SPECTRUM OF BIOLOGICAL ACTIVITY OF INTERFERONS

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Summary. - The interferons comprise a group of proteins first identified by their ability to protect cells against virus infections but also capable of influencing cellular physiology. They are synthesized and secreted by a variety of cell types in response to various inducers. Their effects include antiviral action, inhibition of cell proliferation, modulation of cell differentiation and activation of various cell types in immune system. This review aims to summarize the current state of biology of interferon action with special emphasis on those aspects related to the use of these molecules in antitumoral therapy. The antitumor effects of IFNs results from pleiotropic IFN activity exerted either directly on tumor cells (i.e. antiproliferative effects, effects on oncogene expression, on cell differentiation and enhanced expression of cell surface antigens), or via indirect effects (i.e. activation of effector mechanisms of the host as modulation of the expression of the major histocompatibility antigens, effects on macrophages, NK, T and B cells).

Introduction

Interferons (IFNs) are a family of proteins that act in a species specific manner. Our knowledge of the IFN genes and of the biological effects induced by their expression, is based, predominantly, on studies in humans and mice. An extensive review regarding these molecules and their effects has recently been published [1].

IFNs molecules are classified in three major species, i.e. α-, β- and γ-IFN, based on molecular structure, antigenicity and mode of induction. α-IFN consists of a family of molecules, characterized by specific aminoacidic differences and each subtype is identified with a number (i.e. α1-IFN, α2-IFN and so on). In humans and mice only one β-IFN and one γ-IFN have been identified, the human (hu) β2-IFN molecule being now classified as IL-6. Alpha and β-IFN are usually acid stable and were referred in the past as type I IFNs in contrast to γ-IFN that was referred as type II or immune IFN. For the sake of clarity it must be stressed that the terms α/β-IFN and human leukocyte or lymphoblastoid IFN are referred respectively to: a) a mixture of undefined α-IFN subtypes together with β-IFN, in varying proportion and not precisely identified by specific antigen recognition (this kind of preparation, named α/β-IFN, is obtained by inducing various cell line in vitro, i.e. mouse L or C243 cells, via virus infection or poly I:C treatment); b) hu IFN subtypes derived from Sendai virus-induced buffy coats or from lymphoblastoid (usually Namalva) cells. Hu leukocyte and lymphoblastoid IFNs undoubtedly contain hu α-IFN subtypes, but, until they are totally purified to homogeneity they may contain other cytokines, or other IFN species in low amounts. The term fibroblast IFN, frequently used in the past for β-IFN, is misleading, since fibroblasts can produce more than one IFN species. Production of γ-IFN is restricted to hematopoietic cells involved in immune response and is induced by antigenic or mitogenic stimuli.
In humans and mice multiple genes code for the many different α-IFN proteins, but there is only one gene coding for β-IFN and one for γ-IFN. IFNs are secreted proteins, synthesized as pre-IFNs, and membrane insertion is assured by the leader sequence at the N-terminal end. α and β-IFN genes lack introns and are located on the same chromosome (chr9 in humans and chr4 in mice). In contrast, γ-IFN contains three introns and is located on hu chr12 and on murine (mu) chr10. There are at least 24 non allelic genes or pseudogenes for human α-IFN proteins and at least 12 for the mouse counterpart. Hu IFN genes and molecules have been more extensively studied as they were being developed for clinical applications. Mature α-IFN sequences contain 166-7 amino acids with the exception of hu α2-IFN (165 aa) and mu α2-IFN which has an internal five-codon deletion. Hu α-IFN contrary to mu α-IFN, is usually not glycosylated and it has been subdivided into two classes: hu α- and α2-IFN, based on significant differences in the overall structure (165-166 aa versus 172 aa and higher divergence in the coding sequence between the members of the two families). These differences result in extensive antigenic differences between the two classes of proteins. α1-IFN molecules are also called α-IFN [2], Beta-IFN is a glycosylated protein (166 aa in humans and 161 in mice). The aminoacid homology between hu β-IFN and hu α-IFN proteins is about 30%. Since α and β-IFN genes can be coordinately induced, it is not surprising that there is extensive sequence homology between the 5' flanking regions of the hu β- and α-IFN genes upstream the TATA-box. This region play a critical role in the inducible expression of the IFN genes [3-5]. Gamma-IFN (146 aa in humans and 134 in mice) is a glycosylated protein characterized both in mice and in humans by a remarkably high content of basic residues, that may be related to the acid lability of its biological activity. The 5' flanking region of the hu γ-IFN gene contains nucleotide sequences also found in the 5' flanking regions of other genes that are specifically expressed by T cells, such as the structural gene for IL-2 and the gene encoding the IL-2 receptor. This sequence, about 200 bp long, functions as an inducible transcriptional enhancer and appears to control T cell specific gene expression [6, 7]. Another region of potential regulatory importance is located in the first intron and is 83% homologous to a sequence in the 5' flanking region of the IL-2 gene [8].

Alpha and β-IFN probably originated from a common ancestor by gene duplication. On the contrary the evolutionary relationship of the γ-IFN gene to the other IFN genes is not clear. The only evidence is that murine α-, β- and γ-IFN share at least one common epitope and both human and murine IFNs present sequence homologies within the third exon of the γ-IFN gene.

To exert their different biological activities, IFNs need to bind to specific receptors at the cell surface. Alpha and β-IFN share a common receptor site at the cell membrane, whereas γ-IFN has its own receptor: the average number of receptor sites per cell is in the order of few thousands.

The total number of genes whose expression can either be induced or enhanced by all the different subtypes of α-IFN or by β-IFN is probably the same. We do not know the precise number of these genes: in one system, human fetal lung fibroblasts treated with hu leukocyte IFN, the enhancement or the de novo induction of 17 polypeptides has been identified by two-dimensional electrophoresis. In the same cells, hu γ-IFN, which binds to a different receptor, induces the synthesis of 24 polypeptides, 17 of which are induced by α-IFN, plus another 7 that are not found in α-IFN treated cells [9-11]. Some but probably not all, the IFN-induced proteins appear to be produced via a primary response of the corresponding genes. Several IFN-induced proteins have been described, but only few have been characterized in more detail (i.e. the 2-5A synthetase, the RAS L, the cAMP-independent protein kinase P1, the major histocompatibility complex (MHC) class I and II cell surface antigens and the Mx protein). Other IFN-induced proteins have been identified, but not characterized for their involvement in the mechanism of the IFNs-action (i.e. some guanylate binding proteins, tubulin, metallothionien II, indoleamine 2, 3-dioxygenase) or have been identified solely by means of their molecular weight and cells in which they are induced.

In spite of their number, the biological activities of the various α-IFN subtypes seem to be relatively similar, as well the biological activity of α- and β-IFN. However, the possibility does exist that α- and β-IFN share different biological properties exclusively with respect to the antiproliferative activity. Indication of the existence of differences in the biological activity comes from the human system: it takes 100 x molecules of hu α1-IFN as compared to hu α2-IFN to induce an antiviral effect of comparable magnitude and about 1,000 x to obtain a comparable stimulation of natural killer (NK) activity [12]. On the other hand, hu α1-IFN is able to induce human monocytes to express MHC class II antigens and to produce IL-1, whereas hu α2-IFN is unable to do the same at comparable concentrations [13]. Comparison of the biological activities of different α-IFN subtypes shows that they differ also for the antiproliferative effect [14]. It will be necessary to explore in much greater detail all the possible differences between β-IFN and the various α-IFN subtypes before the reason for the existence of so many α-IFN subtypes becomes clear.

The antiviral effect

IFNs have been discovered by their antiviral action, that remains one of the most widely studied aspects of IFN research [15]. Many different RNA and DNA animal viruses are sensitive to this IFN effect and multiple molecular mechanisms are involved. Type I IFN inhibits the synthesis of viral macromolecules of many viruses (picornaviridae, rabdoviridae, orthomixoviridae, reoviridae, poxviridae, adenoviridae, herpesvirdiae). The inhibition of macromolecular synthesis is often primarily exerted at the step of translation of viral mRNA with some exception
regarding inhibition of earlier or later stages of the multiplication cycle, which include the assembly and the release of the progeny virions. Inhibition of the early stages of life cycle were observed for SV40 and retroviruses. The replication of SV40, a member of the papovaviridae, appears to be inhibited by α/β-IFN in permissive monkey cells at the level of uncoating of the parental virions, thus preventing the subsequent formation of active early transcriptional complexes [16-25]. IFN treatment fails if SV40 infection precedes the IFN treatment. In addition, in the acute exogenous retroviral infection, α/β-IFN appears to act at an early stage in the retrovirus multiplication cycle, preventing either the synthesis or the integration of proviral DNA [26-30]. IFN treatment can prevent the transformation of murine fibroblasts by the Kirsten strain of murine sarcoma viruses by blocking a stage after uncoating, but before the integration of the provirus [31].

