THERAPY-INDUCED ANTIBODIES TO INTERFERON-ALPHA 2A RECOGNISE ITS RECEPTOR-BINDING SITE

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Summary. – Fifty-eight patients with chronic hepatitis B (HB) or C (HC) were treated with recombinant human interferon (rIFN)-alpha 2 and their sera were assayed for antibodies to rIFN-alpha 2c. Twelve of these patients produced low titres and two high titres of the antibodies. We localised the region which was recognised by the high-titre therapy-induced antibodies on the IFN molecule by testing the antibodies with a set of murine monoclonal antibodies (MoAbs) to IFN-alpha 2 in a competitive radioimmune assay (RIA). Only MoAbs with epitopes located in the amino-terminal portion of IFN-alpha 2 could inhibit the binding of radiolabelled IFN-alpha 2 by patients' sera. Our data indicate that the therapy-induced antibodies were directed to the receptor-binding domain of IFN-alpha 2 formed by amino acids (aa) 30–53. In accordance with this observation, human anti-IFN sera inhibited the binding of rIFN-alpha 2 to human cells.

Key words: hepatitis B and C; interferon-alpha 2; therapy; antibodies; epitopes; sequencing

Introduction

rIFN-alpha 2 is a potent antiviral drug which is frequently used in the therapy of chronic HB and HC. For clinical use, three subvariants of IFN-alpha 2 (a, b and c) with subtle antigenic differences (von Gabain *et al.*, 1990; Karayianni-Vasconcelos *et al.*, 1993) are commercially available (Table 1). However, some patients can develop specific humoral response to these preparations that can interfere with the efficacy of the IFN treatment (Antonelli, 1997). Factors contributing to the formation of antibodies to a recombinant analogue of an human protein are still a matter of debate. Clinical studies have demonstrated that the incidence of the therapy-induced antibodies to IFN-alpha 2a was higher than that to IFN-alpha 2b (Antonelli *et al.*, 1991, 1992; von Wussow *et al.*, 1994). The allele coding for subvariant 2b is predominant in human population (Kaluz *et al.*, 1994; Gewert *et al.*, 1995). Therefore it has been suggested that structural differences between the endogenous IFN-alpha 2 subvariant (coded by patient genome) and the exogenous IFN-alpha 2 subvariant (used for treatment) could elicit an antibody response to a „non-self" protein. However, the available data are not fully consistent with this theory. Little is known also about the segments of IFN-alpha 2 toward which the host antibody response is directed. We mapped the epitopes on IFN-alpha 2 molecule which were recognised by the antibodies formed in patients with chronic viral hepatitis undergoing treatment with rIFN-alpha 2. In addition, the clinical relevance of high-titre antibodies to IFN-alpha 2 was briefly addressed. Our findings might contribute to a better understanding of the phenomenon of therapy-induced antibodies to rIFN-alpha 2 in man.

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Abbreviations: aa = amino acid; ALT = alanine aminotransferase; ELISA = enzyme-linked immunosorbent assay; HB = hepatitis B; HBeAg = HBe antigen; HBsAg = HB surface antigen; HBV = hepatitis B virus; HC = hepatitis C; IFN = interferon; PBS = phosphate-buffered saline; RIA = radioimmune assay; rIFN = recombinant IFN

Materials and Methods

Patients and IFN treatment. The trial involved 58 patients with biopsy-proven chronic HB or HC. The patients recieved 1.5 – 5 x 10^6 U of human rIFN-alpha 2a (Roferon A, Hoffmann-LaRoche) or 2b (Intron A, Schering-Plough) intramusculary three times a week in two 6-month intervals. Serum samples were taken from patients at different intervals after the treatment and stored at -70°C.

