IMMUNOGENICITY OF INTERFERON-ALPHA2 IN THERAPY: STRUCTURAL AND PHYSIOLOGICAL ASPECTS

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Summary. – Recombinant human interferon (rIFN)-alpha2 has been approved for therapeutic application in a range of human oncological and viral diseases. However, some patients can develop strictly specific antibody response to rIFN-alpha2, which may diminish its therapeutic potential. Such humoral response appears to be quite complex and obviously depends on multiple parameters. Our review is aimed primarily to factors associated with structural modifications of rIFN-alpha2 that we consider crucial for formation of therapy-induced antibodies. These factors are either related to inherent conformational differences between three IFN-alpha2 subvariants or to immunogenically active contaminating derivatives resulting from production, purification and storage of this recombinant protein. In addition, the role of treatment regimen and physiological variables modulating the immune response to rIFN-alpha2 in the challenged organism are mentioned.

Key words: antibodies; immunogenicity; recombinant interferon-alpha2; therapy

ANTIBODIES AGAINST rIFN-ALPHA2 PRODUCED DURING THERAPY

The recombinant biotherapeutic proteins are now firmly established as a part of modern medicine. Human rIFN-alpha2 was one of the first agents to be approved for the therapeutic use and currently is available for the treatment of various oncological and viral diseases (e.g. hairy cell leukaemia, chronic myelogenous leukaemia, multiple myeloma, non-Hodgkin's lymphoma, malignant melanoma, acquired immunodeficiency syndrome (AIDS)-related Kaposi sarcoma, metastatic renal cell carcinoma and viral hepatitides B and C).

Since the first report in 1982 (Gutterman *et al.*, 1982), many clinical studies have demonstrated the formation of antibodies against rIFN-alpha2 administered to man. The immunogenicity of rIFN-alpha2 can become a serious problem, as its most important therapeutical applications require long-term treatment (usually over several months). As reviewed by Antonelli (1997), the incidence of therapy-induced antibodies to rIFN-alpha2 varies considerably from total absence in the group of hairy cell leukaemia patients to 61% reported in a group of patients with hepatitis C. The resistance to antitumour or antiviral actions of rIFN-alpha2 associated with the development of neutralising antibodies has been found in patients with chronic myelogenous leukaemia, hairy cell leukaemia, renal carcinoma, non-Hodgkin's lymphoma, B-cell leukaemia, cryoglobulinaemia and viral hepatitides B and C (Antonelli, 1997). It is generally accepted that the resistance to further IFN treatment is most likely to occur when neutralising antibodies to rIFN-alpha2 develop early in the therapy and at high titres. A better understanding of this phenomenon would improve the outlook for many patients for whose rIFN-alpha2 remains the primary therapeutic option.

Abbreviations: aa = amino acid; AIDS = acquired immunodeficiency syndrome; HSA = human serum albumin; IFN = interferon; rIFN = recombinant IFN; MAb = monoclonal antibody; MHC = major histocompatibility complex

POSSIBLE CAUSES OF IMMUNOGENICITY OF rIFN-ALPHA2 IN MAN

It surprises that recombinant homologue of natural human IFN-alpha2 can be recognised in human organism as

