

Biological basis for a proper clinical application of alpha interferons

Guido Antonelli

Section of Virology, Department of Experimental Medicine, "Sapienza" University, Rome, Italy

SUMMARY

In recent decades the structure and the function of interferons (IFNs) have been elucidated and it is now clear that IFN is not only a protein but a group of proteins with parallel, but not analogous or identical, biochemical and biological properties. Among them there are at least 12 subtypes of IFN-alpha which are all involved in the control of several cellular functions and are all actively engaged in host defence mechanisms against infections. These acquisitions led to the clinical use of different types of IFN-alpha with appreciable success in several diseases. There are however several possibilities to optimize IFN-alpha treatment which can and must be addressed.

For instance, as regards IFN-alpha biology and therapy issues, we need to understand how the different subtypes can generate similar signalling outputs but also govern specific cellular responses and more in general why the body produces so many of these IFN-alphas.

This paper highlights the complexity of the IFN-alpha system and tries to summarize what is currently known of the distinctive properties of each individual IFN-alpha subtype. The data presented on the functional distinctions between IFN-alpha subtypes suggest that the commercially available IFN-alpha preparations may differ in some biological, pharmacological and therapeutic aspects and that a wiser and more prudent use of the different IFN-alpha preparations may provide more therapeutic benefits to patients.

KEY WORDS: Interferon-alpha, Antiviral agents, Chronic hepatitis, Interferon alpha subtypes, Antibodies to interferon alpha

Received June 15, 2008

Accepted June 30, 2008

INTRODUCTION

In the last few decades interferons (IFNs) have been widely studied from the chemical and biochemical points of view. The mechanisms through which IFNs interact with the cell have been elucidated and many of their biological activities have been characterized. All together these acquisitions led to the clinical use of different types of IFNs with an appreciable success in sev-

eral diseases (Borden *et al.*, 2007), marking a major advance in modern medicine. Indeed IFN represents the first biological response modifiers accepted for clinical use and a model of cytokines used in therapy. Indeed, after more than 35 years of clinical experimentation IFN resists in the pharmacopoeia and this undoubtedly demonstrates that the drug is safe and effective.

Nevertheless, it is now claimed that the predominance of IFNs on the antivirals market is coming to an end (Editorial, no author listed, 2007) due to the approval of new more effective drugs. The availability of these new drugs was certainly awaited and longed for but there are reasons to believe that IFNs can still find place in the modern pharmacopoeia. This justifies the need to further elucidate the mechanisms of biological and pharmacologic effects of IFNs and to gain new

Corresponding author

Guido Antonelli

Section of Virology

Department of Experimental Medicine

"Sapienza" University

V.le di Porta Tiburtina, 28 - 00185 Rome, Italy

E-mail: guido.antonelli@uniroma1.it

insights into the therapeutic potential of IFN in general. Therapeutic options are supported by the following issues referred to below mainly in relation to chronic hepatitis C infection but it is tempting to speculate may also be applicable to other diseases:

- most of the promises of the new drugs have not been kept since many of the antiviral drugs we are awaiting failed to pass phases 2 or 3 due to problem of toxicity and viral drug-resistance (Garber, 2007)
- in most cases IFNs will survive as the drug of choice because it will be used in combination with other newer drugs. Indeed, while IFN therapy may lead to eradication of the infection or cure the disease, in the case of most new drugs (nucleoside analogs, polymerase inhibitors, other synthetic antivirals) we have to expect a very effective therapeutic action (measured for instance by the decrease of viral load) but never, at least so far, eradication of the disease;
- IFN represents a fundamental cellular defence mechanism against viral infections and there are plenty of demonstrations that administrating IFN preparations may lead to a milder course of several diseases.

In the light of the above considerations, it is our firm opinion that IFN will last for further decades so that questions to optimize the treatment of IFNs can and must be addressed:

- What is the role of the hundreds of functionally-unidentified IFN-induced genes?
- What is the current role of IFNs in the development of autoimmune phenomena?
- Do the IFN-induced proteins or components of the IFN system have a role as drugs in future therapies?
- What are the biological and molecular causes of the resistance to IFN therapy?
- What specific and different roles may be played by each individual IFN type?

The last question is one of the main and most intriguing issues which have not been sufficiently addressed in the past and which deserve further attention. Indeed, after more than 50 years since the discovery of IFN it is now time to understand in detail why there are so many types of IFNs with apparently similar activities and to characterize the different types of IFN from the biological, functional and clinical points of view.

This paper focuses on the issue, highlighting the

complexity of the IFN-alpha system and summarizing what is currently known on the distinctive properties of each individual IFN-alpha subtype. Furthermore it attempts to give some information on the features and peculiarity of the different commercially available preparations of IFN-alpha.

THE IFN PROTEIN FAMILY

Only several years after the discovery of IFN did it become clear that IFN was not only a protein but a group of proteins with parallel, but not analogous or identical, biochemical and biological properties. It is now recognized that there are more than 20 non allelic genes which codify proteins possessing properties comparable to those of IFN (i.e. direct antiviral activity, species-specificity, relatively low molecular weights, pleiotropic activity, etc.). All these genes can be divided into three main groups: IFN type I which includes many different IFNs among which many IFN-alphas; IFN type II which includes IFN-gamma; and IFN type III which includes IFN-lambdas. Table 1 depicts the main characteristics of three types of IFN.

