are only sparsely presented to complete the diagnostic approach to the various diagnostic problems of the alimentary canal.

The book is divided logical into 12 chapters. All of them are illustrated with a number of excellent figures. According to the intention of the authors to give the complete presentation of the problems, the first three chapters cover normal anatomy of the alimentary canal, gastrointestinal physiology and radiological aspects of gastrointestinal pathology. All basic data which are of importance for the understanding of the gastrointestinal examinations are presented. In the fourth chapter the authors give very concise description of various techniques of examining of the gut. The precise presentation of the double contrast examination is given, including the basic data about contrast media and pharmacoradiography. To complete the radiological presentation of the gastrointestinal tract, the authors describe all other techniques like CT, MR, ultrasound, arteriography and scintigraphy. Very useful instructions for the evaluation of radiological methods and making the radiological reports are given. Also the role of the intervention radiology is shortly presented.

The main parts of the book are the next eight chapters which describe the various pathological changes in the alimentary tract. In the logical rows the radiological pathology of the pharynks, esophagus, stomach, duodenum, small bowel and colon is described an illustrated with a number of illustrations. All illustrations are of excellent quality. Using more than seventy figures in each chapter, the authors illustrated almost each pathological entity. The special attention was given to the diseases which affect the both small and large bowel, such as intestinal ischemia, infections and infestations, ulcerative colitis and Chron's disease. To present the differential diagnosis between similar radiological symptoms the authors use very simple and instructive tables and diagrams. The diseases of very important localization like ileocecal area and rectum are presented in the separate chapter. That fact indicates that except general knowledge of various problems the authors include in the book their own experience.

Because of very simple and precise presentation of the problems, the concise and illustrative descriptions and excellent functional approach to the gastrointestinal pathology, the book serves an important textbook for residents the source of various data for the radiologists. At the same time it is unavoidable for gastroenterologists, surgeons, pediatricians and medical students.

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Radiol Oncol 1993; 27: 342-3.

Notices

Notices submitted for publication should contain a mailing address, phone and/or fax number of a contact person or department.

Lymphomas

The ESO seminar will be offered *April 21–22, 1994.*

Contact Miss Gollubics, ESO-Vienna-Office, Arztekammer für Wien, Fortbildungsreferat Weihburggasse 10–12, A-1010 Vienna, Austria; or call + 43 1 51501 293. Fax: + 43 1 51501 240.

Oncology

The 11th research workshop (Journees Grenobloises de Cancerologie) "Chemoresistance: from bench to clinical trials", will take place in Grenoble, France, *March 24–25, 1994.*

Contact Pr. C. Vrousos, Hopital Albert Michallon. B. P. 217, 38043 Grenoble Cedex 9, France.

Nuclear medicine

The British Nuclear Medicine Society annual meeting will be held in London, United Kingdom, *March 28–30, 1994.*

Contact Mrs S. Hatchard, The BNMS Conference Secretary, 157 Auckland Road, London SE 19 2RH.

Magnetic Resonance

The 1st Nottingham symposium on Magnetic Resonance in Medicine will be held in Nottingham, United Kingdom, *April 6–8, 1994.*

Contact Julie Mills, University Hospital, Nottingham NG & 2UH, United Kingdom; or call + 44 602 709 951. Fax: + 44 602 424 994.

Brachyradiotherapy

ESTRO teaching course "Modern Brachytherapy Techniques" will take place in Tübingen, Germany, *April 10–14, 1994.*

Contact the ESTRO Secretariat, Radiotherapy Department, University Hospital St Rafael, B-3000 Leuven, Belgium; or call + 32 16 33-64-13. Fax: + 32 16 33 64 28.

Lung cancer – biology and clinical aspects

The 2nd central European conference on lung cancer will be offered in Ljubljana, Slovenia, *June 13–16, 1994.*

Contact The Conference Secretariat, Department of Thoracic Surgery, Medical Center, Zaloška 7, 61105 Ljubljana, Slovenia; or call + 386 61 317 562. Fax: + 386 61 1316 006.

