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MODIFICATION OF THE ANTIGENIC STRUCTURE OF HUMAN INTERFERON ALPHA-2 BY PH 2 TREATMENT: A FURTHER SUPPORT FOR THE ANTIGENIC RELATIONSHIP BETWEEN ALPHA AND BETA INTERFERONS

P. KONTSEK[1], L. BORECKÝ[1], E. KONTSEKOVÁ[1], M. NOVÁK[1], V. KRCHŇÁK[2]

[1]Institute of Virology, Slovak Academy of Sciences, Bratislava and [2]Léčiva Pharmaceutical Company, Praha, Czechoslovakia

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Summary. — We have recently reported that a unique antigenic structure, designated as Common epitope 1, was found to be shared by human recombinant IFN alpha-2 and the human fibroblast IFN beta. The Common epitope 1 was identified with the aid of a synthetic IFN alpha-2 fragment SH 132-137. Based on this observation, we proposed the hypothesis that an antigenic relationship should exist also between natural human leukocyte IFN alpha and natural human fibroblast IFN beta. However, we were not able to detect any Common epitope 1 in preparations of conventional human leukocyte IFN alpha. In the present study, we were looking for a possible explanation of absence of the Common epitope 1 in conventional leukocyte IFN alpha. First, we demonstrated its acid labile nature in the recombinant IFN alpha-2 molecule and second, we proposed that the pH 2 lability of this unique epitope might be responsible for the lack of antigenicity also in pH 2-treated (conventional) leukocyte IFN alpha preparations. Actually, when pH 2 non-treated leukocyte IFN alpha was examined, we succeeded in demonstration of the Common epitope 1 in IFN-preparation. Moreover, antiserum against pH 2 non-treated IFN alpha was capable of neutralizing both the conventional i.e. pH 2-treated leukocyte IFN alpha and fibroblast IFN beta. It is concluded that the nomenclature distinguishing two classes (i.e. alpha and beta as class I and gamma as class II) of IFNs is more appropriate than the current official nomenclature distinguishing three antigenic classes of IFNs.

Key words: *interferon alpha*; *beta*; *antigenicity*; *cross-reaction*; *common epitope*

Introduction

On the basis of antigenicity, biological, and chemical properties, human interferons (IFN) are classified into three major types — alpha, beta, and gamma (Stewart, 1979). However, IFN alpha and IFN beta have several biologi-

cal and physicochemical properties in common and have supposedly evolved from a common ancestral gene (Taniguchi *et al.*, 1980). In spite of a certain grade of homology between IFN-types alpha and beta on the nucleic and amino acid levels, they are considered to be immunologically quite distinct (Hayes, 1981). The axioma of the sharp antigenic difference between alpha and beta types of human IFN has been recently challenged by finding a unique antigenic structure localised on molecules of both recombinant IFN alpha-2 and fibroblast IFN beta (Kontsek *et al.*, 1990). Synthetic polypeptide fragments of IFN alpha-2 aided in its nearer identification. The antibodies directed against this structure, called tentatively Common epitope 1, were capable of neutralizing both IFN alpha-2 and IFN beta with the same efficiency. However, the Common epitope 1 could not be detected in conventional natural leukocyte IFN alpha preparations despite that IFN alpha-2 is considered to represent the major subtype of virus induced human leukocyte IFN alpha (Rubinstein *et al.*, 1981; Levy *et al.*, 1981; Pestka, 1983).

In this paper we show that the acid labile nature of this unique antigenic structure of IFN alpha-2 molecule might be responsible for the absence of the Common epitope 1 in natural virus induced leukocyte IFN alpha since in preparations of pH 2 non-treated leukocyte IFN alpha the presence of the Common epitope 1 has been clearly detected.