Diagnosis of infection with hepatitis B virus (HBV). Viral markers were measured in sera simultaneously using commercially available kits. Serum HBe antigen (HBeAg) and HBeAg antibodies, HB surface antigen (HBsAg) and HBsAg antibodies were determined by an enzyme-linked immunoassay (ELISA) using the Murex Diagnostic (A_{450}) or Sanofi Diagnostic Pasteur (A_{490}) kits. HBV DNA was measured by the Digene Hybrid Capture System HBV DNA Assay kit. Alanine aminotransferase (ALT) level (μcat/l) was determined using an autoanalyzer.

RIA of IFN-binding activity. Serum samples were tested for the IFN-binding activity against IFN-alpha 2c by RIA. Human IFN-alpha 2c (kindly provided by Prof. G. Bodo, Bender, Vienna, Austria) was labelled with $Na^{125}I$ by the chloramin T method (Kontsek *et al.*, 1991). Fifty-μl aliquots of sera diluted 1:2 with phosphate-buffered saline (PBS) were pipetted onto 96-well plates and incubated overnight at room temperature. The plates were washed with 0.05% Tween 20 in PBS and the non-specific binding was saturated by treating the plates with 1% non-fat dry milk for 1 hr. Radiolabelled IFN (10^5 cpm in 50 ml PBS per well) was added to the plates for 1 hr at room temperature. After washing, the bound radioactivity (cpm) in the wells was determined in triplicate. Values at least two times higher than that of the negative control (serum of a healthy person) were considered positive. The antibody titre was defined as the reciprocal of the highest dilution of serum with specific binding over 100 cpm. The specific binding represented the difference (in cpm) between the total binding of labelled IFN by a serum and the non-specific binding by a serum saturated with unlabelled IFN-alpha 2c (1 μg/ml).

Screening for human Ig by ELISA. Plates were coated with IFN-alpha 2c (1 mg/ml in PBS, 50 ml/well) overnight at 37°C. After blocking, sera diluted 1:2 were added in duplicate to the plates (50 μl/well) for 1 hr at room temperature. The plates were washed and the bound human Ig was detected with goat anti-human IgG (Jackson Immunochemicals). The reaction was visualised by orthophenylenediamine and A_{492} was measured. Readings at least two times higher than that of the negative control (serum of healthy person) were considered positive.

Sequencing of human IFN-alpha 2 gene. The genomic DNA from peripheral blood leukocytes was prepared as follows. A blood sample was lysed with 0.84% ammonium chloride (10 mins, 4°C), centrifuged and the resulting pellet (10^8 leukocytes) was boiled for 5 mins. After centrifugation, the resulting supernatant was directly used as template for polymerase chain reaction (PCR) to amplify human IFN-alpha 2. The primers were as follows: a 24-mer (ATG GCC TTG ACC TTT GCT TTA CTG) sense oligonucleotide corresponding to the beginning of the signal sequence of human IFN-alpha 2, and a 27-mer (TCA TTC CTT ACT TCT TAA ACT TTC TTG) antisense oligonucleotide corresponding to the end of a 570 bp region. The amplification and sequencing of the PCR product were performed as described previously (Kaluz *et al.*, 1994).

Competitive RIA for mapping of therapy-induced antibodies to rIFN-alpha 2. The competition between mapped MoAbs to IFN-alpha 2 and IFN-alpha 2 antisera for binding of radiolabelled IFN-alpha 2c was measured. Mouse MoAbs to human IFN-alpha 2 (N22, N27, N40, N27, N22, 2-31 and 2-19) have been characterised previously (Kontsek *et al.*, 1991,1993; Karayianni-Vasconcelos *et al.*, 1993). Immunoplates were coated with antisera (50 μl/well) diluted 1:10 in PBS. MoAbs (culture supernatants, 50 μl/well) were mixed with labelled IFN (10^5 cpm/50 μl/well) at room temperature for 1 hr and then added to the wells precoated with the antisera. After incubation for 1 hr at room temperature, the plates were washed and the bound radioactivity was measured. Each MoAb-antiserum combination was tested in triplicate in two independent experiments. Non-labelled IFN-alpha 2c and an irrelevant MoAb to tau protein were used as positive and negative soluble competitors, respectively.