Magnetic Resonance

The "11th Annual Scientific Meeting and Exhibition of the European Society for Magnetic Resonance in Medicine and Biology" (ESMRMB) will be held in Vienna, Austria, *April 20–24, 1994.*

Contact Vienna Academy of Postgraduate Medical Education and Research, Alser Strasse 4, 1090 Vienna, Austria; or call + 43 1 42 138 313. Fax: + 43 1 42 138 323.

Brachyradiotherapy

The 76th annual meeting American Radium Society will take place in Southampton Princess Hotel, Bermuda, *April 22–26, 1994.*

Contact Office of the Secretariat, American Radium Society, 1101 Market Street, 14th Floor, Philadelphia, PA 19107, USA; or call + 1 215 574 3179. Fax: + 1 215 928 0153.

Radiotherapy

ESTRO teaching course "Principles and Technical Aspects of Clinical Radiotherapy" will be offered in Bari, Italy, *April 24–28, 1994.*

Contact the ESTRO Secretariat, Radiotherapy Department, University Hospital St Rafael, B-3000 Leuven, Belgium; or call + 32 16 33-64-13. Fax: + 32 16 33 64 28.

Neuroblastoma

The international meeting "Ten years of experience

in neuroblastoma. Quo vadis MIBG?" will be offered in Kiel, Germany, *April 25–26, 1994.*

Contact H. Bihl MD PhD, Dept. of Nuclear Medicine, Katharinenhospital, Kriegsbergstr. 60, 70174 Stuttgart, Germany; or call +49 711 278 4300. Fax: +49 711 278 4309.

Skin cancer and melanoma

The ESO teaching course will be offered *May 4–8, 1994.*

Contact Miss Gollubics, ESO-Vienna-Office, Arztekammer für Wien, Fortbildungsreferat Weihburggasse 10–12, A-1010 Vienna, Austria; or call +43 1 51501 293. Fax: +43 1 51501 240.

Brachytherapy

The 11th annual brachytherapy meeting GEC-ESTRO will be held in Linz, Austria, *May 9–11, 1994.*

Contact the ESTRO Secretariat, Radiotherapy Department, University Hospital St Rafael, B-3000 Leuven, Belgium; or call +32 16 33-64-13. Fax: +32 16 33 64 28.

Radiotherapy

The refresher course in radiation oncology will be offered in Toronto, Ontario, Canada, *May 11–13, 1994.*

Contact Continuing Education, Faculty of Medicine, University of Toronto, Medical Sciences Bldg., Toronto, M5S 1A8, Canada; or call +1 416 978 2719. Fax: +1 416 971 2200.

Cancer research

The "25th Annual Meeting American Association for Cancer Research" will take place in Dallas, Texas, USA, *May 11–14, 1994.*

Contact Amer. Assoc. Cancer Res., Meetings Mailing List AACR, Public Ledger Building, 6th and Chestnut Streets, Suite 816, Philadelphia, Pa 19106, USA; or call +1 215 440 9300. Fax: +1 215 440 9313.

Radiotherapy

The 4th annual meeting of the American College of Radiation Oncology will be offered in Washington, DC, USA, *May 14–16, 1994.*

Contact ACRO Office, P.O. Box 12920, Philadelphia, PA 19108, USA; or call +1 215 762 4993. Fax: +1 215 762 8523.

Pediatric neurooncology

The international symposium will be held in M. D. Anderson Cancer Center, Houston, Texas, USA, *May 18–21, 1994.*

Contact MDACC, Univ. of Texas, Conf. Services, Box 131, 1515 Holcombe Blvd., Houston, TX 77030-4095, USA; or call +1 713 792 2222.

Gynaecology

International gynaecological symposium will be offered in Bruges, Belgium, *May 26–28, 1994.*

Contact Hilde Deckers-Van Overmeiren, Vlaamse Vereniging voor Obstretrie en Gynaecologie, Azalealaan 10, Sint Niklaas, Belgium; or call +32 (0)3 776 03 64. Fax: +32 (0)3 766 07 56.

