PORCINE MITOGEN-INDUCED INTERFERON

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Summary. – Induction of porcine interferon (PoIFN) with different lectins (phytohaemagglutinin, *Lens culinaris* lectin, *Arachis hypogea* lectin) in various concentrations (500, 50 and 5 μg/ml) was studied. Kinetics of PoIFN appearance was analyzed by antiviral and antiproliferative tests in human transformed and nontransformed cells. The relation between antiviral and antiproliferative activity of PoIFN in terms of respective units was determined. PoIFN was characterized in comparison with human interferon alpha by the acidostability and thermostability tests.

Key words : porcine interferon; human interferon alpha, gamma; induction; phytohaemagglutinin; *Lens culinaris* lectin; *Arachis hypogea* lectin

Introduction

As a broad family of naturally occurring "biological response modifiers", interferons (IFNs) were defined as antiviral and later also antiproliferative (antitumour) substances (Gresser, 1978; Hubbel *et al.*, 1987). At least two types of IFNs were categorized : type I (virus-induced and/or polyI:C-induced), alpha or beta, and type II (T-cell mitogen-induced), gamma. Mainly human IFNs (HuIFNs) type I and II were produced and analyzed because of their clinical usefulness in humans (Strander, 1982) and to a much lower extent in other species with exception of mouse (Fleischmann *et al.*, 1979). In principle, similar IFN systems as in humans can be found in other animal species such as mouse, rat, horse, calf, pig, monkey etc. Among others namely porcine and bovine IFNs roused interest (Richmond, 1969; Soloviev *et al.*, 1980; Piasecki, 1988) mostly from two reasons : firstly, because the respective animals represented practically unlimited source of blood and spleens, and secondly, beacuse of a relatively high antigenic similarity between porcine and human IFNs (Soloviev *et al.*,1982; La Bonnardiere, 1986). Recently, from both species recombinant forms became available, but with low clinical usefulness (Lefevre *et al.*, 1986; Charley *et al.*, 1987). Based on experimental data, it became more and more evident that for good clinical effect in animals and humans natural IFNs are more suitable (Lavrukhina *et al.*,1981; Jereb *et al.*,1987).

The antiviral and antiproliferative (antitumour) activities of IFNs can be enhanced by combinations of their alpha and gamma types. Experimental data on synergistic effects were obtained in various systems: mouse (Fleischmann *et al.*, 1979), porcine (Piasecki, 1988) and human (Hubbel *et al.*, 1987). A synergism between HuIFN and PoIFN gamma as regards the antiproliferative activity *in vitro* was also observed (Filipič *et al.*, 1991).

The present work was aimed to compare the capacity of different lectins to induce IFN in porcine leukocytes in vitro and to characterize physico-chemically the induced IFNs. Finally, the relationship of the antiproliferative (AP) unit to the standard, antiviral (AV) unit was determined.

Materials and Methods

Cells. Nontransformed human embryonal fibroblasts (HEF) and transformed human amniotic cells (FL) were cultivated in Eagle's medium supplemented with 10 % foetal calf serum (FCS, Flow).

Lectins. The following lectins were used : phytohaemagglutinin (PHA; Krka, Novo Mesto, Slovenia), *Lens culinaris* lectin (LCL; Sigma), and *Arachis hypogea* lectin (AHL; Sigma). They were dissolved in saline in concentrations of 500, 50 and 5 μg/ml.

IFNs. HuIFN alpha (Institute of Immunology, Zagreb, Croatia, and EGIS, Budapest, Hungary), PoIFN alpha (Institute of Microbiology, Medical Faculty, Szeged, Hungary) and PoIFN gamma

prepared as described earlier (Filipič *et al.*,1986) had specific activity 10,000 – 100,000 AV units/mg protein.

Antisera containing polyclonal antibodies to HuIFN alpha, HuIFN gamma (Beringer, Mannheim), PoIFN gamma (donated by Dr. C. LaBonnardiere, France), HuIFN alpha2 and acidolabile IFN (both obtained from the Institute of Virology, Bratislava, Slovakia) were used.