IFN-induced inhibition of the late stages of viral multiplication has been observed in cells infected by Vesicular Stomatitis Virus (VSV), Herpes Simplex Virus-1 (HSV-1), vaccinia virus and in cells chronically infected by retroviruses. The late stages of the life cycle represent the main target of the IFN-induced inhibition of retrovirus multiplication in cells and is observed in the absence of a significant reduction of viral macromolecular synthesis. IFN treatment greatly reduces either the number of retrovirus particles released from the infected cells [32-38] or the specific infectivity of those virions that are released [34-39]. An increase in the number of viral particles associated with the host plasma membrane is also often observed in systems where the number of particles released is reduced. Inhibition or alteration of glycosylation has been proposed as a possible mechanism. It is possible that this inhibitory action involves other IFN-induced cellular effects that determine alteration in cellular components of the plasma membrane or cytoskeleton, including the significant decrease in membrane fluidity [40]. Molecularly cloned type I and II IFNs inhibit the replication of immunosuppressive viruses [41-43].

The three viruses, VSV, HSV-1 and vaccinia, represent three different enveloped viruses that are inhibited at several stages of virus multiplication cycle. In these circumstances the inhibition of the late stages of the replication cycle is observed in some, but not all, cell lines and may reflect the changes in the structure and function of plasma membrane and cell surface induced by IFN independently from virus infection [44, 45]. This may affect the multiplication of those viruses dependent upon host cell plasmamembrane for assembly or release.

The two IFN-induced enzymatic pathways of 2-5A synthetase and of c-AMP-independent eIF2α-protein kinase (pk) are both involved in the inhibition of viral macromolecular synthesis observed with respect to many different viruses. Depending upon its genetic complexity, virus penetration is followed by partial uncoating with formation of subviral particles or nucleocapsid structures containing an activated virion-associated transcriptase or by complete uncoating eliciting free virus genomes available for direct transcription and/or translation by cellular enzymes. The 2-5A synthetase-induced degradation of viral RNA is certainly involved in the inhibition of picornavirus replication [46]. The IFN-induced cAMP-independent pk is certainly involved in the inhibition of VSV life cycle. In fact by inhibiting this enzyme [47] adenovirus infection rescues VSV from the antiviral effect of IFN in cells treated with IFN and coinfected with both viruses. In this respect adenoviruses, like poxviruses, are quite resistant to the antiviral effect of IFN in cell culture probably via their ability to inhibit the activation of the IFN-induced eIF2α-pk, mediated by adenovirus-coded VA RNA.

It is probable that both the 2-5A synthetase and the pk pathways participate in obtaining the inhibition of replication of a lot of animal viruses, but the action of one of them may be prevalent, depending on the peculiar characteristics of the life cycle of each particular class of viruses.

In the case of VSV it has been observed that the major step of virus replication inhibited by the same type of IFN (αA-IFN) is likely dependent upon the type of cell examined. In fact, translation inhibition is observed in epithelial-like amnion U cells [48, 49] and inhibition of accumulation of primary RNA transcripts is observed in human fibroblastic GM 2767 cells [50-52]. Synergism between α/β- and γ-IFN and has been observed with Ectromelia virus (EMCV) and VSV suggesting a unique mechanism. This possibility is supported by the observation that both natural and molecularly cloned γ-IFN inhibit the production of infectious reovirus progeny in human amnion and fibroblast cells, insensitive to the action of hu α-IFN [53, 54]. With respect to reovirus the principal antiviral effect seems to be exerted at the level of translation of primary mRNA transcript.

An host cell gene (Mx gene) induced by α/β-IFN controls susceptibility of the mouse to the antiviral action versus influenza virus. Mx protein alone is sufficient to account for the inhibition of the virus [55]. In this case the site of action of α/β-IFN is probably located between primary and secondary transcription.

HSV-1 and -2 replication is clearly inhibited by α/β-IFN, but contradictory results have been obtained as for the blocked stage. Both natural and molecularly cloned α-IFN and β-IFN, but not γ- inhibit HSV replication in human macrophages [56, 57]. Synergistic antiviral activity between γ-IFN and α/β-IFN has been observed in murine macrophages and in human cells, but not in human macrophages. The principal target in IFN-mediated inhibition of HSV-replication is the inhibition of translation of HSVm RNA into proteins. On the contrary, HSV replication is blocked by type I-IFNs in human fibroblasts at a later stage of the viral cycle. Production of defective virions [58] or inhibition of release of the virions [59, 60] has been observed. In addition IFN plays a crucial role in maintaining persistent infection of macrophages [61].

The site of IFN action and the mechanism described above were determined by in vitro experiment. The nature of the host cell, the type of IFN and the kind of virus that infects the cells are all important parameters that influence
the antiviral effect of IFN both in vitro and in vivo. The modulation of expression of the histocompatibility antigens (see the following paragraphs) induced by IFN represents another element that play an important role in the efficacy of the antiviral effect of IFN in vivo.

Effects of IFNs on the physiology of normal and tumorigenic mammalian cells

Interferon influence the behaviour of mammalian cells in multiple ways. The interaction(s) of IFNs with many other cytokines, growth factors, oncogenes and with the various effector cells of the immune system are essential to understand the regulatory role of these molecules. The effects of IFNs on tumor cells are of interest to address their utilization in human therapy.

Similar to the antiviral activity, the antitumor effect of IFNs results from pleiotropic IFN activity, exerted either directly on the tumor cells or via the host by the complex immunomodulatory action. The mechanism of the antitumor effect and its efficacy is strictly related to the host status and to the specific characteristics of the tumoral cells.

Direct effects of interferons on cellular physiology can be identified as: 1) antiproliferative effects; 2) effects on oncogenes expression; 3) effects on cell differentiation; 4) enhanced expression of cell surface antigens.

Direct effect on tumor cells

Antiproliferative effects. - The antiproliferative effects of interferons are elicited both in normal and in tumor cells even if striking differences in sensitivity are observed. The usual effect of all three interferon species is the inhibition of cellular replication. The majority of the studies on cell growth regulation have been carried out in tissue culture. A wide range of cell types has been used, including normal cells, permanent lines of non tumorigenic cells, such as 3T3 mouse fibroblast, and transformed tumor cells of a variety of histological types. In these cell lines, as in cells freshly isolated from tumors, a wide spectrum of variability is observed. EBV-transformed human lymphoblastoid cells are extremely sensitive to the antiproliferative action of the hu α- and β-IFN, but not to that of hu γ-IFN, while Namalva cells, another EBV transformed cell line, are virtually resistant. Cell clones resistant to the effect of interferons can be selected from sensitive cell types [62-64]. In general, γ-IFN is a more potent inhibitor of cell growth (relative to its antiviral potency) than either α- or β-IFN [65-67]. Furthermore, there is evidence for a synergistic potentiation of the antiproliferative activity between mu γ-IFN and mu α- or β-IFN [67] suggesting again that these classes of interferon have different mechanisms of action. Studies have also been carried out to determine the relative sensitivities of normal versus tumoral cells to the antiproliferative effect of IFNs. Evidence for direct cytostatic effect of interferon on the growth of various human tumor cells in vivo has been provided by experiments in nude mice developing xenografts inoculated with hu lymphoblastoid IFN, hu leukocyte IFN or rec hu α2-IFN. In some cases this treatment lead to complete regression of the tumors [68-72].

In the clinic, direct inoculation of IFN into tumors results in a decreased tumor mass [73, 74] (see "Clinical applications of IFNs as antitumor agents").

