Radiol Oncol 1993; 27: 302-6.

Natural porcine interferon gamma (PoIFN gamma)

Bratko Filipič,[1] Sonja Rozman,[2] Katarina Carlsson[3] and Avrelija Cencič[4]

[1] Institute for Microbiology, Medical Faculty, Ljubljana, [2] BIA Ltd., Ljubljana, Slovenia, [3] Centre of Chemistry, University of Lund, Sweden, [4] College of Agriculture, University of Maribor, Slovenia

The natural porcine mitogen induced gamma interferon (PoIFN gamma) was studied and compared with the human interferons gamma (HuIFN gamma) and alpha (HuIFN alpha). A comparison was performed by the following criteria : pH 2.0 and 56°C stability, molecular weight, dot-blot reactions and cross reactivity (neutralisation index). The biological activity of porcine and human interferons in vitro (antiviral and antiproliferative) was correlated on human nontransformed (HEF) and transformed cells (FL).

Key words: interferon type II-analysis; cells; cultured-drug effects

Introduction

Interferons (IFNs) are defined as proteins/glycoproteins having an ability to protect the infected cells (causing the antiviral state) or to reduce and/or inhibit the growth of transformed cells *in vitro* and tumors *in vivo*.[1, 2] They are divided into at least two classes (Type I - alpha or beta and Type II - gamma) according to their mode of induction (Type I - with viruses or pI:C, Type II - with T cell mitogens or heterologous cells).[3, 4]

Because of clinical use, the main attention was focused on human interferon alpha (HuIFN alpha)[5] and much later on the human interferon gamma (HuIFN gamma).[6] For the IFN gamma production it was thought that a pure population of T lymphocytes is necessary. Latter on, it was found that partially purified spleenocytes can be used as well. The highest titers (IFN units/ml) were obtained when a mixture of peripheral blood lymphocytes and spleenocytes was used for the IFN gamma production, with specific T cell mitogens as inducers.

A comparison between HuIFN alpha and HuIFN gamma shows that they are antigenically completely different: with anti-HuIFN alpha antisera it is impossible to neutralise HuIFN gamma and vice versa.

At the beginning of the clinical use of human IFNs in natural form, the main problem was how to produce enough IFN Concomitantly, the optional solutions arose: to use human-like IFNs. In this respect, many studies were carried out to find the antigenic similarity between human and human-like interferons. Surprisingly, the highest degree of homology was found between murine and human IFNs.[7]

Correspondence to: Dr. Bratko Filipič, Institute for Microbiology, Medical Faculty, 61105 Ljubljana, Slovenia

UDC: 615.281.7.015.44

Similar interferon system as in humans can be found in other animal species, such as rat, horse, cattle, pig, monkey, etc. Porcine and bovine IFNs[8–11] became of interest because of their relatively high antigenic similarity with human one. Such a similarity between Hu and PoIFN is about 78.5 % (at nucleotide level)[12] in the case of IFN alpha. When a recombinant gamma interferons (gamma IFNs) were compared (rPoIFN gamma/rHuIFN gamma) a homology was estimated in 59 % (at nucleotide level).[13] Correlation of non-recombinant forms of porcine alpha and human alpha IFNs shows even higher levels of homology (86.7 % at nucleotide level) between them.[14–15] It seems to be unusual that PoIFN gamma is cross reactive with HuIFN alpha and not with PoIFN gamma. Experiments presented herein are aimed to compare the natural porcine interferon gamma (PoIFN gamma) with the human interferon alpha (HuIFN alpha) by the following criteria: pH 2.0 and 56 °C stability, molecular weight, dot-blot analysis and neutralisation on cell culture *in vitro*.

Material and methods

Blood collection, buffy-coat preparation and interferon induction

Porcine blood was collected aseptically into sterile flasks containing 3.8 % sodium citrate to prevent coagulation. The porcine buffy-coat was separated by introducing 30 % sacha rose.[16-18] The sedimented buffy-coat was washed twice with saline and resuspended in modified Eagle's medium[19] containing 4 % of porcine plasma and antibiotics (Penicillin 5000 U/ml, Streptomycin 5 mg/ml, Gentamycin 10 mg/ml, Neomycin 10 mg/ml). The induction was performed by LCL (Lens culinaris lectin) at a concentration of 25 μg/ml. Cell concentration was adjusted to 10^7-10^8 cells/ml. After three days of cultivation on a spinner (37°C/140 RPM) the cells were sedimented by centrifugation (2000 RPM/20 minutes) and the supernatant representing the crude IFN was harvested.