Induction. To test the inducing capacity of different lectins, the fraction rich for leukocytes and lymphocytes was isolated from porcine blood as described earlier (Filipič *et al.*, 1986, 1990). Cells (10^7/ml) in Eagle's medium with 5 % of homologous plasma were seeded into 10 cm Petri dishes (Nunc) and lectins in concentrations 500, 50, 5 and 0 μ g/ml were added. One ml samples were collected each day during 5 days of incubation in CO_2 atmosphere at 37 °C. Cells were pelleted in Eppendorf tubes by centrifugation (15 mins, 1200 rpm) and supernatants were stored at –20 °C for further analysis. Cells were resuspended in 1 ml of fresh medium with 10 % FCS and seeded into Petri dishes, so that the total cell count remained constant throughout the experiment. Mock-induced controls were made in the same way. All experiments were performed in triplicate in 3 parallels.

Assay of AV activity. Each sample of IFN was tested by the 50 % CPE inhibition assay using herpes simplex virus type 1 (HSV-1) for challenge in both HEF and FL cells (Forti *et al.*, 1986). HuIFN alpha (1 000 AV units/ml) was used as standard. Also the possible direct antiviral effect of lectins (500, 50 and 5 μ g/ml) against HSV-1 virus was tested in FL cells.

Assay of AP activity was peformed in HEF and FL cells as described earlier (Filipič *et al.*,1991). Cells were cultivated in micrototer plates (Nunc) in Eagle's medium with 10 % FCS (Flow). After 1 day of cultivation the medium was changed for a fresh one with 5 % FCS only. Various AV doses of IFNs (1 000, 500, 100, 50, 10, 5 and 1) and concentrations of samples of lectin-induced PoIFNs were added. At this time the initial cell count was determined in untreated controls. Cells were incubated for additional 3 days in CO_2 atmosphere at 37 °C and counted. For cell counts the IFN-treated and control cells were fixed in 25 % glutaraldehyde for 20 mins, washed with PBS and stained with 4 % Methylene Blue for 45 mins at 37 °C. Finally, the plates were thoroughly washed in tape water, air dried and the dye was extracted by adding 100 μ l 0.1 N HCl per well. The absorbance at 570/650 nm was measured (A_{final}) and together with $A_{initial}$ (corresponding to the initial cell count) was used for calculation of the growth index (GI) as follows :

$$GI = A_{final} / A_{initial}$$

By assaying GI for IFN-treated cells (GI_{IFN}) as well as for control, untreated cells ($GI_{control}$), the inhibition of GI in % can be expressed as follows :

$$GI\ inhibition\ (\%) = GI_{IFN} \times 100 / GI_{control}$$

GI ratios were derived from lines generated by dose-response curves for HuIFN alpha and PoIFN gamma. The results were subjected to linear regression analysis. The statistical validity of each experiment was verified by the correlation coefficient (r) for each regression line. All r values were 0.78. Significance (P) values were calculated by three-way analysis of variance.

One AP unit of IFN was defined as a quantity causing 50 % inhibition of GI of given cells (Filipič *et al.*, 1990).

Stability tests. The acidostability and thermostability of IFNs was determined by assaying their AV and AP activities in respective units before and after 30 mins exposition to pH 2.0 and 56 °C, respectively.

Protein content of samples was determined by a modification of the standard method (Filipič *et al.*,1992).

Neutralization test. Neutralization of the AV activity of IFNs by antisera was assayed by the "constant antibody" method (LaBonnardiere *et al.*,1986), in which fixed dilutions of antiserum were applied to FL cells in microtiter plates, followed by serial 3-fold dilutions of IFN and a constant dose of HSV-1 (IFN titer). A parallel titration of IFN was performed without antiserum (IFN control titer). The neutralization index (NI) was calculated as follows :

$$NI = (\log_3 \text{ of IFN titer}) - (\log_3 \text{ of IFN control titer})$$

Results

AP activity of mitogen-induced PoIFN

The AP activity of mitogen-induced PoIFN and HuIFN alpha was studied in FL and HEF cells and the correlation

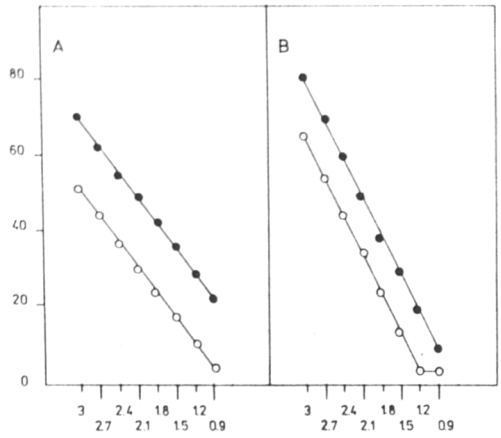

Fig. 1
Inhibition of GI of HEF and FL cells with various AV doses of mitogen-induced PoIFN and HuIFN alpha
HEF cells (A), FL cells (B), PoIFN (●), HuIFN alpha (○).
Abscissa : log AV units. Ordinate : GI inhibition (%).