Effects on oncogenes expression. - Proto-oncogenes (c-ons) are cellular genes which share a high degree of sequence homology with the oncogenes of retrovirus (v-ons). The viral oncogenes thus appear to have arisen by the transduction of the cellular proto-oncogenes from the genomes of vertebrates cells into retroviral genomes [75]. During this process the c-onc is altered, originating an abnormal gene controlled by the viral elements. In this way the v-onc gene product can be functionally different from that of the normal gene and is no longer subject to the stringent regulation operative in normal cells. Other mechanisms of abnormal activation of the c-ons have been observed, namely after retroviral enhancer-promoter or enhancer insertion into the c-onc gene or via DNA rearrangements, chromosomal translocations or point mutations. Abnormal expression and/or abnormal function are two key conditions leading to malignant transformation.

Studies on interactions between oncogenes and interferons, acting as "negative growth factors", have started only a few years ago. Among the over 40 oncogenes identified in humans and mice, so far, only c-myc, c-fos, c-ras and c-mos have been shown to be influenced by IFNs.

The myc oncogene. - The c-myc proto-oncogene is the cellular homologue of the v-myc oncogene of avian myelocytomatisis virus, an acute avian leukemia virus. The c-myc gene product is a nuclear protein that binds to dsDNA and is present in avian, murine and human cells. The gene has been highly conserved throughout evolution and is on chromosome 8 in humans and on chromosome 15 in the mouse [76-78]. The expression of c-myc is correlated with proliferative activity of the cell and plays a role also in malignant transformation. In fact, while c-myc by itself is not transforming in rodent fibroblasts in vitro, it cooperates with the ras oncogene to transform primary rat embryo fibroblast into focus-forming cells that give rise to tumors in nude mice [79].

Jonak and Knight [80] and Einat et al. [81] first reported that β-IFN and α-IFN reduced c-myc mRNA levels in Daudi Burkitt's lymphoma cells. Furthermore Einat et al. showed that this alteration resulted from a reduction in the transcription rate of the c-myc gene and, by comparing wild-type cells with an IFN-resistant Daudi cell variant, they established a tight connection between the decline of c-myc mRNA induced by IFN and the subsequent IFN-mediated G0/G1 arrest characteristic of Daudi cells. On the contrary, α-IFN failed to reduce c-myc mRNA content and to change the cell-cycle distribution in three leukemia cell lines (HL-60, U937 and Friend erythroleukemia) representing different cell lineages of the hemato-
poietic system. Then they extended the screening to a number of other cell lines and found that, while the majority of hematopoietic cells failed to respond to the IFN-induced inhibition of c-myc, several cell lines responded like Daudi, in particular Molt-4 T cells, P3HR-1 Burkitt lymphoma cells and M1 myeloid cells. Thus it is possible to classify the hematopoietic cell lines into two groups according to the responsiveness of c-myc to IFN: "sensitive" cells where c-myc is down-regulated and "resistant" cells, which fail to reduce c-myc in response to IFN.

In addition to c-myc, the genes for β-actin and ornithine decarboxylase and another proto-oncogene, c-fos, are also inhibited by IFN [82]. It is therefore not possible to decide whether the inhibition of the c-myc expression is a direct IFN-induced effect on the c-myc gene, or occurs via a cascade effect resulting from the inhibition of the expression of other competence genes (so called because stimulated by platelet derived growth factor, that renders fibroblast "competent" to engage DNA synthesis) that are activated before c-myc. A decrease in c-myc mRNA level is not always a prerequisite for an impairment of cell proliferation by IFNs, when expression vectors containing the c-myc gene are introduced into Balb/c 3T3 cells (which are sensitive to IFN inhibition of cell growth and c-myc mRNA expression) the transformed cells become partially resistant to the antigrowth effects of IFNs and can partly overcome the inhibition of the G0/G1 to S transition [83]. Since terminally differentiated cells accumulate in the G0/G1 resting phase of the cell cycle, it was interesting to test whether these cells produced IFN during the progressive loss of their proliferative potential. To approach this question Remiztinsky et al. [84] choose, among the various cell differentiation system, one that avoids the use of chemical inducers, i.e. the murine M1 myeloid cells. These cells can be induced to differentiate by lung-conditioned medium enriched in CSF-G. The differentiation inducer gradually stopped the proliferation of the M1 myeloid cells that accumulated in the G0/G1 resting phase of the cell cycle. Neutralizing antibodies against the autocrine IFN, added together with the inducer, abrogated part of this decline in the growth rate, as reflected by the higher cell number and the lower percentage of cells found in G0/G1 phase. Furthermore they reported that the endogenous IFN, immunologically related to β-IFN, is part of the mechanism controlling the decline in c-myc mRNA and that the addition of antibodies against IFN partially abrogated the reduction of c-myc mRNA.

In other cell systems that produce endogenous IFN (e.g. PMA-induced U937 cells and Me2SO-induced mouse erythroleukemia cells), the antibodies against IFN did not abrogate the reduction of c-myc expression observed during the course of the differentiation process. This is likely due to the direct effects on c-myc expression induced by PMA and Me2SO in these cell systems, thus probably bypassing some of the antigrowth effects of autocrine IFN [84].

In light of the involvement of autocrine IFN in c-myc regulation, Kimchi et al. [85] investigated the molecular basis of the loss of sensitivity to IFN of tumor cells and studied how this specific deregulation might affect the process of terminal differentiation. They analyzed the response to IFN of somatic cell hybrids between sensitive and resistant Burkitt lymphoma cells and found that the IFN-mediated transcriptional inhibition of c-myc was dominant over the nonresponsiveness of resistant cells, and that changes in cis-acting regulatory elements of c-myc were not involved in the relaxed regulation.

By isolating IFN-resistant cell variants from the M1 myeloid cell line they demonstrated that the loss of sensitivity to IFN changed the growth kinetics of cells during in vitro induced differentiation. Genetic deregulation which disrupts the suppression of c-myc by IFN results from the loss or inactivation of elements which operate in trans and interferes with the normal growth arrest of differentiating cells.

Although the IFN-mediated arrest in the G0/G1 phase probably involves complex changes in gene expression, apart from the reduction of c-myc, all these data suggest that c-myc inhibition is necessary (but not sufficient) for the development of this type of growth arrest.

The ras oncogenes. - The human and rodent cellular proto-oncogenes c-ras are the homologues of the oncogenes of the Harvey (v-Ha-ras) and Kirsten (v-Ki-ras) sarcoma viruses. There are five members of the human ras gene family, c-Ha-ras (1 and 2), c-Ki-ras (1 and 2) and N-ras. N-ras was first isolated from a human neuroblastoma cell line [86] and has not been associated with a retrovirus; c-Ha-ras was first isolated from three human bladder cell lines (EJ, T24 and J82) [86-88]; Ki-ras has been found in a number of carcinomas but especially in those of lung and colon [88, 89] and also in rhabdomyosarcoma [89] and in a cell line from an intermediate T cell ALL [90]. The five identified members of the ras gene family are located on five different chromosomes. All members of the ras gene family encode antigenically related proteins with a molecular weight of about 21 kDa, which have been named p21s. These polypeptides differ mainly in the carboxyterminal regions. They are membrane bound, largely to the plasma membrane, they bind guanine nucleotides and display GTPase activity. In mammalian cells the function of the p21 c-ras proteins is not known, but there is evidence to suggest that their activity is required for cell division, which can be triggered by ras proteins and blocked by antibodies to these proteins. Ras genes are activated to oncogenes by either point mutations or linkage to a long terminal repeat and occasionally by amplification of their copy numbers.

Like the expression of c-myc, the expression of ras oncogenes, endogenous or transfected, can be influenced by IFNs.

NIH-3T3 cells transformed with the human Ha-ras 1 gene, when cultured in the continuous presence of mu αβ-IFN, show revertant colonies after 10 to 20 cell generations. These revertants do not grow in soft agar and do not give rise to tumors in nude mice, contrary to the transformed line. The c-Ha-ras DNA is still present, but there is a significant reduction of c-Ha-ras mRNA and of the c-
Ha-ras p21 protein. This is the result of IFN inhibition of the transcription of the c-Ha-ras genes in IFN-treated revertants. After cessation of IFN-treatment a small fraction of the cells regains the transformed phenotype, but the majority keep the revertant phenotype during many cell generations, although the transcription of the c-Ha-ras gene and the levels of p21 ras protein are high again [91, 92]. IFN can also affect the expression of endogenous c-ras oncogenes in their normal chromosomal location. Hu β-IFN decreases the expression of c-Ha-ras in human bladder carcinoma cells, but not the expression of the c-Ki-ras gene. The reduced c-Ha-ras transcription occurs prior to the antiproliferative response to IFN treatment of the carcinoma cells [93].