Materials and Methods

Interferons. Conventional human leukocyte IFN alpha (Leu IFNα, 10^6 units per mg) was provided by Dr. N. Fuchsberger from this Institute. The inducer Newcastle disease virus (B1 strain) was inactivated in this preparation by incubation of IFN at pH 2 for 48 hr. Human pH 2 non-treated leukocyte IFN alpha (NT-Leu IFNα, 1.28×10^5 units per ml) was prepared using the same virus for induction as above but the inducer virus was removed from preparation by ultracentrifugation at $100\,000 \times g$ for 60 min (for details see Borecký *et al.*, 1989). Recombinant human IFN alpha-2 (rIFNα-2, 5×10^6 units per ml) was a gift of Dr. G. Bodo, Boehringer, Austria and human fibroblast IFN beta (BM 532, L-0631 FiIFNβ, 10^7 units per mg) was kindly provided by dr. S. Kobayashi (Toray Ind., Japan).

Antibodies. Sheep antisera against human conventional leukocyte IFN alpha (G 026-502-568) and against human fibroblast IFN beta (G 028-501-568) were obtained from National Institutes of Health, Bethesda, Md, U.S.A. Antiserum against pH 2 non-treated human leukocyte IFN alpha-2 were prepared in this Institute by immunization of BALB/c mice. Monoclonal antibody T18, IgM-class, neutralizing the acido labile fraction of pH 2 non-treated human leukocyte IFN alpha (Kontsek *et al.*, 1989*a*) was purified from mouse ascites by precipitation with ammonium sulphate. Monoclonal antibody B6, IgG2b-subclass, prepared after immunization of BALB/c mice with human fibroblast IFN beta (Toray, Japan), was purified from ascitic fluid by Protein A-Sepharose affinity chromatography (Kontsek *et al.*, 1989*b*).

Synthetic hexapeptide SH 132—137. The hexapeptide corresponding to amino acid sequence of human IFN alpha-2 at position 132-137(lys-glu-lys-lys-tyr-ser) was synthesized by the solid phase technique and purified on HPLC to 96% purity (Borecký *et al.*, 1985).

Antiviral IFN assay. The antiviral activity (AV) of human IFNs was determined by cytopathic effect reduction assay on human diploid fibroblasts challenged with vesicular stomatitis virus in microculture plates. Titres of leukocyte IFN alpha (pH 2-treated, and/or pH 2 non-treated) and recombinant IFN alpha-2 were determined by comparison with NIH standard of human IFN alpha (Ga 23-902-530) and titres of IFN beta by comparison with NIH standard of human IFN beta (G 023-902-527).

Neutralization of antiproliferative activities of IFN. The ability of antibodies to inhibit the

antiproliferative (AP) activity of IFN was assayed on human promyelocyte cell line HL 60 in 96-well-plates. RPMI-1640 with 10% horse serum was used for dilution of IFN, antibodies and cultivation of HL 60. In AP assay, 0.05 ml of IFN at concentration of 100 units/ml was mixed with an equal volume of diluted antibody and then 2×10^4 HL 60 cells in 0.1 ml were added to wells immediately. The concentration of cells was estimated microscopically after incubation for 72 hr at 37 °C in 10% CO_2-atmosphere. Each IFN-antibody combination was plated into 4 parallel wells and the mean cell number was calculated. A 100% AP-activity of the tested IFN-preparation corresponded to the difference between concentration of control cells and IFN-treated cells. The neutralization titre was the minimal antibody concentration which inhibited the AP-activity of the assayed IFN preparation to 50% of its initial activity.

Competition assay. The synthetic hexapeptide SH 132-137 was added to diluted antibody to a final 10 μg/ml concentration of hexapeptide. This mixture was preincubated for 30 min at 37 °C. Then 0.05 ml from each sample was incubated with IFN and HL 60 cells, as described in IFN neutralization assay.

Results

Using the IFN-neutralization assay based on inhibition of the antiproliferative (AP) activity of IFNs, a unique antigenic structure (designated as Common epitope 1) was detected which was localized into region 132-137 of the amino acid sequence of human IFN alpha-2. The corresponding structure of human fibroblast IFN beta was formed by amino acid residues at position 134-139 (Pestka, 1986; Kontsek *et al.*, 1990).