Partial purification of PoIFN gamma

Interferon was isolated as follows: to 100 ml of IFN containing supernatant, 3 g of avtoclaved SiO_2 (water glass) were added and incubated overnight at + 4°C. On the next day the suspension was centrifuged at 2500 RPM for 30 minutes to sediment the water glass. The sedimented water glass was resuspended in the 1/8 of the original mixture volume of 50 % ethylene glycol monoethyl ether in 1.4 mM NaCl to elute the interferon. After 2 hours the water glass was sedimented by centrifugation (2500 RPM for 30 minutes). The elution procedure was repeated in 1/16 of the original volume. Both eluates were mixed with 0.1 % of FCS (Fetal calf serum, Flow) and dialysed against distilled water.

Measurement of antiviral and antiproliferative activity

IFNs were tested for antiviral (AV) activity by 50 % cytopathogenic inhibition assay against HSV1 (Herpes simplex virus type 1) as a challenge virus.[20] The antiproliferative activity (AP) was determined by 50 % growth inhibition assay on HEF (human embryonal fibroblasts) and FL (human amniotic cell line) cells.[21] Natural interferons alpha (Institute of Immunology, Zagreb, Croatia; EGIS Budapest, Hungary) at a concentration of 1000 AV (antiviral units/ml) were used as the control standards.

Stability tests

To determine the type of IFNs the samples were exposed to pH 2.0 and heating at 56°C for 30 minutes. Antiviral (AV) and antiproliferative (AP) units were determined before and after such treatment.

Protein content

In each sample, the quantity of proteins was determined by the modified Lowry method.[21]

Serological analyses

To determine the serological similarity and/or differences between porcine and human IFNs (gamma and alpha) the "constant method" according to LaBonnardiere et al.[14] was used. In summary, constant (twofold) dilutions of anti-IFN antisera were added to the FL cells in microtiter plates (NUNC). In the next step, three-fold dilutions of IFNs with the virus (HSV 1) were added. Simultaneously, IFN titration was performed without antisera. The neutralisation index (NI) was calculated as follows: NI = $\log_3$ (antiserum + interferon) - $\log_3$ (interferon).

Antigenic properties

Immunoblot tests were performed according to Pretnar et al.[22] In summary, the antigens HuIFN alpha (Institute for Immunology, Zagreb, Croatia; EGIS, Budapest, Hungary), HuIFN gamma (Sigma, St. Luis, USA), PoIFN gamma (Institute for Microbiology, Medical Faculty, 61000 Ljubljana, Slovenia) in the volume of 10 µl (the concentration of proteins in the samples was 0.9 mg/ml) were spotted on nylon films (Hybond, Amersham) and air dried. The nylon films with the bound antigens were incubated for 60 minutes with the primary antibodies (polyclonal) as follows: Anti-HuAlpha (Boehring, Manheim, FRG; neutralisation titer 10.000 U/mg), Anti-HuGamma (Boehringer, Manheim, FRG; neutralisation titer 10.000 U/mg) and Anti-PoGamma (INRA, Youi-en-Josas, France; neutralisation titer 10.000 U/mg), Anti-HuAlpha1 (Boehring, Manheim, FRG; neutralisation titer 10.000 U/mg), Anti-HuAlpha 2 (Institute of Virology, Bratislava, Slovakia; neutralisation titer 4000 U/mg) and Anti-Acidolabile (Institute of Virology, Bratislava, Slovakia; neutralisation titer 10.000 U/mg). After washing with TTBS (10 mM Tris + HCl, pH 8.0, 150 mM NaCl + 0.05 % Tween 80), the films were incubated in anti-rabbit IgG (Boehring, Manheim, FRG) conjugated with peroxidase. Following three washes in TTBS, the reactions were developed using DAB (diaminobenzidine, Sigma, St.Luis, USA) in the concentration of 50 mg/100 ml TBS and 30 fl of hydrogen peroxide.

Results

Stability tests

Table 1 shows the differences between porcine (PoIFNs) and human interferons (HuIFNs, alpha, gamma) as follows: PoIFN gamma is pH 2.0 stable in contrast to HuIFN gamma which is not. On the other side, PoIFN alpha is pH 2.0 labile, i.e. different from HuIFN alpha which is not. Similar data were found when the resistance to heating to 56°C were compared. It seems that PoIFN gamma is more similar to HuIFN alpha than to its human counterpart.