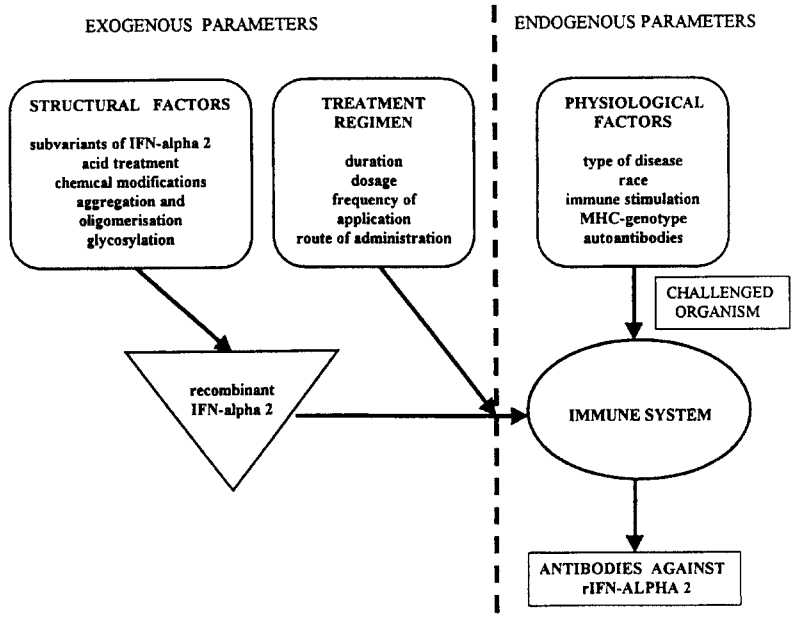

Fig. 1
Interactions of exogenous and endogenous factors affecting the immunogenicity of rIFN-alpha2 in man

„non-self„. The formation of antibodies against the rIFN-alpha2 injected into man appears to be a complex process influenced by broad spectrum of variables (Fig. 1), which can be grouped into two categories: (1) exogenous factors affecting the conformation of rIFN-alpha2 or modulating the basal immunogenic potential of the recombinant protein *via* treatment regimen, and (2) endogenous factors associated with physiological status of the challenged individual and its immune system in particular

At present the role of exogenous factors involved in humoral response to rIFN-alpha2 is much better characterised then that of endogenous factors. A brief description of conformational and antigenic properties of human IFN-alpha2 will facilitate the orientation in the topic discussed below.

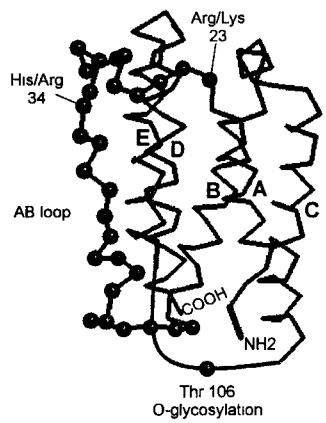

Fig. 2
A three-dimensional model of human IFN-alpha2
The model was constructed from coordinates from Brookhaven Protein Data Bank, Acc. No. 1RH2 (Radhakrishnan *et al.*, 1996). The A-E helices and the N- and C-termini of the molecule are marked. Amino acids (and their positions) differing in individual subvariants of IFN-alpha2, the glycolysation site at Thr106 and the AB loop (aa 23-50) are shown.

Tertiary and antigenic structure of IFN-alpha2

The polypeptide of human IFN-alpha2 consists of 165 amino acids (aa) and structurally belongs to the family of alpha-helical globulins. In contrast to the unglycosylated *E. coli*-derived rIFN-alpha2 is natural IFN-alpha2 a glycoprotein which carries O-linked carbohydrates at Thr106 (Adolf *et al.*, 1991). The conformational model of IFN-alpha2 is composed of five helices (A-E), which are linked by interhelical connections (loops) (Radhakrishnan *et al.*, 1996; Klaus *et al.*, 1997) (Fig. 2). The first three helices form the N-terminal domain, whereas the two others constitute the C-terminal domain. The N-terminal domain of IFN-alpha2 is structurally less stable then the rest of the molecule (Csabayová *et al.*, 1995). In spite of identical overall folding topology of all human IFN-alpha polypeptides, individual IFN-alpha subtypes exhibit distinct antigenic properties (Kontsek, 1994). In human IFN-alpha2 two immunodominant regions have been identified: the structure located around aa 30-50 is involved in high-affinity binding to a

cellular receptor, whereas the domain formed by aa 120-145 seems to play role in signal transduction (Kontsek, 1994; Kontsek *et al.*, 1991).