The synthetic hexapeptide (SH 132-137) corresponding to the amino acid sequence of human IFN alpha 2 at position 132-137, completely inhibited the neutralizing ability of MoAb B6 towards recombinant IFN alpha-2 and fibroblast IFN beta (Table 1). Neither mouse antiserum to IFN alpha-2, nor sheep antiserum to IFN beta were capable of distinguishing IFN alpha-2

Table 1. Effect of synthetic hexapeptide SH 132-137 on the activity of IFN-neutralizing antibodies against various IFN preparations

Antibodies	Hexapeptide SH 132-137 (μg/ml)	Neutralization titre against (100 units) of IFN[a]) Leu IFNα	IFNα-2	IFNβ
Monoclonal B6 to IFNβ	0	> 10 μg/ml	0.01 μg/ml	0.01 μg/ml
	10	> 10 μg/ml	> 10 μg/ml	> 10 μg/ml
Polyclonal to IFNα-2	0	< 25	50 000	50 000
	10	< 25	50 000	< 25
Polyclonal to IFNβ	0	< 10	1 000	1 000
	10	< 10	< 10	1 000
Polyclonal to Leu IFNα	0	10 000	10 000	< 10
	10	10 000	10 000	< 10

[a]) The minimal concentration of antibody which inhibited 50% of antiproliferative (AP) activity of IFN-preparation in concentration of 100 units/ml. The concentration of polyclonal antibodies is shown as reciprocal value of dilution while concentration of monoclonal antibody is given in μg/ml.

Table 2. Effect of pH 2-treatment on AP-activity and antigenic properties of recombinant IFN alpha-2

Antibodies	rIFNα-2 (100 units per ml) pH 2-treated Cell number[a]) × 10^4 ml $\bar{x} \pm s$ (% of contr.)	AP activity[b])	rIFNα-2 (100 units per ml) non-treated Cell number[a]) × 10^4 ml $\bar{x} \pm s$ (% of contr.)	AP activity
None	66.4 ± 4.1 (82%)	+	62.5 ± 5.7 (76%)	+
Monoclonal to FiIFNβ B6 (10 μg/ml)	66.2 ± 4.3 (81%)	+	80.4 ± 4.4 (99%)	−
Polyclonal to rIFNα−2 (dilution 10^{-2})	83.7 ± 5.1 (103%)	−	80.0 ± 5 9 (98%)	−
Polyclonal to FiIFNβ (dilution 10^{-2})	67.1 ± 4.0 (82%)	+	81.7 ± 6.3 (100%)	−
Polyclonal to LeuIFNα (dilution 10^{-2})	82.8 ± 5.1 (102%)	−	83.4 ± 4.8 (102%)	−

[a]) Mean number of cells from 4 wells plated
[b]) + indicates preserved, − lost AP activity

from IFN beta and both showed the same neutralization and cross-neutralization titres against IFN-types tested (Table 1). As indicated by data from competition tests with hexapeptide SH 132-137, a population of serum antibodies directed to Common epitope 1 was responsible for cross-neutralization ability of both antisera. Following interaction with SH 132-137, the antiserum to IFN alpha-2 as well as antiserum to IFN beta completely lost their cross-reactivity. However, their specific neutralizing activity towards immunogen (i.e. IFN alpha-2 or IFN beta, respectively) remained unchanged (Table 1). Polyclonal antiserum to human pH 2-treated leukocyte IFN alpha was capable to neutralize IFN alpha-2, but did not neutralize IFN beta (Table 1) in accordance with the current concept of distinct antigenicity of human IFNs of alpha and beta types. With regard to the proposed existence of a common antigenic structure shared by IFN alpha-2 and IFN beta, the results of tests with polyclonal antisera need explanation. Because IFN alpha-2 is the major subtype present in human conventional leukocyte IFN alpha preparations, we have expected the antiserum to leukocyte IFN alpha to contain also populations of antibodies directed towards the Common epitope 1. If so, this antiserum should be able to neutralize also biological activity of human IFN beta. However, the observed lack of neutralization indicated absence or changes of the antigenic structure of natural IFN alpha-2, at least in the region 132-137.