Inhibition of cellular binding of rIFN-alpha 2. The capacity of anti sera or MoAbs to inhibit the cellular binding of IFN-alpha 2 was tested on human HL60 cells. These cells were grown in Dulbecco's modification of Eagle's Medium (DMEM) with 10% inactivated horse serum. Before experiment, the cells were incubated for 10 mins at 37°C in 0.01% sodium azide and then adjusted to the concentration of 10^6 cells in 50 ml of PBS. The labelled IFN (10^5 cpm/50 ml) in PBS was mixed with an equal volume of the antiserum (diluted 1:10 in PBS) or MoAb (culture supernatant) and incubated for 1 hr at room temperature. The mixture was then added in triplicate to the cell suspension. After incubation for 1 hr at room temperature and washing, the cell-bound radioactivity was measured. In IFN-binding inhibition experiments, non-labelled IFN-alpha 2c and an irrelevant MoAb were used as positive and negative controls, respectively.

Results and Discussion

Detection of therapy-induced antibodies to rIFN-alpha 2

Preparations of rIFN-alpha 2a or rIFN-alpha 2b were administered to 58 patients suffering from chronic HB or HC. Serum samples taken at different intervals after the IFN administration were screened for binding to rIFN-alpha 2c by RIA. Twelve patients showed low IFN-binding activity with titres of 1:4 – 1:16. Sera of two patients (23-year-old (No. 1) and 70-year-old (No. 2) women with chronic HB) exhibited specific IFN-binding with a median titre of 1:64 (range 1:32 – 1:128). Both these high-titre anti-IFN factors were identified as human IgG by ELISA (data not shown). The screening for antibodies developed to rIFN-alpha 2a or rIFN-alpha 2b by use of IFN-alpha 2c as antigen supported an assumption that the therapy-induced antibodies to the particular IFN-alpha 2 subvariant are not specific for

the immunogen but cross-react also with other IFN subvariants (von Wussow *et al.*, 1989; Palleroni *et al.*, 1997).

Determination of the allele coding for IFN-alpha 2 subvariant

In human population, the allele coding for subvariant IFN-alpha 2b is predominant. Thus, the subvariant 2a may be more immunogenic owing to its structural difference from the patient's endogenous IFN-alpha 2 subvariant. Therefore we tried to identify the alleles coding for IFN-alpha 2 in the two patients with high humoral response after the rIFN-alpha 2 a treatment. Sequencing of the PCR product obtained from genomic DNA of patient No. 1 with primers specific for IFN-alpha 2 showed nucleotides G and A at positions 137 and 171, respectively, known as specific for the IFN-alpha 2b allele (Table 1). An attempt to perform a similar analysis for patient No. 2 was unsuccessful. In spite of a demonstration for patient No. 1 that the endogenous and exogenous IFN-alpha 2 subvariants were different, the relevance of such difference for the development of specific antibodies requires further investigation. Only 50% of individuals carrying alleles for subvariant 2b developed neutralizing antibodies after treatment with IFN-alpha 2a (Crowe *et al.*, 1994). On the other side, the frequency of therapy-induced antibodies against IFN-alpha 2b is much higher then the frequencies of alleles coding for other IFN subvariants in the population (Gewert *et al.*, 1993). Taken together, the difference between endogenous and exogenous IFN-alpha 2 subvariants does not seem to play a role in the development of specific host reaction.