Table 1. Relation of AP to AV units for mitogen-induced PoIFN and HuIFN alpha

1 000 AV units of	AP units in cells	
	HEF	FL
PoIFN	10,500	10,000
HuIFN alpha	18,000	20,000

AP units of PoIFN and HuIFN alpha in either type of cells are significantly different ($p \leq 0.005$).

between doses of AV units (from 1 000 to 7.81) and inhibition of GI in % was made (Fig. 1). In the case of PoIFN the ratio of AP to AV units in FL and/or HEF cells was approximately 10, while for HuIFN alpha it was close to 20 (Table 1). On the basis of these data the AP unit of IFN was defined as a quantity causing a 50 % inhibition of GI in FL or HEF cells.

Induction of PoIFN with PHA, LCL and AHL

Three different mitogens were tested for their capacity to induce PoIFN. Fig. 2-A shows the AP activity after days 0, 1, 3 and 5 post addition of PHA in concentrations 0, 5, 50 and 500 μg/ml. The highest activity (16,000 AP units/ml) was obtained with PHA concentration 5 μg/ml. When LCL was tested (Fig. 2-B), again the highest activity (36,000 AP units/ml) was obtained with 5 μg/ml. The situation with AHL as inducer was different from the expected one, namely 500 μg/ml gave the maximum activity (14,500 AP units/ml) in contrast to 50 μg/ml and 5 μg/ml, which yielded much lower activity (2 000 AP units/ml) (Fig. 2-C). When the same lectins were tested for a direct antiviral activity against HSV-1 in FL and HEF cells, none was found (data not shown).

Induction of PoIFN with combinations of PHA, LCL and AHL

Single inducer experiments with different lectins (PHA, LCL and AHL) showed specific induction kinetics. To increase the yield of induction (AP units/ml), different combinations of two lectins in one experiment were used. Fig. 3-A shows the combinations PHA-LCL and PHA-AHL. PHA (5 μg/ml) was added to porcine leukocytes on day 0 and LCL or AHL (500 μg/ml) on day 2. Fig. 3-B shows the combinations LCL-PHA and LCL-AHL. In similarly designed experiment LCL (day 0, 5 μg/ml), PHA (day 2, 50 μg/ml) and AHL (day 2, 500 μg/ml) were applied. Fig. 3-C shows the combinations AHL-PHA and AHL-LCL, where AHL (day 0, 5 μg/ml), PHA (day 2, 50 μg/ml) and LCL (day 2, 500 μg/ml) were used. Samples were collected on day 0, 1, 3 and 5 and assayed for AP activity. The results of these experiments show that the used combinations of lectins did not increase the induction yield, but they prolonged the induction time.

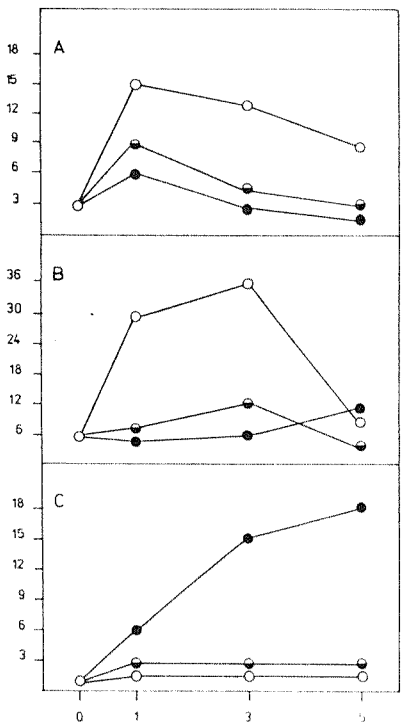

Fig. 2
Induction of PoIFN with mitogens
PHA (A), LCL (B) and AHL (C) were used in concentrations of 5 μg/ml (○), 50 μg/ml (◒) and 500 μg/ml (●). Abscissa : days. Ordinate : AP units/ml × 10^{-3}.