Gynecological endocrinology

The 4th world congress of gynecological endocrinology will be held in Antalya, Turkey, *May 28 – June 3, 1994.*

Contact Medical Congress and Research S. A., Via della pace 5 (Palazzo Centro), CH-6601 Locarno, Switzerland; or call +41 93 322 932. Fax: +41 93 319 836.

Surgical oncology

The tripartite meeting of European Society of Surgical Oncology, British Association of Surgical Oncology and Chirurgishe Arbeitsgemeinschaft für Onkologie, subtitled "Frontiers and Perspectives of Surgical Oncology" will take place in Heidelberg, Germany, *June 8–11, 1994.*

Contact PD Dr. Thomas Lehnert, Department of Surgery, University of Heidelberg, Im Neuenheimer Feld 110, d-69120 Heidelberg, FRG; or call +49 6221 566 207. Fax: +49 6221 565 450.

Update in urological oncology

The ESO teaching course will be offered *June 9–11, 1994.*

Contact Miss Gollubics, ESO-Vienna-Office, Arztekammer für Wien, Fortbildungsreferat Weihburggasse 10–12, A-1010 Vienna, Austria; or call +43 1 51501 293. Fax: +43 1 51501 240.

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Reviewers in 1993

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Editors greatly appreciate the work of the reviewers which significantly contributed to the improved quality of our journal.

LUNG CANCER
BIOLOGY
AND CLINICAL ASPECTS

Under the Auspices of
International Association for the Study of Lung Cancer

13–16 april 1994

Ljubljana – Slovenia

Second announcement

Call for abstracts
& registration

Important dates

Deadline for Early Registration at reduced rate	28 February 1994
Deadline for Cancellation Requests	15 March 1994
Deadline for Guaranteed Hotel Accommodation	21 March 1994
Date of the Conference	13–16 April 1994
Opening Ceremony	13 April 1994

Organizing committee

Chairman: J. Orel
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J. Tobias (United Kingdom)
J. Viale (Italy)
I. Vogt-Moykopf (Germany)
N. van Zandwijk (The Netherlands)

Invited speakers and lectnres

V. Pompe-Kirn (Slovenia)
Epidermiological features of lung cancer in Slovenia

B. Corrin (U. K.)
Premalignant lesions

G. Viale (Italy)
Oncogenes and tumor suppressor genes in lung tumours (With special emphasis on neuroendocrine tumours)

J. Šorli (Slovenia)
Methodology and results of bronchopulmonary cancer detection in Slovenia 1970–1992

D. Ferluga (Slovenia)
Significance of immunohistochemistry for classification of lung cancer

M. Mermolja (Slovenia)
Possibilities and limitations of cytology in the diagnosis of lung tumours

A. Debeljak (Slovenia)
Transbronchial aspiration needle biopsy with flexible and rigid needle in the diagnostic and evaluation of regional spread of lung cancer

P. Rocmans (Belgium)
Limits of surgery for non-small cell lung cancer

M. I. Perelman (Russia)
Reconstructive surgery for tracheobronchial tumours

L. K. Lacquet (The Netherlands)
Complete resection for unsuspected N2 non-small cell lung cancer (Stage III A)

P. Rocmans (Belgium)
Patterns of failure after "complete resection" of lung cancer

J. B. Sørensen (Denmark)
The role of chemotherapy in non-small cell lung cancer

K. Mattson (Filand)
New drugs for non-small cell lung cancer

J. B. Sørensen (Denmark)
Prognostic factors in non-small cell lung cancer

N. van Zandwijk (The Netherlands)
EORTC studies in non-small cell lung cancer

T. Lewinski (Poland)
Staging of small cell lung cancer and its scope according toclinical implications

K. Karrer (Austria)
The importance of surgery in the multimodality treatment of small cell lung cancer

K. Havemann (Germany)
Receptors of the steroid hormone superfamily: a new approachfor the treatment of small cell lung cancer