Epitope mapping of therapy-induced antibodies to rIFN-alpha 2

In an attempt to shed more light on the subtle specificity of humoral response to a recombinant countrepart of natural protein , we identified a segment of rIFN-alpha 2 to which the patient's antibodies were directed. The epitopes recognised on IFN-alpha 2c by human antibodies to IFN-alpha 2a were localised using competitive RIA. To achieve this goal, we exploited a set of murine MoAbs recognising mapped epitopes on human IFN-alpha 2. In competitive assay, the IFN antisera were immobilised and their capacity to bind the labelled IFN-alpha 2c preincubated with each MoAb was measured (Table 2). All MoAbs but one (2–31) with sites located in the aminoterminal region of IFN-alpha 2 (aa 1–64) reduced the reactivity of IFN with the antisera (Fig. 1). The highest competition was exhibited by MoAbs N27 and N40 which were specific for the region of aa 30–53. In contrast, the MoAbs recognising the carboxyterminal region of IFN-alpha 2 (aa 66–166) did not interfere with the binding of human antibodies to IFN-alpha 2. Such a finding was rather surprising because the therapy-induced antibodies appeared to be directed only to the aminoterminal antigenic domain instead of reacting also with the second immunodominat structure of rIFN-alpha 2 located in the carboxyterminal domain (Kontsek *et al.*, 1991), as expected from experiments with polyclonal antibodies. Nevertheless, our results are consistent with those of Nolte *et al.* (1996), who used neutralising assay and hybrid rIFNs for analysis of the epitope specificity of therapy-induced antibodies to IFN-alpha 2a in leukemic patients. Only antibodies specific for the aminoterminal portion (aa 17–64) of the immunogen were identified.

Inhibition of cellular binding of IFN-alpha 2

In type I IFNs, the polypeptide region of aa 29–41 seems to be crucial for their high-affinity interaction with their cellular receptor (Senda *et al.*, 1995). Our experiments indicated that the therapy-induced antibodies to IFN-alpha 2 could bind this receptor-binding structure, because the highest competition was observed with MoAbs recognising the region of aa 30–53. Therefore we compared the capacity of the MoAbs and IFN antisera to inhibit the binding of labelled IFN-alpha 2c to the surface receptors of human HL60 cells (Fig. 2). These experiments demonstrated a decrease in the binding after preincubation of IFN with the positive antisera but not with the

Table 1. Differences between IFN-alpha 2 subvariants at amino acid and nucleotide level

IFN-alpha 2 subvariant	Commercial preparation	Amino acid at position		Nucleotide at position	
		23	34	137	171
a	Roferon A	Lys	His	A	A
b	Intron A	Arg	His	G	A
c	Berofor	Arg	Arg	G	G

Table 2. Competition between MoAbs and antisera in binding to IFN-alpha 2c

Soluble competitor MoAb		^{125}I-IFN-alpha 2c (cpm) bound to immobilised antiserum	
MoAb	Epitope (aa)	Patient No.1 (mean±SD)	Patient No.2 (mean±SD)
2-31	1–64	260 ± 74	1730 ± 238
N40	30–40	134 ± 23	966 ± 40
N27	43–53	121 ± 14	750 ± 29
N54	105–145	298 ± 6	1929 ± 112
2-19	66–166	255 ± 18	2339 ± 163
Myc72 (negative control)	–	258 ± 32	1894 ± 587
Unlabelled IFN (positive control)	–	116 ± 15	435 ± 43

Data from one representative experiment out of two performed are shown.