Type-I IFN

Originally it was thought that IFN type I was induced only by specific cells and then IFN type I was named leukocyte (IFN-alpha) and fibroblast (IFN-beta) to indicate the cellular origin of the molecules. Today it has been formally accepted that almost all nucleated cells may produce type I IFN although there are some specialized cells such as plasmocitoid dendritic cells which are very high producers of type I IFN.

The family of the human type I IFNs now includes 13 non allelic IFN-alpha genes and one single gene of IFN-beta, omega, kappa, epsilon, delta. Additionally in the same region we can find 5 pseudogenes (Chen *et al.*, 2004; Samarajiwa *et al.*, 2006).

The main characteristics of the different IFN-type I are depicted in Table 2.

All these genes, clustered on a specific region of the human chromosome 9, lack introns. All proteins codified by the above genes are proteins containing a 23 aa signal peptide and a 166 aa mature protein. There is an exception represent-

TABLE 1 - Main characteristics of human interferons.

	<i>IFN-α</i>	<i>IFN-β</i>	<i>IFN-ω</i>	<i>IFN-λ</i>	<i>IFN-γ</i>
IFN type	I		II		III
Chromosomal localization	9	9	9	19 or 7	12 or 10
Gene structure	intronless			introns	
N° subtypes	13	1	1	3	1
Pseudogenes	1	0	6	ND	0
Length of predicted mature peptide	165-166	166	172	175 or 174	166 or 155
Acis stability	+	+	+	ND	-
N-Glycosylation	α	+	+	+	+
O-Glycosylation	α	-	ND	ND	ND
Disulfide bond	2	1	2	2	0
Receptor	IFNAR-1 and IFNAR-2		IL- α 28R α and IL-10R β		IFN- γ and IFN- γ R2

ed by IFN-alpha 2 which has the signal peptide but the length of its mature protein is 165 aa since it has a deletion at position 44. Furthermore it has been demonstrated that the allelic form of the IFN-alpha 2 genes does exist. Examples are: IFN-alpha 2a, IFN-alpha 2b, and IFN-alpha 2c (Samarajiwa *et al.*, 2006).

The 13 IFN-alpha genes encode for 12 different proteins. Indeed genes IFN-alpha 1 and IFN-alpha 13 encode for a protein which has shown an identical sequence. Overall the other proteins are different and show a sequence variation between 1 and 24%. Considering the high sequence conservation between the different genes, people assume that the multiplicity of IFN genes is due to gene duplication events. To better understand the importance of such a complex system of type I IFN genes we have to consider that a similar organization has also been demonstrated in avian and other mammalian species.

Individual subtypes may be produced differently by different stimuli, highlighting the importance of the different genes encoding similar molecules (Palmer *et al.*, 2007)

In human, unlike IFN-alpha genes, no subtypes of IFN-beta, omega, kappa, epsilon, delta have been identified whereas in animals different variants

of IFN-xi, tau, delta e nu have been found (Samarajiwa *et al.*, 2006).. Most of the latter human gene products have only been recently characterized and no definite data are available on their specific biological characteristics.

COMMERCIALLY AVAILABLE IFN-ALPHA PREPARATIONS: COMPOSITION, ANTIGENIC STRUCTURE AND IMMUNOGENICITY

Originally the commercial preparations of IFN-alpha currently available for clinical use were either of recombinant IFN (rIFN) alpha 2, obtained from transfected bacteria (namely *E. coli*), or were mixtures of many subtypes of IFN-alpha obtained from transformed cell cultures (namely Namalwa cells) or primary human blood leucocytes (buffy coats) both stimulated by Sendai virus. The composition of the mixtures depended on the methods used for their production and purification. Lymphoblastoid IFN-alpha produced by cell culture has IFN-alpha 2 as its main component while human leukocyte IFN-alpha (LeIFN), mainly derived from monocytes, has IFN-alpha 1 as its main component. Specifically,