Table 1. Stability tests for PoIFNs and HuIFNs.

IFN type	pH 2.0	56°C	M.W.[1] (Daltons)
PoIFN gamma	Stable	Stable	21000
PoIFN alpha	Labile	Labile	20000
HuIFN gamma	Labile	Labile	19000
HuIFN alpha	Stable	Stable	19500

[1] Molecular weight was determined by PAG-SDS electrophoresis

Dot-blot analysis

Using dot-blot tests, the antigenic properties are described (Table 2) in terms of positive or negative reactions. PoIFN gamma shows a positive reaction with antisera against PoIFN gamma, HuIFN alpha and HuIFN alpha 2. HuIFN gamma reacts only with the antiserum against HuIFN gamma, and not with PoIFN gamma.

Table 2. Dot-blot analysis of Po and Hu IFNs.

IFN type	Reaction with Anti[1]				
	Po gamma	Hu gamma	Hu alpha	Hu alpha 1	Hu alpha 2
PoIFN gamma	+	–	+	–	+
PoIFN alpha	–	–	+	–	+
HuIFN gamma	–	+	–	–	+
HuIFN alpha	+	–	+	+	+

[1] Dot-blot reaction: "+" = gives positive reaction
"–" = gives negative reaction

In the case of alpha IFNs, PoIFN reacts with the antiserum against HuIFN alpha and HuIFN alpha 2. HuIFN alpha gave a positive reaction with the antisera against PoIFN gamma, HuIFN alpha, HuIFN alpha 1 and HuIFN alpha 2.

Serological tests

By serological tests in vitro (on FL cells) (Table 3) the level of the neutralisation of IFN's antiviral activity with anti-IFN antisera was quantified by the Neutralisation index (NI). When PoIFN gamma was tested, the following NIs

Table 3. Neutralisation indexes for Po and Hu IFNs.

IFN type	Neutralisation with Anti[1]				
	Po gamma	Hu gamma	Hu alpha	Hu alpha 1	Hu alpha 2
PoIFN gamma	–1.15	0	–1.94	0	–0.29
PoIFN alpha	0	0	–2.43	0	–0.53
HuIFN gamma	0	–0.98	0	0	0
HuIFN alpha	–0.92	0	–2.85	–2.00	–1.60

[1] NI = $\log_3$ (antiserum + IFN) – $\log_3$ (IFN)

were found: With anti-PoIFN gamma – 1.15, with anti-HuIFN alpha – 1.94 and with anti-HuIFN alpha 2, –0.29. PoIFN alpha shows much higher NI for anti-HuIFN alpha (NI = -2.43) as well as for anti-Hu alpha 2 (–0.53). HuIFN alpha shows the following values for NIs: Anti-PoIFN gamma + 0.92, anti-HuIFN alpha –2.85, anti-HuIFN alpha 1, –2.00, and anti-HuIFN alpha2, –1.60

Discussion

PoIFN alpha and HuIFN alpha were found to be antigenically similar, though the differences between them were found when some of the physico-chemical characteristics were tested (temperature stability, acid-resistance).[9-11] Contrarily, natural PoIFN gamma is different from its natural counterpart in humans. Its pH and temperature stability are more similar to that of HuIFN alpha. PoIFN gamma also shows cross reactivity in vitro with HuIFN alpha (complete) and its natural subtype HuIFN alpha 2 When these data were correlated with those obtained by comparison of recombinant IFNs (human, porcine) in the case of gamma interferons, homology was established in 59% (at nucleotide level). It seems possible that in the case of recombinant forms only selected molecules, in contrast to the natural ones (partly purified or purified) when complete molecules composed from different numbers of natural subtypes, were compared.

In this respect it should be mentioned that the comparison by NI (Neutralisation index) in vitro gives a picture of biological activity.

Future experiments with pure porcine interferons (alpha and gamma) are expected to disclose the real level of similarity/differences with human interferons (alpha, gamma), and thus enable clinical use of porcine IFNs in veterinary and human medicine.

Acknowledgment

This work was supported by the grant from the Slovenian Ministry for Research and Technology (URP Molecular Biology, C1-509/381-93; Research field: Biochemistry with Molecular Biology, PI-5064-0381-93).