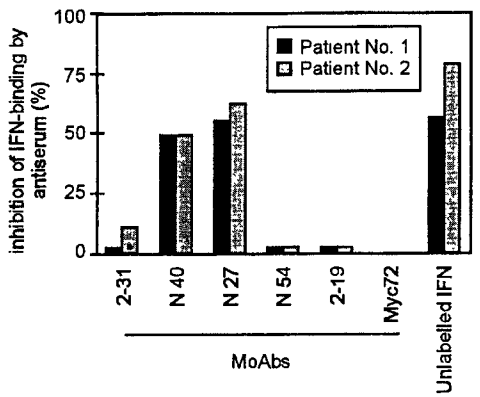

Fig. 1
Effect of MoAbs on the IFN-binding by antisera

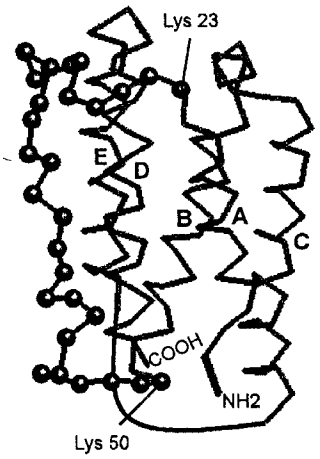

Fig. 3
A three-dimensional model of human IFN-alpha 2
It was constructed from the coordinates from the Brookhaven Protein Data Bank, Accession No. 1RH2 (Radhakrishnan *et al.*, 1996). The helices A-E and the amino and carboxyl termini of the molecule are marked. The loop connecting helices A and B (aa 23–50) and forming antigenic structure recognised by therapy-induced antibodies in IFN-alpha 2a is indicated.

serum of healthy individual. The strongest competitor MoAbs, N27 and N40, demonstrated also the highest potency to inhibit the cellular binding of IFN. In contrast, the only amino-terminally-specific MoAb 2–31 that did not compete with the IFN antisera and all carboxyterminally-specific MoAbs lacked a potency to inhibit the receptor binding. These data corroborated to conclusion from our mapping experiments that the therapy-induced antibodies could be directed to the receptor-binding domain of IFN-alpha 2. In a conformational model of IFN-alpha 2, this segment corresponds to the loop AB formed by aa 23–50 (Fig. 3) (Radhakrishnan *et al.*, 1996).

Clinical relevance of IFN antibodies

It is generally accepted that high level of IFN antibodies could be associated with the diminution of therapeutic efficacy of IFN (Antonelli, 1997). Also we have observed a lower therapeutic effect of rIFN-alpha 2a in chronic HB patients with high level of IFN antibodies. For ilustration, clinical parameters of HBV infection (virological markers and ATL level) in a patient lacking IFN antibodies and in a patient (No. 1) with high titre of IFN antibodies were compared (Fig. 4). A complete remission after IFN treatment in the patient without IFN antibodies could be observed, as indicated by loss of HBV DNA, seroconversion from HBsAg and HBeAg to HBsAg antibodies and HBeAg antibodies, and normalisation of serum ALT level. In contrast, in the patient with high titre IFN antibodies, we demonstrated permanent presence of HBV DNA, continuing Hbs antigenaemia and oscillation of ALT level. Nevertheless, it remains an unanswered question whether a less favorable clinical outcome of IFN-alpha 2 therapy reflected the direct involvement of IFN antibodies or these antibodies merely arose as a result of overall disbalance of the host immune system.

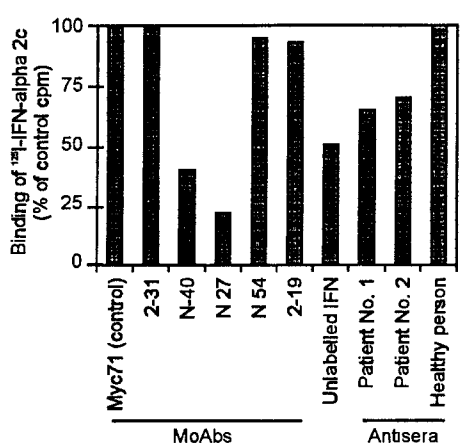

Fig. 2
Inhibition of the IFN-binding to HL60 cells by MoAbs and antisera
Localisation of epitopes for corresponding MoAbs is indicated.