As for the mechanism of action of IFN, since methylation of DNA at specific regions is an epigenetic mechanism for controlling gene expression and since abnormalities in DNA methylation have been implicated in carcinogenesis, Samid et al. [94] treated the persistent revertant cultures with 5-aza-2’d- deoxycytidine (5 AzadC), a nonmutagenic cytidine analogue that inhibits DNA methylation. These cultures underwent rapid retransformation after transient treatment with 5 AzadC. The analogue 6-azacytidine (6 Azac), which does not affect methylation, did not alter the morphology of revertants. The revertants retransformed by 5 AzadC reverted again with IFN treatment. Retransformation of revertants after treatment with 5 AzadC may involve changes in a gene(s) other than ras, the products of which may modulate or interact with the activated ras in persistent revertants, thus restoring the conditions required for oncogenic transformation. The ability of 5 AzadC to induce tumor progression in nontumorigenic cells expressing an activated ras is of special interest, since demethylating drugs are being used in treatment of human cancer [95].

The mos oncogene. - The c-mos proto-oncogene is the cellular homologue of the v-mos oncogene of Moloney sarcoma virus. In mice it is on chromosome 4 and in humans on chromosome 8. The mos gene codes for a cytoplasmic protein, displaying serine/threonine kinase activity. In most mouse tissues the c-mos gene is either not expressed or is expressed at very low levels. Also in this case, the long-term treatment of Moloney murine sarcoma virus-transformed murine fibroblasts with mu αβ-IFN results in the selection of a cell population that has reverted to a nonmalignant state and that is stable even after many cell generations in the absence of IFN. Similar to what is observed in the revertant c-myc- and Ha-ras-transformed cells, also the v-mos gene expression is not inhibited [96].

Effects on cell differentiation. - IFNs promote cell differentiation in some systems and inhibit it in others. In the mouse the susceptibility to IFNs and the ability to produce it, after induction, appears during development [97]. In 1971 Grossberg and Morahan [98] suggested that IFN mechanisms of action where under a repressed state in embryonic life, at a time where control of growth and differentiation is fundamental. All the cells in the adult organism are susceptible to IFNs action, but not all the cells in the organism are identical. In each tissue, cells are characterized by the expression of specific differentiation patterns and may be present as fully differentiated cells or cells at different stages of the differentiation process. There are several experimental models of cell differentiation, in which the effect of IFN have been studied in the past ten years and some specific reviews are available [97-101]. The picture of this aspect appears confusing, probably because many previous results were obtained using partially purified IFN preparations, often containing a mixture of IFN types (i.e., experiment performed with the so called fibroblast IFN, containing α and β species) or a mixture of IFN and other cytokines (i.e., experiment performed with partially purified preparations of natural γ-IFN). It appears more and more evident that IFNs are molecules that belong to the cytokines family, that regulate cellular response and differentiation by a combined action with other cytokines. A clear example is represented by the effects of γ-IFN on the cell of the immune system (see: "Indirect effects on tumor cells via activation of effector mechanism"). These problems will be overcome by the availability of the single IFN species by the recombinant DNA technology and by the purification of the natural and recombinant species using affinity chromatography with specific monoclonal antibodies. In addition conflicting results obtained on this topic may reflect also the different modulation of the gene expression required for differentiation in each specific lineage. For these reasons, to clarify IFNs effect on differentiation one needs to separate the results obtained with respect to: a) the IFN species used and the purity of the preparation; b) the in vitro or in vivo experimental model; c) the differentiation system examined.

Studies on IFNs effect on cell differentiation has been performed in particular on hematopoietic cells, mioblasts, adipocytes and keratinocytes. Table 1 summarizes the results obtained in these systems. Results regarding hematopoiesis, certainly the area in which most data on the effects of IFNs on differentiation have accumulated, needs a more extensive discussion.

The experimental data available so far support a possible in vivo regulatory action of IFNs on the hematopoietic system. In vitro treatment of normal hematopoietic progenitors with IFNs induces inhibitory effects on CFU-GM, -G, -M, BFU-E and CFU-E development [1, 99, 102, 103]. The inhibition of stem cell development is frequently observed at the relatively low IFN concentration of a few units per milliliter, suggesting that IFNs may exert this effect under physiological conditions in the microenvironment of the spleen or bone marrow, in which low amounts of IFN are frequently present. The degree of growth suppression achieved by the mixture of the three IFNs is higher than the additive effects of each IFN separately. The antiproliferative effects suggest a role for IFNs in the physiological control of progenitor cell proliferation. Interestingly CSF-1 enhances poly rI:C-induced production of mu αβ-IFN by murine macrophages and hu monocytes.
Table 1. - Effects of IFNs on differentiating and differentiated cell systems (*)

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<th>Type of IFN</th>
<th>Differentiation system and effects</th>
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<tr>
<td>Mouse IFN</td>
<td>Melanogenesis</td>
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<td>Inhibition of spontaneous and melanocyte hormone stimulated melanogenesis in cultures of murine B-16 melanoma cells [151]</td>
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<td>Hu-rec αA-IFN</td>
<td>Myogenesis</td>
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<td>Acceleration of myotube formation and creatine kinase [CK] isoenzyme transition from CK-BB to CK-MM in normal human myoblast cultures derived from mature skeletal muscle [152]</td>
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<td>p.p. (***) WS influenza virus induced chicken embryos IFN</td>
<td>Myogenesis</td>
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<td></td>
<td>Inhibition of CK isoenzyme transition and myotube formation of chicken embryo myogenic cells [153-156]</td>
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<td>p.p. Mu αβ-IFN</td>
<td>Adipogenesis</td>
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<td>Inhibition of adipose conversion of Balb/C 3T3 [157] and of Swiss 3T3 [158-161] cell clones</td>
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<td>Hu-rec αA-IFN</td>
<td>Keratinocytes</td>
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<td>Decrease of the amount and synthesis of non-covalently bound keratins and increase of the disulphide cross-linked keratins [162]</td>
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<tr>
<td>Hu-rec γ-IFN</td>
<td>Keratinocytes</td>
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<td>Increased percentage of attached cells with a mature-type, differentiated appearance in cultures of normal human keratinocytes [163]</td>
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<td>Mu αβ-IFN</td>
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<td>Increase of keratin synthesis in cultured mouse epidermal cells [164]</td>
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<td>p.p. Hu leukocyte and rat IFN</td>
<td>Nerve cells</td>
</tr>
<tr>
<td></td>
<td>Enhancement of the excitability of cat and rat nerve cells [165]</td>
</tr>
<tr>
<td>Rat embryo fibroblast IFN</td>
<td>Cardiac cells</td>
</tr>
<tr>
<td></td>
<td>Increase of the beating rate in pulsating rat cardiac cultures [166]</td>
</tr>
<tr>
<td>p. Mu αβ-IFN</td>
<td>Cardiac cells</td>
</tr>
<tr>
<td></td>
<td>Increase in the beating frequency of mouse myocardial cells [167]</td>
</tr>
</tbody>
</table>

(*) effect on hemopoietic cells are not listed (see the text); (***) p.p. = partially purified; p = purified.

[104, 105] and CSF-1 itself induces the synthesis of mu αβ-IFN in murine bone marrow cells thereby down regulating its own growth stimulating effects as well as inducing an antiviral state [106, 107]. The IFN-induced suppression of hemopoietic colony formation is reversed increasing the concentration of CSF-1, but not increasing the concentration of erythropoietin, the two growth factors used respectively in the assay for CFU-M or for BFU-E and CFU-E detection. As is usually the case with IFNs, the effects are not exclusively down-regulatory. Hu γ-IFN inhibits hemopoietic colony formation, and on the other hand stimulates monocytes to release GM-CSF and induces T cells to release GM-CSF and IL-3 [108-110].

In 1983 Persussia et al. [111] described that a component of phytohemagglutin stimulates conditioned medium, identified as γ-IFN, induced terminal monocytic differentiation of human immature myeloid cells from normal bone marrow. A preferential stimulatory effect of γ-IFN on monocytic differentiation has also been observed by the same authors. It has been suggested [112] that fibroblast IFN preparations may have two distinct actions on bone marrow cells supplemented with macrophage-derived colony-stimulating factor: a) inhibition of growth of early precursor cells (detected in semi-solid cultures); b) stimulation of growth and differentiation events occurring at a later stage of monocytic differentiation (detected only in liquid cultures).