F. R. Hirsch (Denmark)
Treatment of small cell lung cancer – the Copenhagenexperience

J. Tobias (U. K.)
Ten years of studies in small cell lung cancer from the LondonLung Cancer group

K. Kolarić (Croatia)
Farmorubicin – a new active compound in

the treatment of metastatic small cell lung cancer

N. van Zandwijk (The Netherlands)
EORTC Studies in small cell lung cancer

L. K. Lacquet (The Netherlands)
Surgical treatment of multiple lung cancer

P. Goldstraw (U. K.)
Post pneumonectomy empyema

U. Pastorino (Italy)
Second primary lung cancer: clinical management and chemoprevention

K. Moghissi (U.K.)
Laser in lung cancer – The present and the future

M. Debevec (Slovenia)
The role of radiotherapy in the treatment of lung cancer

I. Vogt-Moykopf (Germany)
Results in surgery on pulmonary metastases

Call for abstract

Free papers

All participants are invited to submit abstracts for Free Papers session. The papers will be arranged and scheduled according to the topics of the Conference.

Posters

Posters will be displayed in the Congress Centre and discussedat poster session. Participants must submit abstracts also for poster presentation. The Scientific Committee will decide whether a submitted paper will be selected for oral or poster presentation.

Video sessions

Video sessions are scheduled to take place during the Conference. Authors of videos are requested to submit the abstract of their video on the same form as for other presentations.

Technical equipment

Double slide projection and overhead projection will be madeavailable in all sessions. Video projection in video sessions and in other sessions on request.

Slides

Speakers are kindly requested to hand their slides and check them through at least one hour before the beginning of their session, at the slide center.

Abstracts

Abstracts must be mailed together with 3 photocopies to the Conference Secretariat.

Department of Thoracic Surgery,
Medical Center,
Zaloška 7,
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Tel.: + 386 61 317 582
Fax: + 386 61 1316 006

Conference information
DATE

The Conference will be held on 13–16 April 1994, Ljubljana, Slovenia

Venue

CANKARJEV DOM
Cultural and Congress Centre,
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Conference organizers

CANKARJEV DOM
Congress Department,
Prešernova 10,
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Tel.: + 386 61 210 956
Fax: + 386 61 217 431

Conference secretariat

Department of Thoracic Surgery
University Medical Centre,
Zaloška 7,
61105 Ljubljana, Slovenia.
Tel.: + 386 61 317 582
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Conference language

The language of the Conference is English. There will be no simultaneous translation facilities.

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INFORMATION

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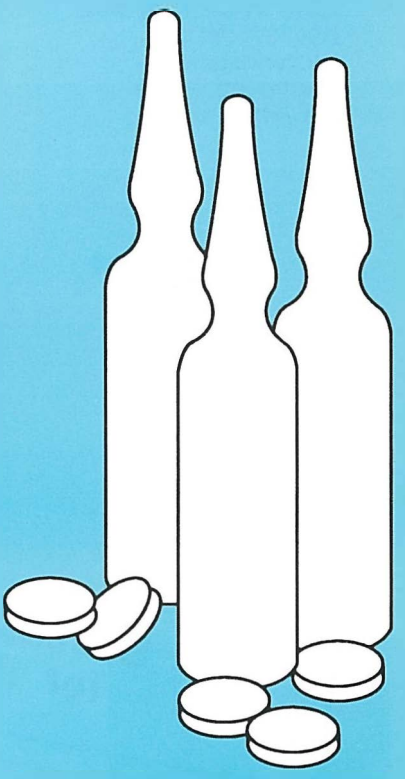

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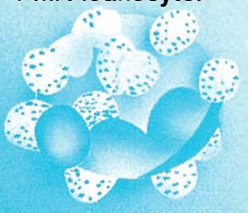