TABLE 2 - *IFN type I: genes and proteins.*

<i>Subtypes</i>						
	<i>Gene</i>	<i>Protein</i>	<i>Older denomination</i>	<i>Notes</i>	<i>Length (a.a.)*</i>	<i>% identity with human IFN-alpha</i>
IFN α	IFN-A1	IFN- α 1	IFN-alpha D	identical to IFN- α 13	166	N.A.
	IFN-A2	IFN- α 2	IFN-alpha A	There the allelic forms (2a, 2b and 2c)	165	N.A.
	IFN-A4	IFN- α 4	N.A.		166	N.A.
	IFN-A5	IFN- α 5	IFN-alpha G		166	N.A.
	IFN-A6	IFN- α 6	IFN-alpha K		166	N.A.
	IFN-A7	IFN- α 7	IFN-alpha J		166	N.A.
	IFN-A8	IFN- α 8	IFN-alpha B		166	N.A.
	IFN-A10	IFN- α 10	IFN-alpha C		166	N.A.
	IFN-A13	IFN- α 13	N.A.	identical to IFN- α 1	166	N.A.
	IFN-A14	IFN- α 14	IFN-alpha H		166	N.A.
	IFN-A16	IFN- α 16	N.A.		166	N.A.
	IFN-A17	IFN- α 17	IFN-alpha I		166	N.A.
	IFN-A21	IFN- α 21	IFN-alpha F		166	N.A.
	YIFNaE	IFN- α P22	N.A.	pseudogene	166	N.A.
IFN- β	IFN-B1	N.A.*	N.A.		161	25-32
IFN- ω	IFN-W1	N.A.	N.A.	there are multiple IFN-W genes but only one is functional	172-174	55-60
IFN- κ	IFN K	N.A.	N.A.		180	27-32
IFN- ϵ	IFNE1	N.A.	N.A.		185	similar to that of IFN- β
IFN- ξ	IFNz	N.A.	N.A.	at the moment identified only in mice		
IFN- τ	N.A.	N.A.	N.A.	at the moment identified only in the ungulate ruminants		
IFN- δ	N.A.	N.A.	N.A.	at the moment identified only in pigs		
IFN- ν	N.A.	N.A.	N.A.	at the moment identified only in felines		

*N.A. Not applicable

in LeIFN-alpha at least 15 subtypes have been isolated; the main components are IFN-alpha 1, IFN-alpha 2, IFN-alpha 4 and IFN-omega, altogether accounting for 95% of LE- IFN-alpha. The remaining subtypes, including IFN-alpha 5, IFN-alpha 7, IFN-alpha 8, IFN-alpha 10, IFN-alpha 14, IFN-alpha 17, IFN-alpha 21 are defined as minor components since they are present in small amounts, although they may play an important functional role (Viscomi *et al.*, 1995). LeIFN preparations are marketed in several countries as Alfaferone in Europe or as Alferon in the USA (in this last country it is indicated only for the intralesional treatment of refractory or recurring external condylomata acuminata).

In an attempt to optimize the pharmacological action of IFN-alpha another type of IFN-alpha has been produced in *E. coli* and commercialized as Infergen. It is a formulation of recombinant, non glycosylated consensus IFN-alpha whose amino acid sequence is designed by selecting at each position the most commonly occurring amino acid at that position among the various subtypes of natural IFN-alpha. Some minor changes had to be made for stability and structural reasons.

Today some of these preparations are no longer used in clinical practice (i.e. Wellferon produced by the Namalwa cell line) and recombinant preparations of IFN-alpha are also being originated in yeast. Such expression systems are generally preferred since, in contrast to *E. coli*, they produce a glycosylated protein through a more physiological secretory pathway, (Muller *et al.*, 2006).

The short half-life of IFN-alpha has led to the development of IFN-alpha preparations which have a longer half-life. This was achieved by the attachment of a polyethylene glycol (PEG) molecule to the IFN-alpha molecule. Two different commercial preparations of pegylated IFN have been developed for clinical use: PEG-Intron, a 12-kDa linear pegylated IFN-alpha 2b; and Pegasys, a 40-k-Da branched pegylated IFN-alpha 2a. Both have long half-lives (Pegintron: 40 h; Pegasys: 80 h) compared to non-pegylated forms of IFN. This allows a once weekly administration. These preparations have been demonstrated to be effective for the treatment of patients with hepatitis C and results of clinical trials have shown that both of the pegylated molecules produce sus-

tained viral response rates superior to those achieved with their respective standard IFN-alpha (Luxon *et al.*, 2002; Zeuzem *et al.*, 2000)

More recently another approach, called genetic fusion, has been developed to enhance the half-life of the bioactive molecule. This technology led to the production of Albuferon which is the result of the fusion between a recombinant form of IFN-alpha 2b and the human albumin, currently at phase 3 development step (Subramanian *et al.*, 2007).

It should be emphasized that whereas in general the linkage process has proven to be highly effective for slowing the clearance of IFN-alpha, thus improving the pharmacologic characteristics of the drug (i.e. increasing the plasma half-life), it has been also shown that this process may modify the drug's *in vitro* biological activities. For instance, it has been reported that pegylation of IFN-alpha 2 (both linear PEG and branched PEG) results in a preparation that has a lower specific activity compared to standard IFN-alpha 2 although the linkage process does not modify the structure of the IFN-alpha molecule (Grace *et al.*, 2001; Bailon *et al.*, 2001; Foser *et al.*, 2003). Further, the pegylation process results in production of a heterogeneous mixture of isomers each having a different specific activity and a specific molecular weight (van der Auwera *et al.*, 2001).

Although there is evidence that the different commercially available preparations of IFN-alpha have comparable effects (at least when compared for class, i.e. standard IFN-alpha 2a vs. standard IFN-alpha 2b or Pegasys vs. PEG-Intron), it should always be considered that the IFN preparations currently used *in vivo* are structurally distinct. This consideration has found its major application in the study and management of the *in vivo* development of antibodies to IFN able to neutralize or bind to the IFN molecule. Although initially unexpected, it was clear from the first years of clinical use of IFN preparations that the patients treated with IFN-alpha may develop antibodies to the administered IFN-alpha during treatment (Antonelli, 1997; Kessler *et al.*, 2006). Interestingly some reports showed that commercial recombinant preparations tend to produce a detectable level of antibodies in a larger number of patients than the non-recombinant preparations (Antonelli *et al.*, 1991; Antonelli *et al.*, 1992; Foster *et al.*, 1998).