According to the inhibitory effect on the growth of hematopoietic progenitors, erythropoiesis and/or leukopoiesis, have been frequently observed [113] in clinical trials carried out with hu α-IFN. The involvement of hu γ-IFN or α-IFN in the development of some cases of aplastic anemia has been suggested [1].

In the past it has also been observed that reticulocytes resemble cells that came in contact with some IFN species. In fact, Hovanessian and Kerr [114] showed that the 2'-5' oligoadenylates synthetase in rabbit reticulocyte lysates proceeds as in extracts from IFN-treated cells.

The 2-5A synthetase appearance is considered a valuable marker for a cell response to IFN treatment. Recently, 2-5A synthetase appearance and induction in chicken embryo erythrocytes during development were observed
by Sokawa and Sokawa [115]. Enzyme activity first appeared in the embryos on the 15th day of incubation, a marked increase being seen 1 or 2 days after hatching. The 2-5A synthetase enzyme is distributed widely in mammalian cells. Basal levels can vastly differ among various cell lines and tissue [116]. In a given cell line 2-5A synthetase activity may vary depending to log vs stationary growth phases. In some truly differentiating systems, the basal levels of 2-5A synthetase were found to be higher: i.e., in the mature and immunocompetent peripheral T splenocytes versus the immature T cells obtained either from the thymus of BALB/c mice or from the spleen of athymic nude mice [117]. This effect might be due to the production of IFN molecules involved in the modulation of the immune response (see below).

Several lines of evidence indicate in vitro that IFNs modulate the differentiation potential of leukemic cells. In the myeloid lineage, hu α-IFN (both native and recombinant) or β-IFN induce cells of the histiocyte lymphoma line U937 to move along the monocytic pathway of differentiation [118]. Recently it has been shown by Testa et al. [119] that hu α- or β-IFN may participate in the inhibition of cell proliferation occurring during cellular differentiation of U937 cells, but only γ-IFN may be involved in the induction of expression of specific monocytic markers involved in cellular immunoregulation (HLA-antigens, Fc and C3b receptors). Perussa et al. [120] also showed that a component of a PHA-CM identified as γ-IFN induces cells of the peripheral blood of patients with chronic myelogenous leukemia to differentiate to monocyte-like cells. Even myeloid cells as mature as metamyelocytes (but not mature neutrophils) can be induced by this γ-IFN preparations to undergo monocytic differentiation.

These observations may offer new insights into the understanding of monocytic recruitment and differentiation during virus infections or immune reactions. Induction of differentiation of the human promyelocytic leukemia cell line HL-60 (non inducible to differentiate by α- or β-IFN treatment alone), exerted in vitro by chemical inducers (12-o-tetradecanoyl-phorbol-13 acetate (TPA), retinoic acid, etc.), can instead be stimulated by α-, β- or γ-IFN [121-123]. It is of interest that purified r-Hu IFN-γ[120] can induce the expression of antigens characteristic of monococytes as well as changes in morphology consistent with monocoid differentiation. Data indicate that de novo protein synthesis is induced by γ-IFN to exert its effects. Antibodies anti-γ-IFN block the effect. Human γ-IFN alone is also able to induce IL-2 receptors on hu peripheral blood monocytes [110].

Similarly to HL60 cells, mouse myeloid leukemic M1 cells could not be induced to differentiate into macrophages and granulocytes by IFNs, which instead “stimulate” the differentiation induced by a number of compounds (D-factor, lipopolysaccharide, poly I:C) [124].

In the erythroid lineage the effects of mouse IFNs on Friend virus-induced erythroleukemic cells (FLC) have been studied by Rossi et al. [99]. Friend-virus (FLV)-induced leukemic cells (FLC) are erythroid precursor blocked in their differentiation pathway at the stage of proerythroblast (late CFU-E). The addition of dimethylsulfoxide (Me2SO) (or of several other compounds) to FLC cultures causes a massive shift towards the normoblast stage of erythropoiesis. This is a bona fide example of terminal differentiation as a complete cessation of cell division occurs even though the cells are still nucleated [125].

Low doses of mu αβ-IFN are able to increase the erythroid differentiation induced by Me2SO or other inducers [126, 127]. Mu β-IFN is certainly responsible of this increased differentiation, in that the effect is obtained using mu recombinant β-IFN and is neutralized by a monoclonal antibody, that recognizes this IFN species [128]. Mu β-IFN is unable per se to induce the differentiation process. When natural and recombinant preparation of mu γ-IFN are used, doses in the range of 0.1-100 U/ml do not affect the process, indicating that no enhancing effects is induced by γ-IFN in this system [128].

Cells of the line K562 may be considered the closest human counterpart of the murine FLC. They respond to hemin or butyric acid induction with a differential expression of the embryonal globin genes. Treatment of K562 cells with hu IFNs results in a modulation of hemoglobin production induced by butyric acid or hemin treatment [129].

All together the results obtained in vitro treating leukemic cell culture with IFNs suggest that they might exert a differentiation inducing effect in vivo in close correlation with other differentiation factors.

The spontaneous production of a β-like IFN molecule (followed by the induction of 2-5A synthetase gene expression) during the late stage of hematopoietic differentiation has been observed by Kimchi et al. and it might play a role as an autocrine mechanism of inhibition of cell growth during terminal differentiation [130-132]. In fact, α- and β-IFN can inhibit cell growth. Inhibition of c-myc expression is probably involved in this effect (see the paragraph "Effect on oncogenes expression"). In a Friend erythroleukemia cell line, Me2SO-induced differentiation is accompanied by the increase of the 2-5A synthetase enzyme that appears to be mediated by FLC secretion of a β-like IFN [133]. However the observation that an α/β IFN-resistant FLC clone [134, 135] is unable to "induce" the 2-5A synthetase system to any detectable level even when exposed to 10,000 U/ml of mouse fibroblast IFN and yet, cells of these clones do differentiate upon induction with Me2SO or hexamethylenemethyacetamide (HMBA) indistinguishably from parental wild type FLC, suggest that 2-5A synthetase and IFN do not play an essential role in the process of induction of differentiation.

IFN production apparently occurs also in human U937 leukemic cells differentiating towards macrophage-like cells. The levels of 2-5A synthetase activity and, incidentally, those of HLA-mRNAs also increase [136]. In these conditions, the administration to the cultures of anti-β-IFN antibodies prevents the elevation of the levels of both
HLA-mRNAs and 2-5A synthetase activity [137]. Mouse myeloid leukemic M1 cells exhibit detectable 2-5A synthetase activity only when they differentiate to phagocytic cells after incubation with conditioned medium (CM) from rat embryonic cells, in the absence of detectable IFN activity in the supernatants. Anti-IFN serum abolished enzyme activation by CM; yet, it did not influence M1 cell differentiation to phagocytes. IFN added exogenously per se neither induced phagocyte activity of these cells nor did enhance the CM-induced differentiation of the cells. Moreover, dexamethasone, which induces differentiation of the same cells, did not induce the enzyme activity [138].

Interesting observations come from IFNs treatment of hairy cell leukemia patients, a form of B cell derived leukemia that can be successfully treated with α-IFN. In many patients the α-IFN treatment results in the disappearance of hairy cells from the peripheral blood, sometimes also from the bone marrow and restores the normal levels of platelets, granulocytes, monocytes and hemoglobin. This effect has been ascribed to the capacity of hu α- and β-IFN to stimulate the lymphomyeloid stem cells of these patients toward the myelomonocytic lineage, which reduces the excessive formation of partially mature B cells with the phenotype of hairy cells. Hu γ-IFN which has no therapeutic effect in hairy cell leukemia, does not exert this effect on lymphomyeloid stem cells [139-142].

In Daudi cell line, a B cell lymphoblastoid line transformed by EBV and highly sensitive to IFN, cell growth inhibition induced by hu lymphoblastoid or rec-α2-IFN is accompanied by plasmacytoid differentiation [143]. Cells from other leukemias of B cell lineage can also be directed toward differentiation by IFN treatment. Hu lymphoblastoid IFN as well as hu β-IFN and γ-IFN can induce blast transformation and plasmacytoid differentiation in chronic B lymphocytic leukemia cells, which is another indication that abrogation of maturation arrest contributes to the IFN-induced remissions obtained in some patients with B cell malignancies. It is important to stress that such effects are obtained with cells from some, but not from all, patients [144, 145].