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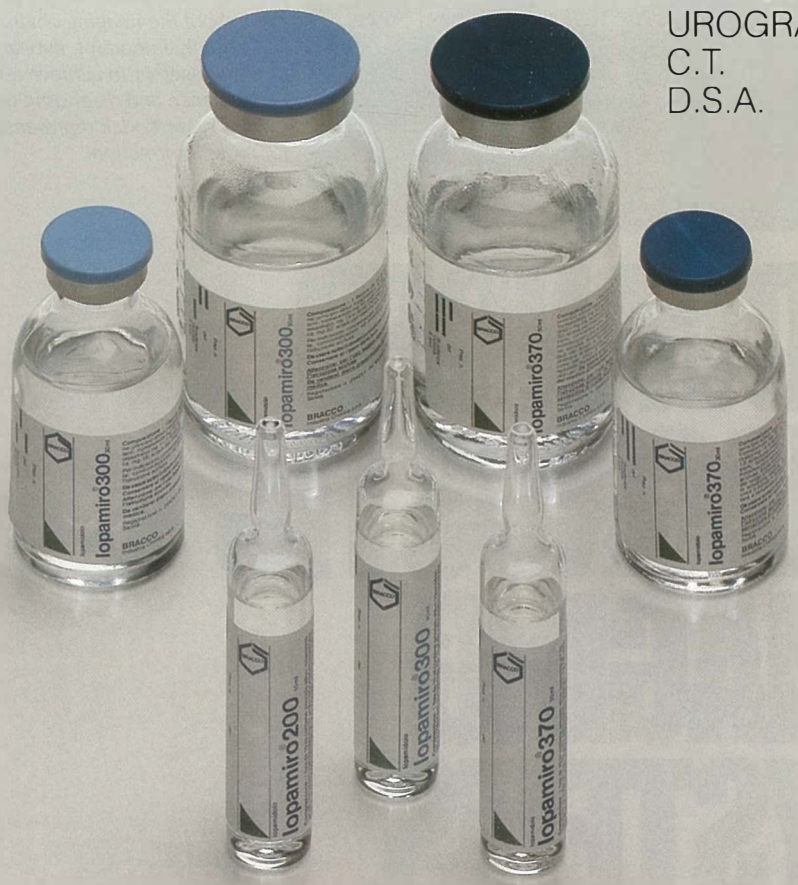

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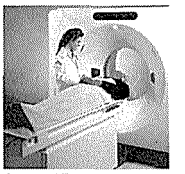

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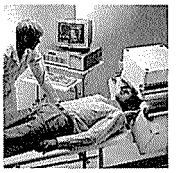

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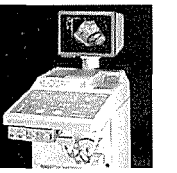
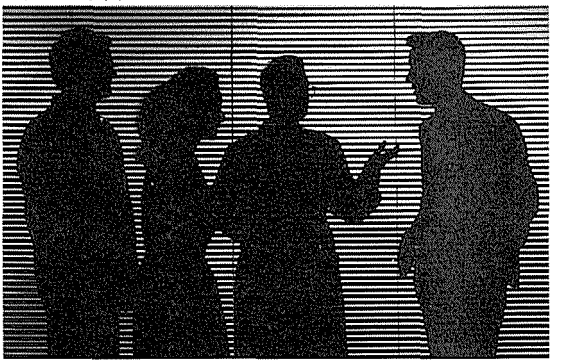
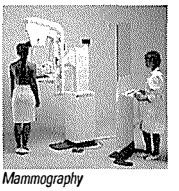


Ultrasound

Multi Modality





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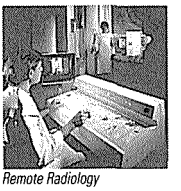

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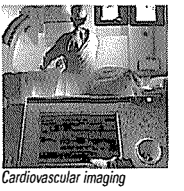