Since the commonly used method for inactivation of inducer virus in preparations of human leukocyte IFN is incubation at low pH, we decided to study the possible effect of acidification on biological characteristics of recombinant IFN alpha-2. The preparation of rIFN alpha-2 (in concen-

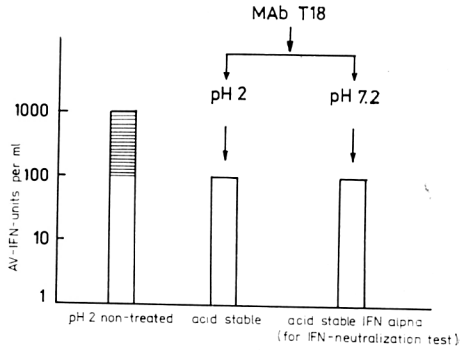

Fig. 1
Selective removal of acid labile fraction of pH 2 non-treated leukocyte IFN alpha by MoAb T18
MoAbT18 is directed against the acid labile Leu IFN alpha fraction (Borecký *et al.*, 1989). The IFN sample was exposed to MoAb T18 for 30 min at 37 °C, then divided into 2 aliquots: one was kept at pH 2, while the other at pH 7.2 for 48 hr at 4 °C. The residual AV activity and/or neutralization with anti-IFN alpha and anti-IFN beta sera were examined subsequently.
Acid labile IFN alpha (hatched column)
Acid stable IFN alpha (empty column)
1 — pH 2 non-treated IFN alpha
2 — acid stable IFN alpha
3 — acid stable IFN alpha (for IFN-neutralization test).

tration 10^5 units per ml) was acidified to pH 2 with 6 N HCl and, after 48 hr at 4 °C, the pH was reverted to pH 7.2 using 5 N NaOH. As shown in Table 2, the AP activity of rIFN alpha-2 was only slightly decreased after pH 2 treatment, however, its antigenic properties showed significant alterations. The pH 2-treated rIFN alpha-2 has lost its binding capacity to MoAb B6 and anti-IFN beta serum (Table 2). Because these antibodies were shown to neutralize IFN alpha-2 only through the Common epitope 1, the acid pH seems to be responsible for the rearrangement in conformation of rIFN alpha-2, which resulted in a diminished antigenicity of the region 132-137. In contrast, antisera to IFN alpha-2 and to leukocyte IFN alpha could not differentiate between pH 2 treated and non-treated forms of rIFN alpha-2, since they neutralized both IFN forms with the same efficiency (Table 2). These data demonstrated the existence of both acid stable and acid labile antigenic structures on the molecule of rIFN alpha-2. In the same time, the results of these experiments indicated the acid labile nature of Common epitope 1 on IFN alpha-2 molecule and suggested that the presumed distinct antigenicity of leukocyte alpha and fibroblast beta IFNs might be only the secondary consequence of pH 2 induced conformational changes in preparations of leukocyte IFN alpha.

To support this hypothesis, we prepared human leukocyte IFN alpha, but instead of conventional pH 2 treatment the inducer virus was removed from preparation by ultracentrifugation. Such IFN preparation was used in experiments which were designed a) to confirm the existence of the Common epitope 1 in pH 2 non-treated IFN alpha, and b) to detect the antibodies towards Common epitope 1 in immune serum raised against pH 2 non-treated leukocyte IFN alpha. The following results were obtained:

a) The preparations of pH 2 non-treated leukocyte IFN alpha are known to consist of two, i.e. acid stable and acid labile subpopulations (Chadha, 1985; Borecký *et al.*, 1986). We have found these two IFN alpha types to

Table 3. Neutralization of acid stable fraction carrying the Common epitope 1 in pH 2 non-treated leukocyte IFN alpha by anti-IFN alpha and beta sera, respectively

Antibody	Hexapeptide SH 132-137 (μg/ml)	Acid stable fraction with Common ep. 1 (units/ml)	Cell number[a]) × 10^4/ml x ± s (% of control)	AP activity[b])
None	0	0	70.6 ± 4.3 (100%)	—
None	0	100	50.8 ± 3.7 (72%)	+
Polyclonal to Leu IFNα (dilution 10^{-2})	0	100	70.8 ± 4.4 (100%)	—
	10	100	67.9 ± 5.6 (96%)	—
Polyclonal to rIFNα-2 (dilution 10^{-2})	0	100	70.5 ± 4.1 (100%)	—
	10	100	70.7 ± 3.9 (100%)	—
Polyclonal to FiIFNβ (dilution 10^{-2})	0	100	69.3 ± 5.1 (98%)	—
	10	100	48.9 ± 3.7 (69%)	+
Monoclonal to FiIFNβ B6 (10 μg/ml)	0	100	70.3 ± 3.9 (99%)	—
	10	100	47.6 ± 4.0 (67%)	+