References

1. Gresser I, Tovey MG. Antitumour effects of interferon. *Biochem Biophys Acta* 1978; **516:** 231-47.
2. Hubbell RH, Graft AJ, Leibowitz JP, Gillespie DH. Synergistic antiproliferative effect of recombinant gamma-interferons. *J Biol Resp Mod* 1987; **6:** 141–53.
3. Bonnem EM, Spiegel RJ. Interferon-alpha: Current status and future promise. *J Biol Resp Mod* 1984; **3:** 580–94.
4. Toth M, Endresz V, Toth S, Beladi I. Human interferon alpha and beta have more potent priming activities than interferon-gamma. *J Gen Virol* 1985; **66:** 893-6.
5. Borden EC. Interferons: In pursuit of the promise. In: Mirand EA,Hutchinson WB,Mihrich E. Eds. 13th International Cancer Congress, Part E., Cancer Management. New York: Alan R. Liss 1983: 287–296.
6. Osborne LC, Georgiades JA, Johnson HM. Large scale production and partial purification of mouse immune interferon. *Infect Immunol* 1979; **23:** 76–80.

7. Soloviev VD, Pokidysheva LN, Volodnina TN. On the antigenic similarity of some alpha interferons. *Vopr Virusol* 1982; **27:** 526-8.

8. Richmond JY. An Interferon-like inhibitor of foot and-mouth disease virus induced by phytohaemmaglutinine in swine leukocyte cultures. *Arch Ges Virus Forsçh* 1969; **27:** 282–9.

9. Soloviev VD, Ogarkov VI, Marchenko VI, Monastyreva LN, Parfenov VV, Bukharova II, Preobrazhenskeja NK, Zubanova NA Swine blood leukocytes, a new source of production of interferon active in human cells. *Vopr Virusol* 1980; **25:** 716–20.

10. Soloviev VD, Pokydisheva LN, Volodnina TN.On antigenic similarity of some alpha-interferons. *Vopr Virusol* 1982; **27:** 526–8.

11. Piasecki E. Properties of natural porcine interferons. *J IFN Res* 1988; **8:** 61–73.

12. Lefevre F, LaBonnardiere C. Molecular cloning and sequencing of a gene encoding biologically active porcine-interferon. *J IFN Res* 1986; **6:** 349–60.

13. Charley B, McCullough K, Martinod C. Antiviral and antigenic properties of recombinant porcine interferon gamma. *Vet Immunol Immunolopathol* 1987; **7:** 357–68.

14. LaBonnardiere C, Laude H, Berg K. Biological and antigenic relathionship between virus-induced porcine and human interferons. *Ann Inst Pasteur/ Virol* 1986; **137 E:** 171–80.

15. Lavrukhina LA, Gutman NR, Manakhanova LS. Antiviral effect of swine leukocyte interferon in experimental mice. *Vopr Virusol* 1981; **20:** 414– 8.

16. Filipič B, Golob A, Toth S, Mecs I, Beladi I, Likar M Interactions between human and porcine interferons. *Acta Virol* 1991; **35:** 19–26.

17. Filipič B, Golob A, Golec-Wondra M, Vitez Lj, Kešе D, Likar M. Preparing and partially purification of swine immune interferon. In: Filipič B. ed. Yugoslav Colloquium on IFN. Ljubljana: Slovenian Microbiological Society 1986: 137–9.

18. Filipič B, Golob A, Sinadinovska R, Babič D, Struna A, Pretnar G. Preparing and purification of porcine immune interferon. In: Likar M. ed. Posvetovanje ob 45. obletnici Instituta za Mikrobiologijo, Medicinske Fakultete v Ljubljani. Ljubljana: Institute for Microbiology, Medical Faculty: 1990: 71–80.

19. Filipič B, Carlsson K, Zupan S, Knežević M, Raspor P. Preparation and partial purification of porcine interferon gamma (PoIFN gamma). *Acta Pharm* 1993; **43:** 71-4.

20. Forti LR, Schuffman SS, Davies AH, Mitchell MW. Objective antiviral assay of the interferons by computer assisted data collection and analysis. In: Pestka S. ed. *Method in Enzimology,* vol. 119, part C. London, New York: Academic Press 1986: 533-40.

21. Filipič B, Carlsson K, Hartman-Pretnar K, Podgornik A, Koselj P. A novel protein adetermination micromethod. *Acta Pharm* 1992; **42:** 355-60.

22. Pretnar G, Filipič B, Golob A, Škodič A, Toth S, Mecs I, Suhar A. Electroinduction of Interferon-like proteins. *Bioelectrochem Bioenerg* 1991; **25:** 183–92.