Hu leukocyte IFN, administered to nude mice bearing xenograft of human osteosarcomas, induces growth arrest of the osteosarcomas cells. Inhibition of tumor growth is only maintained when IFN is given daily, and in some cases the tumor tissue is replaced by normal bone and marrow tissue. The most likely explanation is that the block of cellular proliferation by IFN leads secondarily to tumor differentiation [146].

The differentiation-stimulating activities of IFNs are theoretically relevant to their antitumor action, since inducing differentiation they redirect cells toward normality. However except in the case of hairy cell leukemia, until now there are no direct indications that stimulation of differentiation plays an important role in the antitumor activity of IFNs in vivo. Instead a clear involvement of host-defence mechanisms has been demonstrated, even if direct effects on tumor cells are also possible [147]. The experimental approach used to distinguish between direct and indirect (i.e. host mediated) effect has been: 1) the use of transplatable tumor cell lines sensitive and resistant to IFN treatment in vitro and 2) the performance of xenograft of hu tumor cells sensitive to IFN in mice treated with hu or mu IFNs.

Friend erythroleukemic cells represent a good model to analyse whether the antitumor effect of IFNs may be related to the differentiation enhancing properties of fibroblast IFN. In fact, Friend cells inoculated intraperitoneally (i.p.) in syngeneic DBA/2 mice can develop ascitic tumors, whereas they do form subcutaneous tumors resembling reticulum cell sarcoma, when inoculated subcutaneously (s.c.). Daily i.p. injections of mu αβ-IFN result in a drastic decrease in the number of Friend cells recovered from the peritoneal cavity in the days immediately following tumor cells inoculation, but the effect was as marked in mice injected with αβ-IFN-sensitive cells as in mice injected with αβ-IFN-resistant cells. Subcutaneously administration of mu αβ-IFN also inhibited the growth of either IFN-sensitive or αβ-IFN-resistant cells. In mice inoculated with γ-IFN, administration of IFN treatment induced extensive necrosis of these tumors and in some instances even caused complete regression. These results indicate that the antitumor effect induced by αβ-IFN in this tumor model system is mainly host-mediated [148-150].

Enhanced expression of cell surface antigens. - Stimulation of major histocompatibility antigens (MHC) expression at the cell surface results from a direct effect of interferons on normal and tumor cells. Studies in animal models indicate that enhanced expression of MHC class I antigens can augment tumor rejection.

Indirect effects of IFNs on tumor cells via activation of effector mechanisms of the host

The elimination of tumor cells in vivo involves several effector systems, most importantly macrophages, cytotoxic T cells and natural killer (NK) cells. Any of these systems may be involved in transplatable tumor rejection by IFN-treated mice [68, 168, 169]. There is no unifying hypothesis to account for the ensemble of observations dealing with the antitumor effects of IFNs in mice. The main emphasis of the past years has been on the immunomodulating properties of γ-IFN. This is because γ-IFN, as a product of activated T cells, is an obvious mediator of the immune system. In addition, γ-IFN is often many times more potent as immunomodulator than the other IFN species on the basis of the antiviral titer. However, β- and γ-IFN, produced during infections, are able to modify immune response increasing NK activity, suppressing or enhancing antibody formation (according to the timing used), activating macrophages for cytotoxicity and monokine secretion and enhancing the expression of MHC class I antigens and sometimes of class II.

Modulation of the expression of the major histocompatibility antigens. - Modulation of the expression of antigens of the major histocompatibility complex (MHC) is
the chief effect by which interferons influence the immune system. Class I antigens are involved in the restriction of cytotoxic T cells and are expressed in the majority of the cells in the body. Class II antigens are principally expressed at the surface of the cells of the immune system and are important in cell-to-cell recognition and interaction. Class III are not cell surface antigens but components of the complement cascade.

The expression of class II antigens is limited to certain cell types; it is constitutive on Langherans and dendritic cells and on B cells and is transient but inducible on monocytes, macrophages and some T cells. Quantitative variation of these antigens at the cell surface plays a central role in immunoregulation [170, 171].

Interferons modulate expression of both class I and class II antigens.

Stimulation of the expression of class II antigen is important to ensure efficient antigen presentation and immunization. Gamma-IFN is one of the most powerful T cell-derived soluble mediators that induce the enhanced expression of class II antigen on accessory-cells [172-177]. A selective increase of class I and II antigen, not only on macrophages but in many tissue, has been shown after intravenous administration of recombinant mu γ-IFN in mice. In the lymphatic-hematopoietic system, bone marrow cells show the most significant increase of both class I and class II antigens. The increased expression of class II antigens by γ-IFN on monocytes has functional implications since results in an accelerated immune-response. Similar results have been obtained in vivo for the human system [178].

Alpha- and β-IFN are in general powerful stimulators of class I antigens on accessory cells. There are however examples of enhanced expression of this antigens on macrophages and monocytes [179, 180].

Differential effects of individual α-IFN subspecies can occur [181]. Under certain conditions, however, α- and β-IFN can down-regulate class II antigens induced by γ-IFN [182, 183]. This is one of the few examples of antagonistic relationships between γ-IFN and αβ-IFN. Usually IFNs act synergistically on the immune system as in the case of their antiviral and antineoplastic effects [184]. The possibility that the constitutive production of αβ-IFN by murine macrophages [185] is one of the natural mechanisms that down-regulate the expression of class II antigens is intriguing.

Induction of class I and II antigens by α- and β-IFN is also observed on T and B cells [186-188]. The mayor effect of α- and β-IFN is however the induction of class I antigens [189, 190]. This result is not limited to in vitro system, but can be obtained by systemic administration of IFN to animals [191].

IFNs can modulate the expression of MHC antigens also in cells not belonging to the immune system. These cells may be normal or tumor. Expression of class I antigens in fibroblast is stimulated by αβ-IFN in the murine system [192]. Mu γ-IFN enhances this expression in practically every tissue in the body. In the brain, astrocytes are induced to express class I by mu αβ-IFN and class II by γ-IFN. The enhanced expression of class I can favor interaction of virus-infected cells with cytotoxic T cells [193] and appears a good way to favor their elimination [194], whereas induction of class II suggests a role of these cells in antigen presentation. This is true also for endothelial cells [195, 196].

Recombinant hu γ-IFN induces the expression of class II antigens on tumor cells of various origins such as melanoma cells (which often already express these antigens), glioma cells and cells from several human carcinoma cell lines [197-199]. Also specific melanoma antigens are increased by γ-IFN [200]. No evidence, however exists that shedding of melanoma and/or class II antigens on these cells is immunostimulatory in vivo [201] and can account for antitumor effect of IFNs.

Class I antigens are important for the interaction of cells bearing foreign antigens with effector cells. These antigens disappear on many tumor cells but when their expression is reinduced, oncogenic potential of these cells can diminish [202, 203]. The importance of the enhanced expression of class I antigen for the antitumor effect of interferons is also directly shown by the observation that increased expression of these antigens by hu αβ-IFN on adenovirus transformed cells reduces the tumorigenicity of these cells in immunocompetent hosts [204].

Effects on macrophages. - Macrophages derive from myeloid stem cells of the bone marrow and are the most mature member of the mononuclear phagocytic differentiation lineage. They play a key role in the host defense against microorganisms and neoplasia and are ubiquitous in the body, both as circulating and as resident cells. Many of the functions of macrophages are modulated by soluble mediators secreted by themselves or present in the environment. Some of the most important ones are secreted by lymphocytes, γ-IFN being one of the most potent.

Alpha- and β-IFN can be produced by macrophages. They influence growth and differentiation of macrophages themselves and their effector functions i.e. non receptor-mediated phagocytosis, receptor-mediated phagocytosis, tumor cell killing. Mu αβ-IFN stimulates non specific phagocytosis and increases the number and density of Fc receptors [205-208]. It seems, however, clear that the enhanced expression of Fc receptors and of high affinity binding sites can be dissociated from the enhanced phagocytosis, which is also present. In differentiated monocytes, the effect of γ-IFN on the receptor for the complement protein C3bi is opposite to that on Fc receptor. Affinity for the ligand is instead reduced [209].

In contrast to cytotoxic T cells, but as for natural killer cells, macrophages can destroy tumor cells without MHC restriction.