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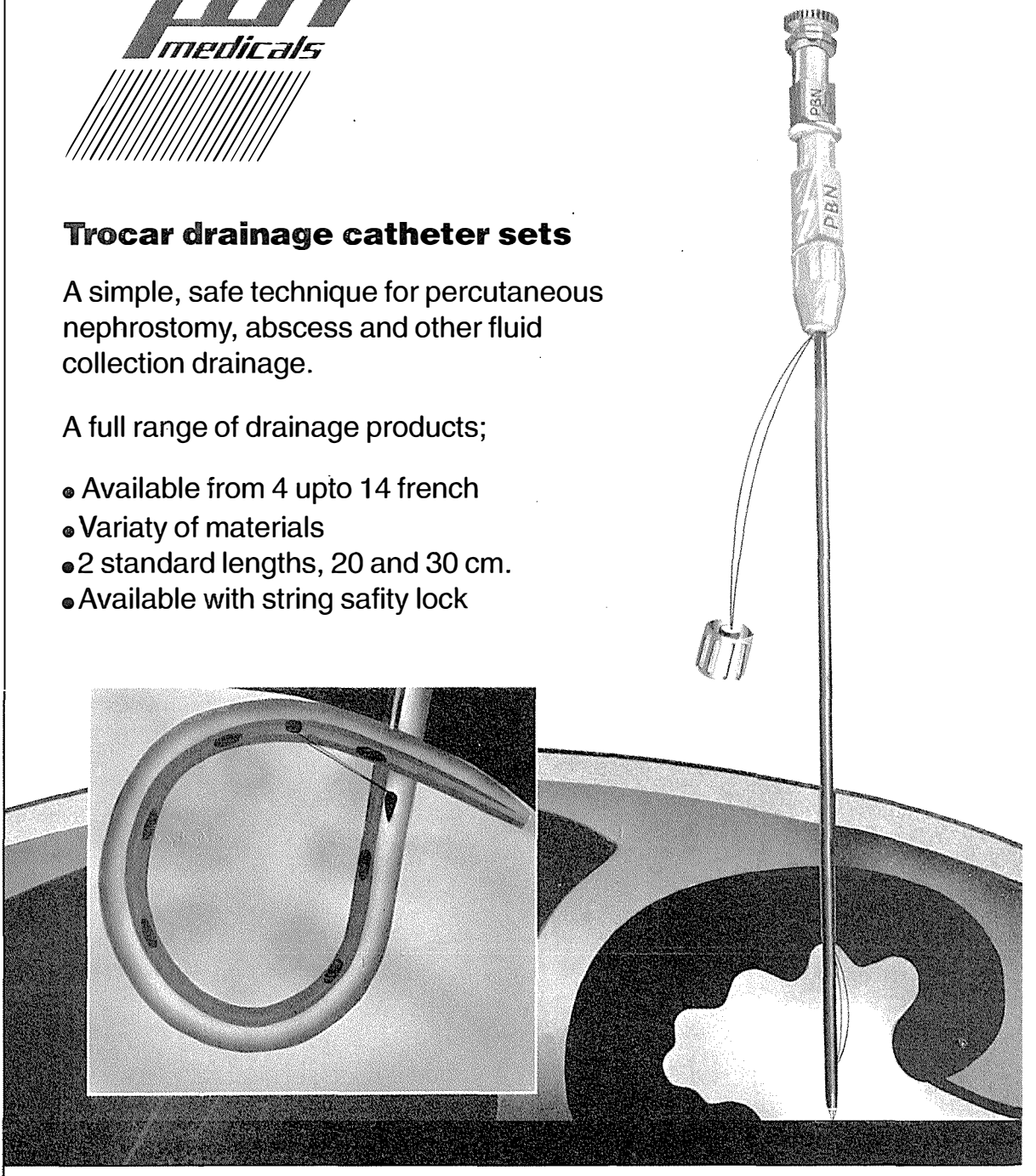

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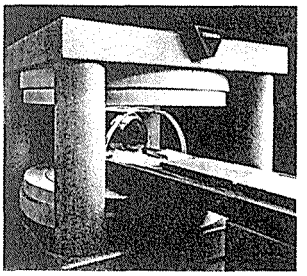