In human genome three allelic variants of IFN-alpha2 gene have been detected (Gewert *et al.*, 1995). At the amino acid level they differ from each other only at one or two positions. The structural differences are as follows: IFN-alpha2a has Lys23 and His34, IFN-alpha2b has Arg23 and His34, and IFN-alpha2c has Arg23 and Arg34 (Table 1). These substitutions located in the biologically relevant loop connecting helices A and B (loop AB) lead to minor structural alterations detectable by monoclonal antibodies (MAbs) (Siemers *et al.*, 1988; von Gabain *et al.*, 1990; Andersson *et al.*, 1991; Karayianni-Vasconcelos *et al.*, 1993a).

EXOGENOUS FACTORS AFFECTING ANTIBODY RESPONSE TO rIFN-ALPHA2

Exogenous factors of two types determine the production of antibodies to rIFN-alpha2 in treated patients: structural factors influencing directly the conformation of IFN-alpha2 molecule, and non-structural factors (treatment regimen) affecting the contact between the immunogen and the host immune system.

Structural factors

Humoral response to administered rIFN-alpha2 might reflect its structural differences from the host endogenous IFN-alpha2. The immune system is an extremely sensitive tool for recognition of even minor structural alterations in proteins and therefore subtle but immunologically relevant conformational differences between recombinant and natural IFN-alpha2 may elicit specific humoral response in human organism. The following alternatives are considered: (1) differences between subvariants of IFN-alpha2, and (2) modifications of the molecule of rIFN-alpha2 during production and storage.

Structural differences between IFN-alpha2 subvariants

All three recombinant subvariants of human IFN-alpha2 are available for clinical use: IFN-alpha2a (Roferon A, Hoffmann-LaRoche, Switzerland), IFN-alpha2b (Intron-A, Schering-Plough, USA) and IFN-alpha2c (Berofor, Basotherm, Germany). The capacity of exogenous IFN-alpha to raise an immune response in man varies among different preparations (Itri *et al.*, 1989; Weck *et al.*, 1989; Antonelli *et al.*, 1991, 1992; Steinmann *et al.*, 1992; von Wussow *et al.*, 1994; Oberg and Alm, 1997; McKenna and Oberg, 1997) (Table 1). In general, a minimal humoral response to natural (leukocyte or lymphoblastoid) IFN-alpha has been observed. With recombinant IFNs, an immunogenic potency comparable to that of natural IFN-alpha was reported for IFN-alpha2c, whereas application of two other subvariants is associated with substantially higher antibody formation. It was hypothesised that Arg23 and Arg34 contribute to the lower immunogenicity of IFN-alpha2c (Steinmann *et al.*, 1992). However, this finding may merely reflect a lower number of patients treated with IFN-alpha2c compared to those treated with subvariants 2a or 2b. The incidence of neutralising and binding antibodies is significantly higher against subvariant IFN-alpha2a than subvariant IFN-alpha2b.

Table 1. Clinical immunogenicity of different IFNs-alpha

IFN	Amino acids at positions		Frequency of antibodies (%)	
	23	34	neutralising	binding
rIFN-alpha2a	Lys	His	20-50	45-61
rIFN-alpha2b	Arg	His	0-24	15-51
rIFN-alpha2c	Arg	Arg	1	data not available
natural leukocyte IFN-alpha	–	–	0-1	data not available
natural lympho-blastoid IFN-alpha	–	–	0-6	9

Considering IFN-alpha2b gene as the predominant subvariant of IFN-alpha2 gene present in the normal population (Kaluz *et al.*, 1994; Gewert *et al.*, 1995) it has been proposed that antibodies (1) are formed to subvariant 2a because of minor antigenic differences between these subvariants, and (2) are directed to an epitope associated with this modification. However, available experimental data are not always consistent with this hypothesis. Therapy-induced antibodies from patients treated with rIFN-alpha2a or rIFN-alpha2b can cross-react with all three rIFN-alpha2 subvariants with similar titres (von Wussow *et al.*, 1989; Antonelli *et al.*, 1991; Nolte *et al.*, 1996). These experiments indicated that antibodies to any IFN-alpha2 subvariant do not specifically recognise epitopes associated with the respective immunogen but are directed to determinants common to all subvariants. In addition, no direct link between antibody formation against rIFN-alpha2a and the presence of its allele in genotype was detected (Crowe *et al.*, 1994). In transgenic mice bearing the human IFN-alpha2b gene, no antibody response was obtained following immunisation with either IFN-alpha2a or IFN-alpha2b (Palleroni *et al.*, 1997).