TABLE 3 - Some of the IFN type I-stimulated genes, functions and action of the proteins.

Gene	Protein function and mechanism of action	Main <i>in vitro</i> action
IRF7, RIG1, MDA5, STAT1	Signalling to IFN- α/β genes or to interferon stimulated genes. Induction of type I IFNs	Many
OAS	RNA cleavage, degrades viral and cellular RNA induces IFN- α/β	Antiviral, anti-growth
PKR	EIF2 α phosphorylation, blocks protein synthesis	Antiviral, anti-growth
MxA	Interferes with intracellular virus trafficking	Antiviral
p56	Binds EIF3, Blocks protein synthesis	Antiviral, anti-growth
PLSCR1	Phospholipid migration, DNA binding	Amplification of expression of some ISGs
TRAIL	Ligand of death receptor	Pro-apoptosis
XAF1	Blocks inhibitor of apoptosis (XIAP)	Pro-apoptosis
G1P3	Inhibits caspase 3	Anti-apoptosis
GBP1	GTPase	Antiviral, angiogenesis inhibitor
ISG20	3'- exonuclease for RNA and DNA	Antiviral
ADAR1	Adenosine deaminase for dsRNA RNA editing, altered translation	Antiviral
iNOS	Nitric oxide synthase	Antiviral
Viperin	Not Known	Antiviral

As already stated, the rIFN preparations in clinical use all contained only IFN-alpha 2 while the non recombinant preparations contained multiple subtypes. The differences in the incidence of antibodies to IFN-alpha development could be associated with the formulation of the IFN-preparations and not the IFN molecules themselves. Indeed two very similar IFN molecules, namely rIFN-alpha 2a and rIFN-alpha 2b, were significantly different from the immunogenic potential activity point of view. The above hypothesis was also confirmed by the fact that substantial changes in rIFN preparations led to a significant increase in the stability of the preparation and to a decrease of immunogenicity (Hochuli, 1997). Another important aspect of the antibodies to IFN issue is the fact that patients who do become resistant to rIFN-alpha treatment because of antibodies could be effectively treated with LeIFN preparations (Casato *et al.*, 1991; von Wussow *et*

al., 1991; Roffi *et al.*, 1995; von Wussow *et al.*, 1988.). This is not unexpected, as the results obtained *in vitro* indicate that LeIFN can overcome the neutralizing activity displayed by sera from patients treated with rIFN-alpha 2 (Steis *et al.*, 1988; Antonelli *et al.*, 1991).

Although the incidence of anti-IFN antibody seroconversion is lower in Pegylated IFNs-treated patients the issue is still important because cases of patients treated with pegylated IFN who become resistant to IFN therapeutic efficacy have been reported (van der Eijk *et al.*, 2006) and patients who become resistant to rIFN or pegylated IFN-alpha can be efficaciously treated with LeIFN (Santantonio *et al.*, 2006) (see below).

The difference in the induction of IFN-alpha antibodies, their cross-reactivity pattern toward individual IFN-alpha subtype and the response of anti-IFN seropositive patients resistant to a new treatment with LeIFN during therapy strongly in-

dicating that, other than the importance of the process of stabilization of the preparation, there may be differences in the antigenic profiles of the different IFN- α commercial preparations. In this regard several reports have clearly demonstrated that despite the high homology of the IFN- α subtype sequences, each IFN- α subtype showed a distinct antigenic profile (Foster *et al.*, 1998). In particular one report evaluated neutralization and immunoactivity of a variety of IFN preparations with various monoclonal antibodies (IFN- α mAb). Specifically, 9 IFN- α mAb obtained through immunization with rIFN- α (rmAb), lymphoblastoid IFN- α (Ly mAb), and LeIFN (Le mAb) were tested. The MoAb were evaluated for their ability to neutralize the antiviral activity of 11 rIFN- α subtypes, two rIFN- α hybrids, and lymphoblastoid and LeIFN preparations. The results of the neutralization assay revealed that the IFN- α mAb significantly differ in their ability to neutralize the individual IFN- α species. Interestingly, none of the IFN- α mAb was able to neutralize all the IFN- α species. In particular, rmAb were unable to neutralize LeIFN or LyIFN, whereas LE mAb and LY mAb efficiently neutralized rIFN- α 2. The reaction between the different IFN- α molecules and IFN- α MoAb is unpredictable despite the identity of the sequences of the regions where the antigenic determinants are located (Viscomi *et al.*, 1999). This indirectly demonstrates that IFN- α derived from different sources (i.e. *E. coli*, lymphoblastoid cell lines, leukocytes) and subjected to different purification and formulation processes may exhibit different antigenic profiles and different immunogenicity when administered to human even if the sequences of the protein are identical.

ARE ALL TYPE-I IFNS BIOLOGICALLY EQUIVALENT?