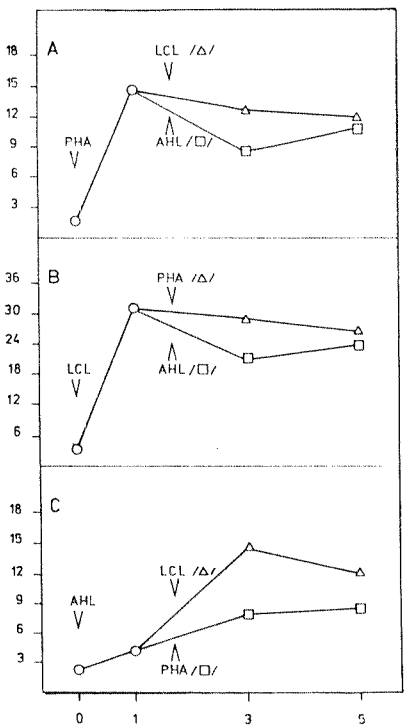

Fig. 3
Induction of PoIFN with combinations of mitogens
Combinations PHA-LCL and PHA-AHL (A), LCL-PHA and LCL-AHL (B), AHL-LCL and AHL-PHA (C). Mitogens were added at times shown by vertical arrows in concentrations described in Results. Abscissa: days. Ordinate : AP units/ml × 10^{-3}.

Table 2. Characteristics of mitogen-induced PoIFN

IFN type	Tested at		NI with antibodies to					
	pH 2.0	56 °C	PoIFN gamma	HuIFN gamma	HuIFN alpha	HuIFN alpha1	HuIFN alpha2	Acidolabile IFN
PoIFN								
PHA-induced	+	+	–1.15	0	–1.94	0	–0.29	0
LCL-induced	+	+	–1.22	0	–1.98	0	–0.31	0
AHL-induced	+	+	–1.19	0	–1.96	0	–0.31	0
PoIFN gamma	+	+	–1.23	0	–1.96	0	–0.31	0
PoIFN alpha	–	–	0	0	–2.43	0	–0.53	0
HuIFN gamma	–	–	0	–0.98	0	0	0	0
HuIFN alpha	+	+	–0.96	0	–2.85	–2.0	–1.60	0

NI = neutralization index (see *Materials and Methods*).
(+) = stable, (–) = labile.

Characterization of mitogen-induced PoIFN

Samples of PoIFN obtained after mitogen induction were further characterized by acidostability, thermostability and neutralization tests (Table 2). The obtained data show that the neutralization cross-reactivity between PoIFNs induced by different lectins is the same regardless of the antibodies used (anti-PoIFN gamma, anti HuIFN alpha and anti-HuIFN alpha2). Mitogen-induced PoIFNs were characterized also for their molecular weight by PAGE-SDS and for their serological cross-reaction by immunoblot (dot blot) analysis (data not shown).

Discussion

According to the obtained data, differences in the inducing capacity of PHA, LCL and AHL could be found. It seems that the highest induction could be obtained with LCL in concentration of 5 μg/ml. The average yield of IFN obtained by LCL induction was approximately 36,000 AP units/ml, which is comparable to 45,000 AP units/ml (4 500 AV units/ml) achieved by LCL induction of HuIFN gamma (Cantell *et al.*, 1986). When combinations of PHA, LCL and AHL were used, they did not increase the yield of induced IFN, but they prolonged the induction time. This result indicates that combinations of lectins induced a higher total amount of IFN. The dual character of AHL induction remains unsolved. On one hand, a marked induction takes place with high concentration of the inducer (500 μg/ml), but a poor induction with low concentrations (5 or 50 μg/ml). On the other hand, IFN induced with low concentrations (5 or 50 μg/ml) of AHL promoted the proliferation of HEF and/or FL cells, whereas IFN induced with high concentration (500 μg/ml) of AHL inhibited the proliferation of the cells.

The comparison of properties of mitogen-induced PoIFN and of HuIFN gamma shows that both natural forms show differences mainly in acido- and thermostability. On the contrary mitogen-induced PoIFN and HuIFN alpha are similar, both are acido- and thermostable. An interesting similarity can be found in neutralization properties : PHA-induced PoIFN reacted with antibodies to PoIFN gamma (NI = –1.15), HuIFN alpha (NI = –1.94) and HuIFN alpha2 (NI = –0.29).

Further experiments with purified mitogen-induced PoIFN together with C-terminal sequence comparison will apparently show the real level of similarity between natural PoIFN and HuIFN alpha.

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