On the other side, a higher incidence of therapy-induced antibodies to rIFN-alpha2a and epitope mapping of these antibodies seem to support the immunogenic differences between subvariants 2a and 2b. Using a set of hybrid rIFNs-alpha in biological neutralisation assay Nolte *et al.* (1996) localised the epitopes for antibodies from cancer patients treated with rIFN-alpha2a within aa 17-64 and

possibly within aa 22-31. An alternative approach based on the competition between mapped MAbs to IFN-alpha2 and antibodies developed in hepatitis B patients treated with rIFN-alpha2a corroborated the assumption that the produced antibodies were directed against the site(s) located in the N-terminal region of aa 30-50 and inhibited cellular binding of IFN-alpha2c (Liptáková *et al.*, 1998). Both approaches led to the conclusion that the therapy-induced antibodies to rIFN-alpha2a selectively recognised only the N-terminal domain, which is essential for the binding of IFN-alpha2 to a cellular receptor (Senda *et al.*, 1995). The antigenic variability in AB loop between subvariants 2a and 2b was demonstrated by Siemers *et al.* (1988) using murine MAbs.

In summarising these controversial data we assume that antigenic differences between subvariants of IFN-alpha2, experimentally proved at the protein level, provide a firm support for a hypothesis that individual IFN-alpha2 subvariants exhibit a different immunogenic potential in human organism. It appears that Lys23 enhances the immunogenic potential of AB loop without changing overall antigenic structure of this domain. For analogy, MAbs cross-reacting with both human IFN-alpha1 and alpha2 were obtained only by immunisation with IFN-alpha1, whereas immunisation with IFN-alpha2 led exclusively to generation of IFN-alpha2-specific MAbs (Kontsek *et al.*, 1991; Karayianni-Vasconcelos *et al.*, 1993a). This indicated that even epitopes shared by several IFN-alpha subtypes may have different immunogenic potential in each subtype.

Structural modifications of rIFN-alpha2 during production and storage

Modifications of the structure of rIFN-alpha2 during its production and aggregate formation during its storage in particular have a major impact on its immunogenic potential.

Exposure to low pH

It has been hypothesised that conformational changes in rIFN-alpha2 may be induced by treating the bacterial culture with sulphuric acid during fermentation. However, no differences in the tertiary structure of „acid-treated„ and „non-treated„ IFN-alpha2a were observed by three different methods (ion-mass spectroscopy, CD and NMR) (Hochuli, 1997). It was concluded, that the acid exposure does not influence the immunogenicity of IFN-alpha2. In this context it should be noted that IFN-alpha retains its bioactivity after the pH 2-treatment and, therefore, any significant changes in the overall conformation of IFN-alpha2 molecule after acidification are not probable. On the other side, the immune system has an extreme ability to recognise a minimal structural modification of the protein surface. Using MAbs we have demonstrated that the exposure of rIFN-alpha2 to pH 2 caused structural modification of its antigenic surface (Kontsek *et al.*, 1991; Kúdela *et al.*, 1996). Based on these findings we assume that antigenic properties of pH 2-treated rIFN-alpha2 can slightly differ from those of natural human counterpart. Therefore a potential effect of low pH on the immunogenicity of rIFN-alpha2 should still not be neglected.