The induction of all IFNs type I is activated by viruses (both RNA and DNA viruses) or by microbial products (either bacteria and protozoa) through a series of events which are now at least in part known and that require the activation of Toll-like receptors (TLR) present on the cell surface or on endosomal cell membranes. TLRs then

activate specific transcription factors which leads to the derepression of IFN type I genes which are normally silent. IFNs are then produced by the cell and can interact with specific receptors of adjacent cells. Binding of IFN type-I to specific cell surface receptors, which comprised two subunits denoted IFNAR-1 and IFNAR-2 belonging to the class II cytokine receptor superfamily, results in the activation of multiple intracellular signalling cascades which involves mainly JAK-STAT, MAPK, Crkl, IRS pathways. The diverse pathways differently contribute to the activation of the biological activities of type-I IFN. For instance, there is evidence that activation of the JAK-STAT pathway is important for control of viral replication while the IRS-PI3K plays a role in mediating virus-induced cellular death. Together these pathways coordinately lead to the synthesis of proteins that mediate antiviral, growth-inhibitory and immunomodulatory responses (Brierley *et al.*, 2006).

Type-I IFN induces the expression of more than 100 proteins. Only some of them have been molecularly and functionally characterized (see table 2). Few of these proteins have been used as biological markers of the *in vivo* action of IFNs and are currently used in pharmacodynamic studies. Over the last 30 years increasing evidence has been published demonstrating that although type I IFNs act through a single cell surface receptor composed of the IFNAR1 and IFNAR2 subunits, IFN- α subtypes exhibit differences in biological activities and/or potencies.

Many groups have compared the antiviral effects of different human and animal IFN- α subtypes. Almost all contributions indicate that the antiviral activity exerted by some subtypes is greater than that of other subtypes. Sometimes a difference more than 1000 fold can be recorded. Differences in antiviral activity of IFN- α subtypes in cells can be related to their different ability to induce some of the effectors of IFN action such as 2',5'-oligoadenylate synthetase (2-5 OAS), protein Kinase R (PKR) and myxovirus resistance A protein (MxA).

Just some examples can be taken into account to substantiate the above considerations.

The antiviral activities of several subtypes of rIFN (IFN- α 1, 2, 6, 7, 8, 10) were evaluated against rhinovirus types 39 (RV 39) and 1A (RV 1A), or vesicular stomatitis virus (VSV). The 50% in-

hibitory concentrations (IC₅₀) ranged from 4±3 pg/ml for IFN-alpha 10 to >3000 pg/ml for IFN-alpha 1 against RV 39 or >3,000 pg/ml for IFN-alpha 1 against RV 1A. IFN-alpha 10 was the most active of the IFNs and was more active than IFN-alpha 2, against RV 39, RV 1A, and VSV (Sperber *et al.*, 1993).

In another study a few subtypes of IFN-alpha were compared in their activity against VSV and human immunodeficiency viruses (HIV). The IC₅₀ against HIV was 37±14 pg/ml for IFN-alpha 2, and >90,000 pg/ml for IFN-alpha 1. In general, relative activity against HIV was similar to relative activity against VSV on WISH cells, suggesting that IFN-alpha 1, compared to the other subtypes, possesses a lower antiviral activity on human cells (Sperber *et al.*, 1992).

More recently the effects of five human rIFN-alpha subtypes 1, 2, 5, 8, and 10 were compared in their ability to inhibit hepatitis C virus (HCV) replication using HCV subgenomic replicon system as a model. The results showed that each IFN-alpha subtypes suppressed HCV replication in a dose-dependent manner. Among them, IFN-alpha 8 was the most effective, while IFN-alpha 1 was the least effective with IC₅₀ of 0.123 IU/ml versus 0.375 IU/ml, respectively. The differential effects against HCV replication did not correlate with levels of the IFN-responsive ISRE or GAS reporter activities, nor they did activate the other known reporters such as AP1, NF-kappa B, CRE and SRE. This suggests that the divergent effects of IFN-alpha subtypes against HCV replication may depend on JAK-STAT-independent pathways (Koyama *et al.*, 2006).

Basically the above results had been already obtained by Yamamoto (Yamamoto *et al.*, 2002). Also in this study IFN-alpha 8 was the most potent while IFN-alpha 2, 5, and 10 were intermediately active, and IFN-alpha 1 was the least potent in all cell lines. The observed differences between the IC₅₀s of IFN-alpha 1 and - alpha 8 ranged from 250 - to 2200 - fold. The same authors also demonstrated that the relative antiviral activities of the subtypes were associated with the different induction of expression of 2,5-OAS in a typical hepatocellular carcinoma cell line (Yamamoto *et al.*, 2002).

Consistent with this finding is the more recent observation that a disparity in MxA-inducing activity of IFN-alpha subtypes does exist suggest-

ing that differences in anti-VSV-induced cytopathic effect of IFN-alpha subtypes in WISH cells may be related to their different ability to induce MxA (Schanen *et al.*, 2006).

All together these findings strongly indicate that the different IFN-alpha subtypes display the antiviral activity against specific viruses to a different and distinctive extent.