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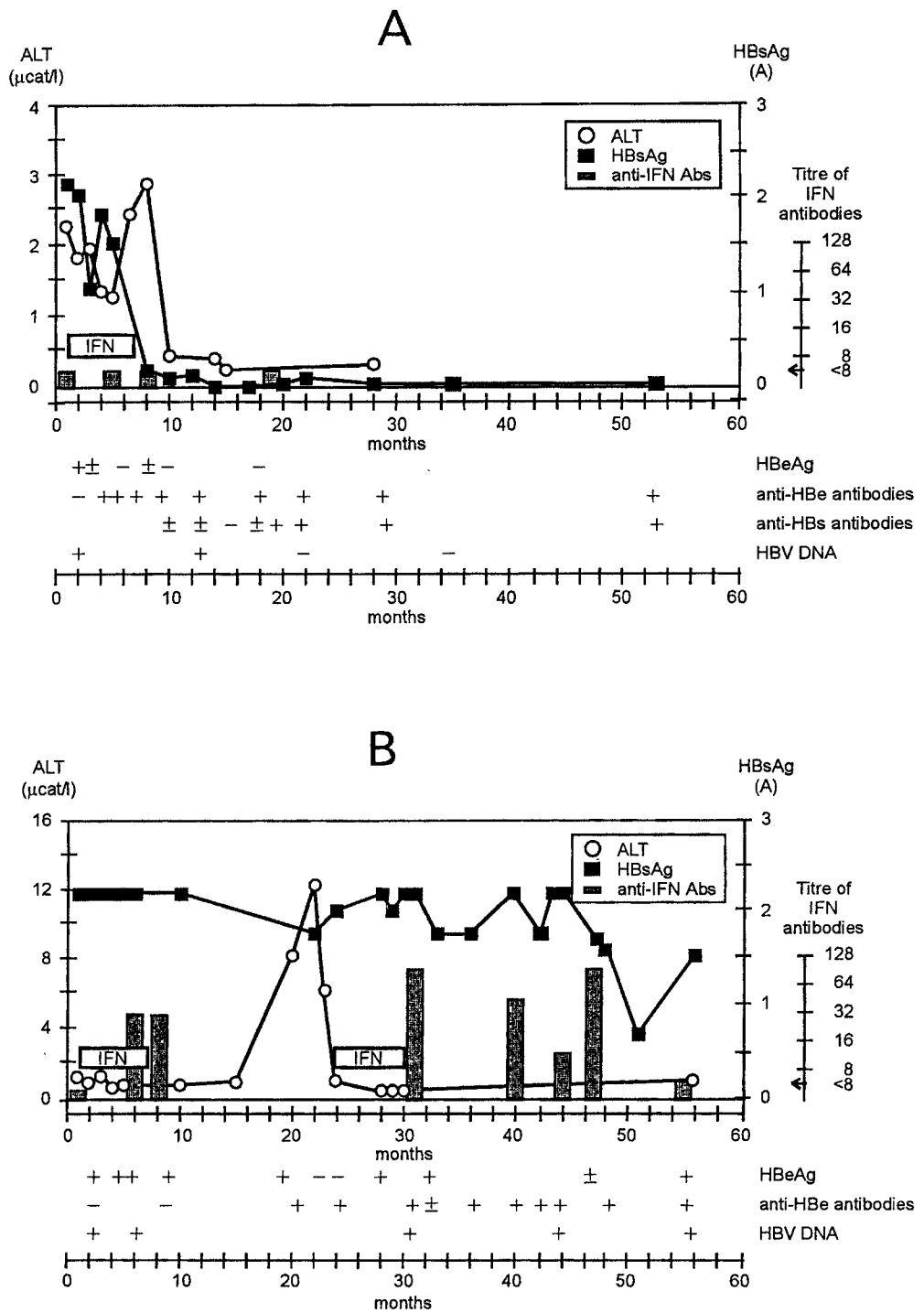

Fig. 4

Dynamics of HBV serum markers during IFN treatment in a patient without the therapy-induced IFN antibodies (A) and in a patient with high-titre IFN antibodies (B)

In the upper part, the presence (+), absence (–) or ambiguous status (±) of HBeAg, HBe antibodies, HBs antibodies and HBV DNA is indicated. The boxed IFN marks the period of IFN treatment.

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