[a]) Mean number of cells from 4 wells plated
[b]) + indicates preserved, — lost AP activity

be antigenically distinct, and, using MoAb T18 which selectively neutralized the acid labile IFN alpha, the acid stable fraction of pH 2 non-treated leukocyte IFN alpha could be separated. This procedure is summarized in Fig. 1. It shows a preparation containing 90%, i.e. 900 AV units per ml of acid labile IFN alpha and 10%, i.e. 100 AV units per ml of acid stable IFN alpha type. After preincubation with an excess of MoAb T18 (10 μg per ml), for 30 min at 37 °C a final IFN preparation with antiviral activity of 100 units per ml was obtained. This preparation was divided into two parts. One part was pH 2-treated to demonstrate the acid stability of biological (AV and AP) activity (Fig. 1), while the second (pH 2 non-treated) one was used in AP IFN-neutralization assay (Table 3). In accordance with our hypothesis, MoAb B6 and anti-IFN beta serum recognized the Common epitope 1 and neutralized completely the AP activity of investigated pH 2 non-treated IFN-preparation. The specific interaction of both antibodies used with Common epitope 1 found support in competition experiments using hexapeptide SH 132-137 which completely inhibited the neutralizing activity of MoAb B6 and anti-IFN beta serum. The AP-activity of the acid stable fraction of leukocyte IFN alpha was neutralized also by antisera to IFN alpha-2 and conventional leukocyte IFN alpha, but the hexapeptide SH 132-137 did not significantly affect their neutralizing activity, as expected from the polyclonal nature of these antisera (Table 3).

b) The next experiments were performed using mouse polyclonal antiserum against pH 2 non-treated leukocyte IFN alpha (Table 4). The results of experiments with this antiserum lend also support to the presence of the

Table 4. Neutralization activity of antisera against pH 2 non-treated leukocyte IFN alpha

Antibody	Hexapeptide SH 132-137 (μg/ml)	rIFNα-2 (100 units per ml) Cell number[a]) × 10^4 ml $\bar{x} \pm s$ (% of control)	AP activity[b])	FiIFNβ (100 units per ml) Cell number[a]) × 10^4 ml $\bar{x} \pm s$ (% of control)	AP activity[b])
None	0	68.3 ± 4.4 (79%)	+	73.6 ± 5.1 (86%)	+
Polyclonal to pH 2-NT Leu IFNα	0	85.7 ± 5.0 (100%)	−	86.1 ± 6.3 (100%)	−
	10	86.7 ± 4.7 (101%)		72.8 ± 4.9 (85%)	+

[a]) Mean number of cells from 4 wells
[b]) + indicates preserved, − lost AP activity

Common epitope 1 in the immunogen because it reacted with the unique acid labile structure present in pH 2 non-treated IFN alpha. In addition to neutralization of rIFN alpha-2 and leukocyte IFN alpha, the antiserum cross-neutralized also the fibroblast IFN beta. In competition assay with hexapeptide SH 132-137, only the cross-reactivity of antiserum with IFN beta was inhibited. This fact suggested that only one population of serum antibodies recognizing the Common epitope 1 was responsible for cross-neutralization of IFN beta.