It is well known that type I IFN does not have only a direct antiviral activity but it may also play a role in both initiating and maintaining immune response. Again there is evidence that IFN-alpha subtypes may behave in different ways in this respect. For instance studies on peripheral blood mononuclear cells (PBMC) showed that IFN-alpha 1, but not all subtypes of IFN-alpha, is able to increase the expression of HLA class II antigens (Rhodes *et al.*, 1986). On the other hand, IFN-alpha 2 is less effective than other type I IFNs in antagonizing the increased expression of the above antigen when induced by IFN gamma (Dhib-Jalbut *et al.*, 1996). Further, it has been proposed that IFN-alpha 8, among the examined subtypes, had the strongest ability to upregulate HLA class II antigen expression (Yanai *et al.*, 2001). When the Th1-type cell percentages and Th1/Th2 ratios were examined it was found that both parameters were increased in PBMC of chronic hepatitis patients after cultivation *in vitro* with the IFN-alpha subtypes. In this respect significant differences between IFN-alpha 5 and 8 have been recorded (Ariyasu *et al.*, 2005).

It is also known that IFN-alpha induces chemokinesis of both CD4(+) and CD8(+) T cells together with functional changes that result in enhanced T cell motility, including up-regulation of the integrins LFA-1 and VLA-4, and subsequently, increased ICAM-1- and fibronectin-dependent migration. There is evidence that IFN-alpha 2 and IFN-alpha 8 significantly differ in the induction of the above indicated immunologic activities despite having similar effects on the induction of the antiviral A (MxA) protein. However, the same authors demonstrated that transcription of other IFN-stimulated genes seems to be selectively activated by IFN-alpha 2, but not IFN-alpha 8, in T cells (Foster *et al.*, 2004). Other differences in immunomodulatory activities are displayed by various IFN-alpha subtypes: for instance, it has been demonstrated that IFN-alpha subtypes may differ in their ability to affect the function of lympho-

cytes B. Specifically it is known that in co-stimulation with antibodies to IgM, IFN-alpha induced the cells to proliferate (but not to differentiate) and that IFN-alpha subtypes differed greatly in their relative proliferative effects. Specifically, IFN-alpha 8 at 0.1-0.5 ng/ml induced proliferation, whereas most of the other subtypes were active only at concentrations >5 ng/ml, and IFN-alpha 1 was substantially inactive (Hibbert and Foster, 1999). These differences do not parallel the expression of some of the IFN-alpha-induced gene such as those codifying HLA class I antigens.

Furthermore, the capacity of 3 IFN-alpha subtypes (alpha 1, alpha 2 and alpha 4) to augment human natural killer cytotoxicity after *in vitro* exposure was shown to differ among subtypes. Stimulation of NK activity by IFN-alpha 2 was consistently and significantly greater than by IFN-alpha 4 or IFN-alpha 1 (Verhagen *et al.*, 1990). This seems to be a general phenomenon: as far as NK-inducing activity of IFN is concerned, it has been demonstrated that there is a lack of correlation between NK-stimulatory and other activities of the IFN-alpha subtypes, suggesting that different biological/immunological activities may be mediated by different regions of the IFN-alpha molecule (Verhagen *et al.*, 1990).

It is also interesting to mention that augmented NK cytotoxicity did not correlate with the anti-proliferative effects of IFNs. In anti-proliferative assays, the hierarchy of activity is IFN-beta greater than IFN-alpha 2 greater than IFN-alpha 4, whereas, in the NK augmentation assay IFN-beta and IFN-alpha 2 were of equivalent activity (Losinno *et al.*, 1992). In contrast to its deficient ability to augment NK activity, IFN-alpha 7 has potent antiviral and antiproliferative activities (Ortaldo *et al.*, 1984).

All together the above findings suggest that the different IFN-alpha subtypes display the immunomodulatory activity to a different and unique extent.

Over the last three decades it has been also established that type I IFNs may inhibit the growth of many normal and transformed cells. This was why IFNs were used in clinical trials in different types of tumours. Indeed, for some forms of malignant diseases IFNs became the drug of choice (alone or in combination with other anticancer agents). Also for this definite activity, several re-

ports have shown that when the different IFN subtypes were compared on an antiviral unit basis for their antiproliferative effect their activity varied significantly (Foster and Finter, 1998). It is likely that the different subtypes do have differential effects on cell proliferation but the difference in the antineoplastic effects of the different IFN-alpha subtypes is far from being known in detail.

There are many other examples demonstrating a peculiarity of antiviral, immunomodulatory and antiproliferative actions of the different subtypes which have not been mentioned here. All together these findings strongly indicate that subtypes of IFN-alpha, often quantitatively and, sometimes, qualitatively, display disparate biological activities *in vitro*. The fact that the biological activities are distinct and definite is further demonstrated by the observation of a synergistic action among different IFN-alpha subtypes. For instance, a synergistic antiviral activity induced by IFN-alpha 2 and - alpha 8 in the hepatocellular carcinoma cell line HepG2 has been reported (Yamamoto *et al.*, 2002). This phenomenon is considered to reflect the synergistic induction of 2',5'-OAS. More importantly from this point of view is the observation that nHuIFN-alpha is a more potent suppressor of the response to profibrotic stimuli in human dermal fibroblasts than rIFN-alpha. This finding has been interpreted as due to the synergism between different IFN-alpha subtypes and antifibrotic cytokines and factors (Santak *et al.*, 2007).