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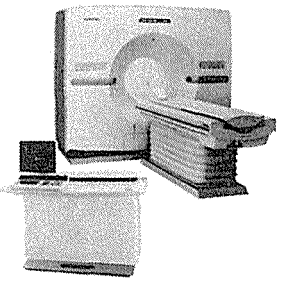

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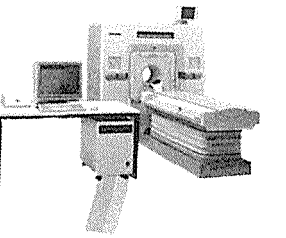

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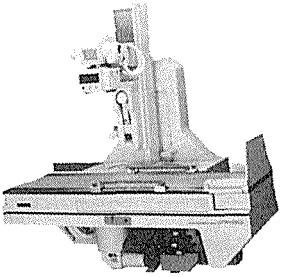

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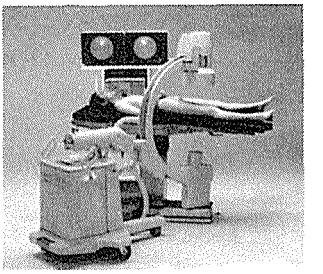

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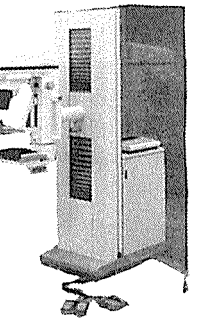

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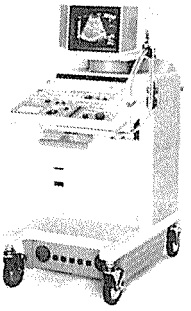

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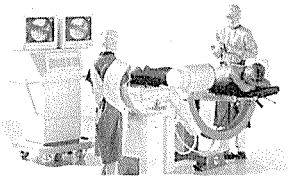

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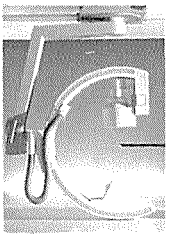

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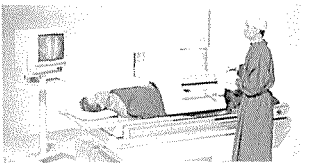

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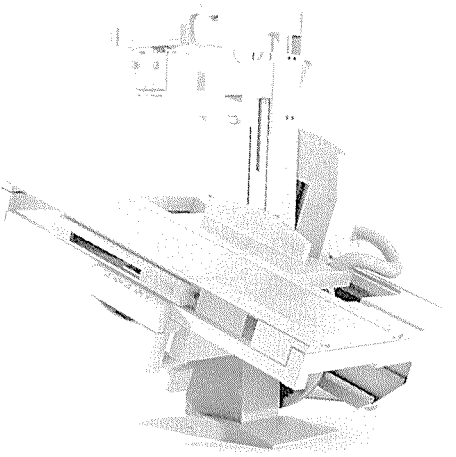

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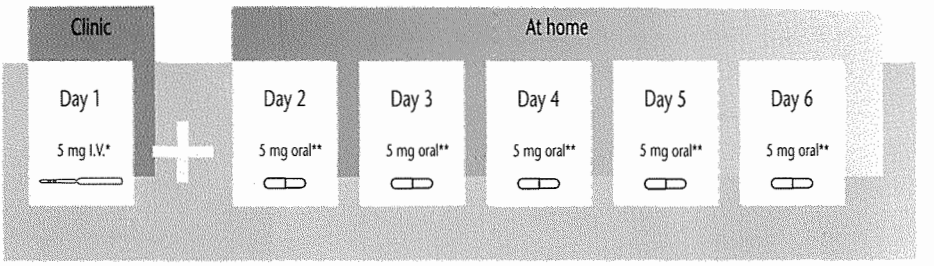

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