Cytolytic process to be carried out needs the activation of macrophages. This activation is a multistep process [210]. Lymphokines, released from activated T cells, are capable of priming macrophages and this activity named MAF (macrophage-activating factor) can be ascribed to
γ-IFN. In fact MAF copurifies with γ-IFN, is inactivated by highly specific antibodies to γ-IFN and its effect can be reproduced by recombinant mu γ-IFN treatment [211-219].

Alpha- and β-IFN, in addition to γ-IFN, have also activating capabilities [220, 221]. However they are less efficient when compared with γ-IFN on the basis of the antiviral titer. [222-224]. Mu α/β-IFN enhances in vitro and in vivo cytolytic activity of macrophages against tumor cells [225, 226]. Treatment of monocyte from human peripheral blood with hu leucocyte IFN or β-IFN increases their cytolytic activity against malignant cells [220].

Also oxygen-dependent and -independent microbidal products are produced by macrophages in response to stimulation by γ-IFN [227]. Other cytokines as α-IFN, β-IFN, CSF-1, TNF or IL-2 lack this ability [228-229].

Once macrophages are activated, it is essential that they retain their capacity to kill. In vitro, when removed from activating stimuli, activated macrophages quickly lose their ability to kill. A cause of such loss is the negative regulatory effect that prostaglandin E has been shown to have on activation [230, 231]. Gamma-IFN is able to prevent this disactivation [232] therefore it seems that γ-IFN acts not only as an activator, but also as a lymphokine that maintains cytolytic activity once this activity has been induced. In addition γ-IFN stimulates intracellular parasites (Leishmania and Toxoplasma gondii) killing and restores the capacity of human monocytes to kill Malaria parasites.

Effects on NK cells. - NK cells [233] are capable of mounting a cytotoxic response against malignant virus-infected cells and also against normal cells without the need for prior immunization and without MHC restriction. Moreover NK cells are not thymus-dependent, since they are present in nude and neonatally thymectomized mice [234, 235]. Morphologically NK cells have been identified as large granular lymphocytes (LGL) with a high cytoplasmic-nuclear ratio and azurophilic granules in their cytoplasm [236, 237] which contain cytolitic proteins involved in target cell destruction by a cell lysis mechanism also present in cytotoxic T cells. It is interesting that LGL appear as a heterogeneous population when their surface antigens are analysed with monoclonal antibodies. Since NK cells are not MHC-restricted, target cells can be syngeneic, allogeneic and even xenogeneic. Moreover because NK can be activated without previous sensitization, they, like macrophages, are believed to be the first step of defense against tumor cells and infectious agents. Along with this view point, activation of NK cells by all the three species of IFNs has been considered as an antitumor effect. However clinical data do not show a good correlation between tumor regression and NK stimulation [238-241].

NK cell activity can be up or down regulated by IFNs either directly, enhancing NK cells activity, or indirectly via protective effects on the target cells. It is not yet clear why a cell become a target for NK activity. Normal, tumor and infected cells can be targets. In viro treatment with IFN of normal cells confers protection against killing, whereas tumor cells may be protected or not by this treatment [242-247]. Both in vivo (intraperitoneal in mice) and in vitro (using mouse spleen cells or human peripheral blood leukocytes) there is a marked increase of NK cell activity upon administration of an optimal dose of IFN [248-251]. Above the optimal dosage, instead of being enhanced, NK activity is often decreased. The mechanism underlying these opposite effects is unknown.

IL-1, IL-2, thymosin and β endorphins regulate in vivo NK cell activity [252-255], it is therefore difficult to extrapolate the results of in vitro experiments and define the effect of the simple administration of IFNs. NK cells can be induced to produce α- and β-IFN by virus infection. Interestingly, they can also be stimulated to produce γ-IFN, as mature T cells. Cloned lines of murine NK cells, or freshly isolated human large granular lymphocytes produce γ-IFN if cultivated in presence of IL-2 [256, 257]. However it needs to be underlined that the cell killing capacity of NK cells is boosted by IL-2, via a direct effect on NK cells and probably not via the IL-2-induced γ-IFN production [258].

Effects on T cells. - IFNs profoundly effect T helper cells function. This is obtained both by an indirect enhancing effect via enhanced expression of class II antigens on accessory cells and by direct effects on these cells [259]. Mu α/β-IFN enhances the specific cytotoxicity of sensitized lymphocytes against allogenic tumor cells [260]. Similarly hu α-IFN and hu β-IFN increase the cytotoxicity of human lymphocytes in in vitro experiments [261, 262]. On the other hand, IFNs treatment can either enhance or down regulate T suppressor cell activity. The apparent contradictory evidence between these opposite effects can be explained by the observation that IFNs are only a component of a complex network of interactions among a number of cytokines.

Effects on B cells. - Maturation toward antibody-producing plasma cells is a very complex process which requires many cytokines. Several cytokines influencing and regulating growth and differentiation of B cells have been identified in humans (IL-1, IL-2, IL-4, IL-6 = β2-IFN) and in mouse (IL-1, IL-2, IL-4, IL-5 and BCDF-m) [263-267]. In both humans and mice all IFN species can also influence B cell differentiation and growth.

In vivo IFNs can influence antibody formation indirectly by effects on antigen-presenting accessory cells and by effects on T helper and suppressor cells. Studies on the effects on antibody formation have frequently given contradictory results of either suppression or enhancement [268, 269]. The generation of antibody-forming cells is depending on the timing of IFN administration [270]. In general (but this is not always the case), inhibition in vivo and in vitro is obtained when IFNs act before sensitization, and enhancement when they act after sensitization.
Interferons influence also proliferation and differentiation of B cells in vitro. Hu γ- and β-IFN stimulate Ig synthesis by B cells. Gamma-IFN has different degrees of activity on B cell function depending on the dosage. Low concentrations of a few hundreds of units of rec hu α2-IFN enhance pokeweed mitogen-stimulated Ig synthesis by peripheral blood mononuclear cells, but concentration of 10⁷ Units suppress Ig production in absence of effects on B cell proliferation [271].

The observation that the addition of antibodies against γ-IFN to activated T cell abrogates the capacity of cell supernatants to stimulate B cell production of antibody-forming cells in a Jerne-assay [272, 273] suggests that γ-IFN is one of the natural B cell differentiation factors. Gamma-IFN stimulation of Ig synthesis is isotype specific. In resting murine B cells stimulated by LPS, rec mu γ-IFN enhances the synthesis of IgG2a, but suppresses the synthesis of IgG3, IgG1 and IgG2b [274]. IgG2a is known as the most efficient antibody for the induction of antibody-dependent macrophage and killer cell cytotoxicity for tumor cells. In addition γ-IFN blocks the stimulating effect of IL-4 on resting B cells probably acting as natural down regulator of the effects induced by IL-4 [275-277]. IL-4 (=BSF-1=BCGF-1) is a lymphokine produced by helper cells, instrumental in B cell activation and proliferation which can induce a state of activation in mast cells and T cells. IL-4 enhances B cell proliferation and the synthesis of some Ig by LPS-stimulated B cells. This enhancing effect appears isotype specific, in fact only the synthesis of IgG1 and IgE is enhanced [274, 277-279]. The enhancement of IgE and IgG1 synthesis by the supernatants of the cloned T helper cells is inhibited by γ-IFN [277].

Clinical application of IFNs as antitumor agents

The discovery of IFN in 1957 induced a lot of enthusiasm about this magic tool that would eliminate viral diseases. IFN was not toxic at the doses sufficient to protect from viral infections, and was very selective against a variety of viruses. It was a natural therapeutic agent that would destroy almost all viruses as effectively as penicillin killed bacteria. Moreover around 1970 a relevant antitumor action was seen in some animal experimental models. The opportunity to test these molecules in clinical trials became possible with the production of large amounts of human leukocyte IFN produced from buffy coats by Kari Cantell [280]. Initial results with this material, used both as antiviral and as antitumor agent, were positive [281]. Clinical trials in cancer with IFN purified from buffy coat leukocytes began in 1970 with good results in the treatment of post surgical osteogenic sarcoma [282].

After an initial period of limited availability of the natural material, production of bulk quantities of pure IFNs from procaroytic cells resulted from recombinant DNA technology (Table 2).