As already stated, the above findings indicate that IFN-alpha subtypes possess distinctive biological properties, reinforcing the consideration that the IFN-alpha system represents an evolutionarily conserved group of secreted cytokines that co-ordinately act as mediators of host defence and homeostasis.

ARE ALL TYPE-I IFNS PHARMACOLOGICALLY EQUIVALENT?

The previous section provided evidence that the different IFN-alpha subtypes may possess distinct biological activities. All reported studies however referred to *in vitro* experiments. In the last decades evidence has been accumulating that the above differences can also be recorded in *ex vivo*

or *in vivo* situation. Below we provide some examples to support such a view. Transgenic mice expressing the IFN- α 1 gene had significantly lower cytomegalovirus (CMV) titers in the inoculated muscle than mice expressing either the IFN- α 4 or the IFN- α 9 transgenes. Furthermore, IFN- α /beta receptor knockout mice had markedly higher levels of CMV replication in the tibialis anterior muscles than the wild-type parental strain following IFN- α 1 transgene inoculation, suggesting that the protection observed is due to host cell-mediated IFN signalling. These data provide some of the first clear evidence indicating that differences in the antiviral efficacy of the IFN- α subtypes do also exist *in vivo* (Yeow *et al.*, 1998).

More recently the capacity of individual type I IFN multigene family members to abolish influenza virus replication in a vaccination/challenge mouse model was assessed. Differences in antiviral efficacy were found among the subtypes. IFN- α 5 and IFN- α 6 are the most effective, while IFN- α 1 is the least effective in reducing lung virus replication (James *et al.*, 2007).

Individual IFN- α subtypes and preparations of lymphoblastoid IFN were tested in three human tumour cell lines derived from liver, lung, and neuroblasts from patients. Their relative antiviral activities differed markedly; as far as their antiproliferative activity is concerned subtypes IFN- α 10, IFN- α 17, IFN- α 21, and IFN- α 5 were the most active, and IFN- α 2 was the least active. IFN- α 1 and IFN- α 8 had comparable intermediate activity (Foster *et al.*, 1996).

More importantly from the applicative point of view, there are some observations in patients indicating that in some conditions natural preparations of IFN- α , containing most of the natural mixture of IFN- α subtypes, may display a higher therapeutic efficacy than preparations of rIFN- α 2 or PEG-IFN- α , which are composed by only one subtype of IFN- α . This statement is justified by the following experimental data.

In 2003, Scagnolari *et al.* (2003) studied the pharmacodynamic profiles of different IFN- α preparations by measuring the serum neopterin levels and the levels of expression of protein MxA mRNA in *in vivo* PBMC in 2 patients with essential mixed cryoglobulinaemia whose resistance

to rIFN- α 2a treatment developed concomitantly with the production of serum neutralizing antibodies to IFN. These markers were measured before treatment and 24 and 48 h after a single injection of rIFN- α 2a, consensus IFN [(C)/IFN], or LeIFN. No increase or only a slight increase in MxA mRNA levels was detectable after administration of rIFN- α 2a or (C)IFN, whereas a significant increase (≥ 10 -fold) in MxA mRNA expression was recorded following administration of LeIFN. The neutralizing antibodies to rIFN- α 2a cross-react with (C)IFN. Sera from these patients neutralized most but not all of the subtypes present in the LeIFN mixture, and no significant increase in neopterin levels was observed after these patients were switched to LeIFN treatment. The data demonstrate that the issue of neutralizing antibodies still exists and that LeIFN may induce an increase in the level of MxA mRNA expression in patients resistant to treatment with rIFN- α 2a or (C)IFN and this is associated with a clinical response (Scagnolari *et al.*, 2003). Then the superiority of LeIFN compared to other IFN preparations may be explained at least in part by the difference in the antigenicity profile of the molecules included in the preparations.

Indeed, according to a recent publication, the appearance of antibodies neutralizing PEG-IFN antibody should be considered one of the causes of a lack of antiviral response (van der Eijk *et al.*, 2006). In this regard the recently reported case concerning the clinical outcome of a chronic hepatitis C patient with several favourable response factors to antiviral therapy (such as low viral load, 2a/2c genotype, and no co-morbidity) is also interesting. Despite the existence of such factors, two treatment regimens with PEG-IFN plus ribavirin, with and without amantadine, failed, most likely due to anti-IFN antibody development production which neutralized the PEG-IFN activity. The lack of IFN-induced side-effects during PEG-IFN treatments and the evidence of an antiviral response and typical IFN-related adverse events while being re-treated with LeIFN, which is known to be antigenically different from IFN- α 2, support this hypothesis (Santantonio *et al.*, 2006).