Discussion

In a previous paper we reported on the until now unobserved antigenic relationship between human recombinant IFN alpha-2 and human fibroblast IFN beta (Kontsek *et al.*, 1990). A unique antigenic structure, designated as Common epitope 1, was found to be responsible for the detected antigenic similarity. The Common epitope 1 on IFN alpha-2 molecule was formed by amino acid residues at positions 132-137, while the corresponding structure on IFN beta was localized in the region 134-139. This followed from competition tests employing the synthetic hexapeptide SH 132-137. Only antibodies directed against the suspected Common epitope 1 were able to neutralize both the recombinant IFN alpha-2 and the fibroblast IFN beta with the same efficiency (Table 1). Based on this, we speculated that human IFNs of alpha and beta types could be antigenically more closely related than admitted earlier. We proposed that the divergence of class I IFNs from the common ancestor went through IFN beta to IFN alpha family, and, IFN alpha-2 could represent the antigenic "missing link" between the two IFN types. However, this hypothesis was handicaped by the lack of evidence for the presence of the Common epitope 1 in natural (conventional) leukocyte IFN alpha (Table 1). Since preparations of human conventional leukocyte IFN alpha are mixtures of several IFN alpha subtypes, with alpha-2 and alpha-1 IFNs as major subtypes (Rubinstein *et al.*, 1981; Levy *et al.*, 1981; Pestka, 1983), we expected at least a partial neutralization of the AP-activity of natural IFN alpha by antibodies such as MoAb B6, anti-IFN alpha-2 serum and anti-IFN beta serum. However, the neutralizing effect of these antibodies could not be detected (Table 1).

In the same time, these experimental data could not exclude the possibility that conformational changes might have occurred in population of IFN alpha-2 present in natural leukocyte IFN alpha preparation during preparation or storage leading to altered antigenicity. We assumed that pH 2 treatment, a step commonly used for inactivation of inducer virus, could be crucial for conformational changes of natural IFN alpha-2. The acid pH did not alter the biological activity (such as AP or AV) of recombinant IFN alpha-2. However, in contrast to AP and AV activity, the altered antigenic properties of rIFN alpha-2 after pH 2-treatment demonstrated the possibility of acid labile nature of the Common epitope 1 (Table 2). In the next test, using pH 2 non-treated leukocyte IFN alpha, we succeeded to detect the presence of a functioning Common epitope 1.

We choose two different attitudes for its screening. The first consisted in detection of antibodies to the Common epitope 1 in an antiserum directed against pH 2 non-treated leukocyte IFN alpha. As indicated by the neutralization of both IFN alpha-2 and IFN beta, with this antiserum we succeeded in demonstration of a population of antibodies directed against the Common epitope 1 (Table 4). In the second experiment, we tried to show that the AP-activity of pH 2 non-treated IFN alpha was neutralized with antibodies, such as MoAb B6, antiserum to IFN alpha-2 and/or antiserum to IFN beta that reacted with the Common epitope 1. The design of such an experiment proved more complicated because of presence of two antigenically different alpha species (acid stable and acid labile) in preparations of natural pH 2 non-treated leukocyte IFN alpha (Borecký *et al.*, 1989). But after treatment with MoAb T18, which neutralizes only the acid labile IFN species, only the acid stable alpha-fraction which represents the pH 2-treated conventional leukocyte IFN alpha, remained active (Fig. 1). This persisting acid stable IFN activity proved then neutralizable not only by antiserum to conventional leukocyte IFN alpha and/or antiserum to IFN alpha-2, but, most important, also by MoAb B6 and polyclonal antiserum to fibroblast IFN beta (Table 3). The results of these experiments support the proposed existence of the Common epitope 1 in preparations of pH 2 non-treated IFN alpha. Moreover, competition tests with synthetic hexapeptide SH 132-137 demonstrated a complete inhibition of pH 2 non-treated acid stable fraction of IFN alpha also by antiserum to IFN beta (Table 3). Taking into consideration the limited sensitivity of the IFN-neutralization assay used (about 10 AV units) as well as the fact that antisera to IFN alpha or IFN beta and MoAb B6 completely neutralized the acid stable species of IFN alpha in concentration of 100 units per ml when the acid treatment was omitted, we propose that natural IFN alpha-2 was responsible for at least 90% of AP-activity of this preparation.

We conclude that the presented experimental data support our hypothesis that an antigenic relationship exists between natural human IFNs of alpha and beta types. In support of this hypothesis we succeeded in detection of a unique antigenic structure called Common epitope 1 which most probably represents the missed antigenic link. This antigenic relationship between both IFN types was not detected until now because of the acid labile nature of the Common epitope 1 which escaped detection when conventional (i.e. pH 2-treated leukocyte IFN alpha preparations) were examined. These results support the proposition of Rubinstein (1982) that the nomenclature defining alpha and beta IFNs as "class I" and gamma IFN as "class II" interferons, seems to be more preferable than the current official nomenclature.

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