Clinical trials with recombinant IFNs began about ten years ago and have employed with few exceptions [283] α2-IFN (or αA-IFN) one of the major α-IFN subtypes in IFN produced from buffy coats. Trials with purified β-IFN and γ-IFN began only in the latter part of 1983 and only phase I trials have been conducted to determine the maximum tolerated dose and the biologically optimum dose. Beta-IFN from both natural and recombinant sources seems to have a spectrum of antitumor activity and side effects similar to those of α-IFN, even if weaker.

Table 2. - Interferons used in human therapy

<table>
<thead>
<tr>
<th>Type I</th>
<th>Recombinant</th>
<th>Natural</th>
<th>Producer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subtype α</td>
<td>α2a or αA (Roferon A)</td>
<td></td>
<td>Hoffman-La Roche</td>
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<tr>
<td></td>
<td>α2b (Intron A)</td>
<td></td>
<td>Shering-Plough</td>
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<tr>
<td></td>
<td>α2c</td>
<td>αM1 (Lymphoblastoid IFN)</td>
<td>Boehringer</td>
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<tr>
<td></td>
<td></td>
<td>αL.C (Leucocyte IFN)</td>
<td>Wellferon</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>several preparations</td>
</tr>
<tr>
<td>Subtype β</td>
<td>βser</td>
<td>β1</td>
<td>Toray, Rentshler, Serono</td>
</tr>
<tr>
<td>Type II</td>
<td>γ</td>
<td></td>
<td>Genentech, Biogen, Ciba/Geigy, Kyowa Hakko</td>
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</table>
Gamma-IFN has been shown to have antitumor activity and side effects different from those of α-IFN [284]. Gamma-IFN may prove to be useful as an immunomodulator in the treatment of infections due to intracellular parasites, such as toxoplasmosis, atypical mycobacterium and leprosy [285].

Several side effects occur during the α2-IFN treatment such as fever, chills, myalgias and headaches [286, 287]. These effects have been recently reviewed by Quesada et al. [287]. Reported cardiovascular effects include hypotension, cardiac dyshrhythmia, tachycardia and premature ventricular extrasystole. Transient leukopenia has also been observed. The effects on the central nervous system, even if less common, are the most problematic and can be dose limiting [288-290].

The knowledge of pharmacokinetics phase I studies of different types of IFN is indispensable in choosing treatment schedule. The fate of endogenous and exogenous interferon is determined largely by the type of IFN, the site of its induction or administration and the amount administered. Beta-IFN and γ-IFN differ in their pharmacological behaviour from α-IFN. For γ-IFN neither intramuscular, nor rapid intravenous infusion resulted in sustained serum levels [291, 292]. Hu β-IFN has therapeutic activity after intramuscular injection despite low or absent serum levels [293-295]. Alpha-IFN has a very short life in the plasma. Intravenously administered α-IFN disappears rapidly from plasma; the relatively small size of the molecule leads to an efficient filtration by renal glomerulus; it is reabsorbed almost quantitatively by the tubules, where it is destroyed by proteolysis [296]. Bocci [297] estimates that the kidneys eliminate at least 2% of the plasma α-IFN/min, thus clearing the plasma pool in less than an hour. Megadose of this IFN can overcome this problem but high plasma levels of IFN produce severe central nervous system toxicity and suppress the immune system. Intramuscular administration delays the catabolism of the molecule, therefore this route of administration has been preferred for α-IFN use in clinical trials.

Phase II clinical trials have defined effectiveness of pure α-IFN as single agent in several malignancies refractory to established therapies. Last year the U.S. Food and Drug administration licensed two firms to sell recombinant α-IFN for the treatment of hairy cell leukemia, and its local administration in condyloma acuminata is the application most likely to be licensed next in the United States. A response rate with IFN of more than 90% in hairy cell leukemia [298] allowed the drug to be licensed.

To briefly summarize effects of IFN therapy in several kind of tumors, we can distinguish between a) solid tumors and b) hematological malignancies. Generally in any kind of tumor both Buffy coat leukocyte IFN and recombinant species have been tested.

Solid tumors in which IFN treatment has lead to various degrees of tumor regression are malignant melanoma, Kaposi’s sarcoma, breast carcinoma, bladder papillomatosis, nasopharyngeal carcinoma, malignant glioma. Nega-
tive results have instead been obtained in the treatment of colorectal carcinoma, lung cancer, glioblastoma multiforme.

In malignant melanoma, the use of recombinant α2-IFN shows a partial response, however not higher than that obtained with the best chemotherapeutic agents imidazol, carboimide and nitrosoareas [299, 300].

Partial responses were also obtained in renal cell carcinoma patients, treated with α2-IFN and in phase I trials with naturally produced β-IFN [301] and βγ-IFN (an engineered modified molecule) [302]. Although response rate have remained < 50% in this kind of tumor, these results have been encouraging in a disease which has not generally been responsive to other forms of systemic therapy.

Preclinical animal model systems have suggested antitumor potential for treatment of patients with breast carcinoma [303]. A combined analysis of two clinical trials with buffy coat IFN indicate 50% or greater reduction in the diameter of the measurable tumor [304]. Negative results have been obtained in the phase II trial with recombinant α- and α2-IFN in contrast to preclinical data and data with naturally produced α-IFN. Whether these differences between natural and recombinant IFN result from IFN preparation, dose, schedule, or patient characteristics remains to be established.

Relatively high doses of αA-IFN and α2-IFN gave partial responses in patients with Kaposi’s sarcoma in the setting of acquired immunodeficiency syndrome (AIDS) [305, 306]. The rate of opportunistic infections was lower after IFN treatment of responding patients. In both trials the higher dose seemed more efficacious than the lower in inducing regression of the neoplastic disease.

Very good results have been obtained in bladder papillomatosis. Complete regression in eight to eight patients, lasting from 4 to 46 months, were reported when the tumor base or the surrounding tissue was injected transurethrally with α-IFN and intravesically with α2-IFN [307]. The advantage in this kind of tumor is in fact the possibility of local (intravesical) administration without systemic toxicity. Partial responses with α-IFN [308] and significant disease regression with intravenous β-IFN [309] have been obtained in the treatment of nasopharyngeal carcinoma and of cervical carcinoma, another tumor associated with viruses. The treatment with leukocyte IFN gave total or partial regression in about 40% of patients [310].

In hematological malignancies, hairy cell leukemia is the tumor in which the most dramatic response has been obtained i.e. complete remission in 19 of 20 patients treated with α-IFN [311-314]. The role of β-IFN and γ-IFN are currently under investigation. Similarly, the treatment of early stages of multiple myeloma with other αA-IFN or partially purified leukocyte interferon, results in tumor regression in a good percentage (40-50%) of patients [314, 315].

In lymphomas no response of clinical significance occurred in Hodgkin’s disease patients treated with leukocyte IFN and in non Hodgkin’s lymphomas with unfa-
rable histologies [316]. On the contrary, non-Hodgkin's lymphomas with favorable histologies respond well to partially purified α-IFN [317, 318], to partially purified β-IFN [319] and to α-IFN [320]. A greater than 50% response frequency occurred in a trial with α-IFN [321]. Moreover relatively low doses of α2-IFN can be administered, if given subcutaneously, instead of intramuscularly [322].

In chronic myelogenous leukemia some effectiveness of α-IFN has been observed [323].

In retrospect, mouse study have largely predicted what has been found clinically. The mouse studies summarized by Gresser [324] indicate that IFN is most effective when injected repeatedly and during the period of tumor growth; efficacy is inversely related to the tumor load and roughly proportional to the dose; antitumor effects are maximal when direct contact between tumor cell and IFNs is maximal. The study of the clinical use of IFN has just begun. Few of the numerous recombinant α-IFN have been tested. There have been few trials with β-IFN and testing of γ-IFN has only reached phase I level. Dose, frequency and route of administration of IFN i.e. and ideal schedule of treatment has not yet been defined in any of the tumor tested. Moreover, the role of IFNs in combination with other modalities of cancer treatment such as chemotherapy and other biological response modifiers, has just began to be tested, and seems to be the best way to use IFNs in therapy. Although the side-effects of fatigue and anorexia are troublesome, when compared with cytotoxic agents, no residual toxicities for vital organs occur.

In order to identify more rational dose schedules and to improve patient tolerance, a not delaying challenge is to define: a) the mechanism of antitumor action, and b) the therapeutic role of single IFNs species and combinations.

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