Chemical modifications

A possible cause of enhanced immunogenicity is also chemical modification of IFN-alpha2 molecules, which potentially occurs during preparation, formulation and storage of the final product. In particular, the increased immunogenicity might be associated with oxidation of sulfhydryl groups that takes place when rIFN-alpha2 is stored at ambient temperature (Hochuli, 1997). When tested in mice, monomers of rIFN-alpha2 with two of the methionine residues oxidised to methionine sulfoxid had enhanced immunogenicity compared to the native molecule (Hochuli, 1997).

Aggregate formation

The oxidised forms of IFN tend to form oligomers or aggregates with human serum albumin (HSA) routinely added to IFN preparations. The amount of aggregates is temperature-dependent but does not depend on the time of storage at 4°C (Hochuli, 1997). In the mouse, the relative immunogenicity of IFN-alpha2a correlated with the increase in IFN oligomers and IFN-HSA aggregates (Palleroni *et al.*, 1997). These findings indicate that high molecular mass aggregates of rIFN-alpha2 may contribute to the immunogenicity of this protein injected into man.

To reduce the risk of formation of immunogenic contaminants during storage, rIFN-alpha2 should be stored at 2 – 8°C. In addition, a new formulation without HSA eliminating the possibility of IFN-HSA aggregation has been developed (Hochuli, 1997).

Carbohydrate moiety

It has been hypothesised that carbohydrates present in the native IFN-alpha2 may influence its immunogenicity by masking immunogenic sites, because the administration of natural IFN-alpha to man elicits none or minimal antibody response (Galton *et al.*, 1989). The immunogenic potential of *E. coli* – derived IFN-alpha2 may be then boosted by the lack of carbohydrate moiety. However, a direct proof supporting this hypothesis is still missing. No significant antigenic differences between glycosylated natural and non-glycosylated rIFN-alpha2 proteins have been identified (Adolf *et al.*, 1991;

Karayianni-Vasconcelos *et al.*, 1993b). Moreover, patients' antibodies to *E. coli*-derived IFN-alpha2 did not discriminate between glycosylated natural IFN-alpha2 and rIFN-alpha2 (Nolte *et al.*, 1996). The therapy-induced antibodies are primarily raised to the AB loop of IFN-alpha2, but the glycosylated Thr106 is situated in the CD loop on the opposite side of the molecule (Fig. 2). In spite of high flexibility of the region of aa 102-106 in IFN-alpha2 (Radhakrishnan *et al.*, 1996) it seems unlikely that the bound carbohydrates could mask epitopes located in the N-terminal domain.

In this context it should be pointed out that natural IFN contains at least 14 different molecular species but only a few of them are glycoproteins. IFN-alpha14 and IFN-omega are N-glycosylated and IFN-alpha2 is O-glycosylated (Labdon *et al.*, 1884; Adolf *et al.*, 1991). Therefore a more plausible explanation of lower immunogenicity of natural IFN-alpha suggests that this IFN may be less immunogenic because individual subtypes are present at lower (sub-immunogenic) concentrations than single recombinant species in an equal dose of rIFN-alpha2 (Antonelli, 1997). This issue is still controversial, nevertheless, the role of glycosylation should at least be considered.

Alternative treatment of patients developing antibodies against rIFN-alpha2

It is interesting that some patients who became resistant to rIFN-alpha2 treatment could be cured effectively with a natural IFN-alpha preparation which was a mixture of functionally homologous but antigenically different subtypes (von Wussow *et al.*, 1991a,b). Distinct antigenic properties of single IFN-alpha subtypes may explain this phenomenon. The therapy-induced neutralising antibodies against rIFN-alpha2 were highly specific for this particular subtype and neutralised other IFN-alpha subtypes only marginally (Gutterman *et al.*, 1982; Nolte *et al.*, 1996). Therefore the neutralising capacity of patients' antibodies raised to rIFN-alpha2 seems to be unable to inhibit biological potency of all subtypes in natural IFN-alpha.

Another solution for treatment of patients responding to rIFN-alpha2 may be provided by application of rIFN preparations with analogical spectrum of bioactivity but with distinct antigenic properties (e.g. IFN-alpha1 or IFN-omega).