Interesting results have also been obtained on the tolerability and efficacy of LeIFN preparations in the re-treatment of patients poorly tolerant or non-responders/relapsers to rIFN- α preparation.

tions. For instance, some studies (Cacopardo *et al.*, 1998; Cagnoni *et al.*, 2000) demonstrated that the good compliance with LeIFN administration shown by chronic hepatitis patients poorly tolerant or non-responders/relapsers to rIFN-alpha allowed retreatment with full IFN-alpha doses to be started, thus increasing the chance to obtain a larger number of sustained responses.

In another study, Dughera *et al.*, (2002) demonstrated that in about 90% of the patients with haemophilia A or B or von-Willebrand's disease and chronic post-transfusional active HCV hepatitis who developed major side effects during the course of a previous treatment with rIFN-alpha, treatment with human LeIFN yielded a biochemical and clinical response. Interestingly, throughout the treatment with LeIFN none of the patients had to discontinue therapy due to severe adverse reactions (Dughera *et al.*, 2002).

The influence of the type of IFN-alpha commercial preparations on the variations in platelets and white blood cells was studied in patients suffering from chronic viral C hepatitis. The decrease was lower for patients treated with LeIFN than for the other IFNs again indicating the higher tolerability of LeIFN compared to rIFN-alpha preparations (Colombatto *et al.*, 1997; Toccaceli *et al.*, 1998).

Lastly, a couple of case reports merit discussion. The first presented the case of a patient with histologically proven chronic hepatitis C and chronic hepatitis B and additional compensated cirrhosis of the liver who achieved sustained complete biochemical and viral response following several months of therapy with LeIFN. Prior to this treatment, various other therapy approaches including rIFN-alpha 2b or a combination of natural IFN-beta (nIFN-beta) and IFN-gamma (rIFN-gamma) had been administered (Musch *et al.*, 2004).

The second report was the case of patient with pulmonary metastasis from renal cell carcinoma (RCC) treated with rIFN-alpha 2b. A complete response was achieved within 4 months and thereafter persisted for 5 years until he developed another lung lesion. Several types of alternative therapy were then administered without any response. The patient was then treated by LeIFN. The pulmonary lesion achieved a partial response after 11 months of treatment (Oya *et al.*, 2005). The explanation for the superiority of LeIFN-al-

pha compared to rIFN-alpha in the above cases has not been addressed, but it can be speculated that it is due to the above described peculiar characteristics of LeIFN. To explain the great tolerability (and efficacy in some instances) of LeIFN we have to consider again that LeIFN preparations contain a natural and physiologically weighted mixture of different IFN-alpha subtypes which can act in a coordinate manner, possibly each one being individually able to exert additional or synergistic effects other than the main activity for which IFN is being used.

This assumption found several experimental supports: for instance, there are findings showing that IFN-alpha 1, one of the major constituents of the LeIFN, poorly induces IFN-gamma-inducible protein-10 (IP-10) a key chemokine in Th1-type inflammatory diseases, compared to IFN-alpha 2. This observation may have clinical importance because IFN-alpha 1 may be more beneficial in cases where Th1-mediated side effects (e.g., exacerbation of autoimmune diseases) are not desirable (Hilkens *et al.*, 2003). Indeed, use of LeIFN treatment has been described as tolerable in populations with autoimmune hepatitis (Malaguarnera *et al.*, 2004) or inflammatory bowel disease (Bargiggia *et al.*, 2005).

FINAL CONSIDERATIONS AND CONCLUSIONS

It is now evident that IFN type I is a heterogeneous family of structurally and functionally related proteins. Among them there are at least 12 subtypes of IFN-alpha which are all involved in the control of several cellular functions and are all actively engaged in the host defence mechanism against infections but whose biological activities, which are not completely known in detail, are not the same. Over the last 10 years the structure of various human IFN-alpha subtypes has been elucidated by combining protein chemistry and molecular biology but some central issues in IFN-alpha biology remain to be clarified. Specifically, it is crucial to understand how different subtypes can generate similar signalling outputs but also govern specific cellular responses; how the different IFN subtypes activate the same cell surface receptor complex to mediate variable responses and, more in general, why the body pro-

duces so many of these IFNs). In this regard it has been proposed that IFN-alpha subtypes may exhibit differences in biological potencies based on their affinity interactions with the IFN receptor subunits, IFNAR1 and IFNAR2. Indeed, increasing evidence indicates that distinct differences in critical amino acid residues among the different IFN-alpha determine the nature of the ligand-receptor interaction and the subsequent responses. Another possibility is that a distinct binding site(s) on the IFN-alpha receptor and some IFN-alpha does exist.

Modern techniques in molecular biology and biochemistry have made it possible to generate highly purified IFN-alpha preparations for therapeutic purposes. Specifically IFN-alpha preparations used in the treatment of viral and neoplastic diseases consist of single or multiple IFN-alpha subtypes. From the data reported in the present review it seems possible to indicate that administering IFN-alpha 2 and LeIFN may lead to different *in vivo* action. Indeed, several reports have shown the pharmacological superiority of natural mixtures of IFN-alpha compared to single IFN-alpha preparations and this may be due to the fact that IFN-alpha subtypes are different from a structural and functional point of view and may have synergistic effects. All the considerations concerning the functional distinctions between IFN-alpha subtypes should prompt us to further address the possibility that such a difference may be more appreciated in clinical practice.

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