Nonstructural factors

Treatment regimen

Humoral response to an immunogen can be affected by the treatment regimen. In principle, these variables are determined by interactions between rIFN-alpha2 and the host immune system and their effects might modulate the level of immunogenicity of the administered preparation in organism.

Duration of treatment

A higher incidence of antibodies has been reported in patients long-term treated with rIFN-alpha2 compared with patients treated for a shorter period of time (Figlin *et al.*, 1986; Steis *et al.*, 1988). Most patients developed antibodies against rIFN-alpha2 within one year after the treatment, and the median time varied from 6 – 12 months for IFN-alpha2a (von Wussow *et al.*, 1994; Russo *et al.*, 1996) to 13 – 25 months for rIFN-alpha2b (von Wussow *et al.*, 1989; Oberg and Alm, 1997).

Dosage

Higher doses of rIFN-alpha2 are more likely to precipitate the antibody formation than lower doses (Dianzani *et al.*, 1989), even though some data indicated a higher immunogenicity of lower doses (Porres *et al.*, 1989).

Frequency of application

The probability of the antibody development in patients increases with the number of times the immunogen is introduced (Itri *et al.*, 1989), as confirmed also experiments in the mouse model (Palleroni *et al.*, 1997).

Route of administration

The seroconversion rate was reported to be significantly higher in patients treated with rIFN-alpha2 subcutaneously then intravenously (Larocca *et al.*, 1989; Oberg and Alm, 1997). An analogical conclusion was drawn from animal experiments (Palleroni *et al.*, 1997).

ENDOGENOUS FACTORS AFFECTING ANTIBODY RESPONSE TO rIFN-ALPHA2

Response to a given immune challenge is determined not only by structural properties of the immunogen but also by physiological status of the challenged individual. It is reflected by a fact that only a portion of treated patients develop antibodies to rIFN-alpha2. The physiological factors given below might modulate the immune system:

Type of disease

With regard to the type of disease of patient challenged with rIFN-alpha2 the incidence of antibodies against rIFN-alpha2 was lower in patients with leukaemia, lympho-

ma and melanoma (13 – 22%) than in patients with renal carcinoma (42%) (Itri *et al.*, 1989). In general, oncological patients produce antibodies to rIFN-alpha2 to a lesser extent than patients suffering from infectious diseases (Jacobs *et al.*, 1989).

Race

Although the immune response against rIFN-alpha2 is not influenced by sex and age, there seems to be difference between racial groups. Chinese patients with chronic hepatitis B developed neutralising antibodies against rIFN-alpha2a more frequently (39%) than Caucasian patients (14%) (Lok *et al.*, 1990).

Immune stimulation

The immunomodulatory effect of administered rIFN-alpha2 may exert concomitant stimulation of host antibody response to other molecules. Experiments on mice showed that the immunomodulatory effect of mouse IFN-alpha may raise the antibody production against human rIFN-alpha2 (Palleroni *et al.*, 1997).

Major histocompatibility complex (MHC)-genotype

MHC affected the antibody-response to human rIFN-alpha2 in mice (Palleroni *et al.*, 1997). High-titre neutralising antibodies against IFN-alpha2a were detected in mice with H-2^d haplotype, but no specific antibody response was detected in mice with H-2^b haplotype Analogically, the MHC genotype of patients may likewise influence the antibody response to rIFN-alpha2.

Autoantibodies

Natural autoantibodies of low prevalence against cytokines seem to contribute to regulation of the cytokine network in healthy individuals (Bendtzen *et al.*, 1990). An increased level of IFN-alpha2 after injection of a recombinant cytokine into human organism might trigger the physiological response resulting in production of antibodies against IFN-2 to restore the disturbed homeostasis. Such immune reaction might result in some patients with the organism weakened by the disease in extremely high secretion of autoantibodies against IFN-alpha2.

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