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**EFFECT OF TYPE I INTERFERONS ON THE EXPRESSION OF FELINE  
LEUKAEMIA VIRUS.**

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**ABSTRACT (132 words)**

The efficacy of interferons (IFNs), used empirically to treat retrovirus-infected cats has been shown *in vivo*, but the direct effect on infected cells is largely unknown. Tenfold serial dilutions of three recombinant IFNs available for therapy, human IFN $\alpha$ (2a), IFN $\alpha$ (A/D) and feline IFN $\omega$  were added to the chronically FeLV-infected cell line FL74. IFNs did not apparently affect viral protein expression. However, reverse transcriptase activity (RT), directly proportional to the amount of infectious free virions, decreased with increasing concentrations of IFN and longer treatment times. The induction of apoptosis by IFN was suspected. Results of its evaluation by annexin V-Fluos staining showed that IFNs decreased the viability of treated FeLV-infected cells, and increased apoptosis, but not of non-infected cells. According to the IC<sub>50</sub>, rHuIFN $\alpha$ (A/D) appeared to be the most effective IFN in inhibiting RT.

**1. Introduction**

Feline leukaemia virus (FeLV), an important pathogen of cats, is an oncogenic retrovirus belonging to the genus Gammaretrovirus of the family *Retroviridae*. When a cat is infected by FeLV, after a period of initial replication in the oronasal region, the virus spreads throughout the body. Unless the immune response in the cat is potent enough to eliminate the virus completely, virus infected cells are frequently secluded in the bone marrow. FeLV infects blood cell precursors in the bone marrow and lymphoid tissues, and induces neoplastic (mainly lymphoma and leukaemia) and non-neoplastic (anaemia, immune-related diseases) syndromes. Most common hematological signs are aplastic anaemia, leukopenia and thrombocytopenia (reviewed by Hartmann, 2006). Immune suppression, which develops frequently, allows also secondary infections to establish. It causes cachexia, progressive weakness and, eventually, death.

There is no effective method to eliminate the virus. The infection may be controlled with antiviral chemotherapy, mainly Zidovudine (AZT). Therapeutic efficacy of AZT appears to be less promising in FeLV-infected than in FIV-infected cats, and it seems to be effective only when cats are treated at the beginning of the infection. In addition, AZT produces non-regenerative anaemia and other secondary effects such as neutropenia, vomiting or anorexia, which worsens the clinical outcome (reviewed by Hartmann, 2006).

Interferons (IFNs) are cytokines that regulate immunity in infectious diseases and tumours (Belardelli et al., 2002; Baldwin et al., 2004). Presently, two main types of IFN are considered: Type I (IFN- $\alpha$ , IFN- $\beta$ , and IFN- $\omega$ , amongst others), and type II (IFN- $\gamma$ ) (Pestka et al., 2004). Different interferons are produced by different cells: leukocytes produce IFN- $\alpha$ , fibroblasts produce IFN- $\beta$ , trophoblasts produce IFN- $\omega$ , and activated T-cells and natural killer cells IFN- $\gamma$  (Balkwill, 1989). Also, target cells are different depending on the type of interferon: lymphocytes contain functional receptors for IFN- $\omega$ , whereas a wide variety of cells respond to signals from IFN- $\alpha$ , IFN- $\beta$ , and IFN- $\gamma$  (Tizard, 2004).

Type I IFNs induce antiproliferative and antiviral responses, and not only play an important role in the innate immune response, but also influence the generation of the adaptative immune responses (Gerlach et al., 2006). IFN- $\alpha$  regulates multiple biological functions, including antiviral activity, immune regulation, cell differentiation, and cell survival or death in a variety of cell types. Type I IFN acts through IFN-stimulated response elements (ISRE) in the promoter region of interferon-stimulated genes (ISGs) (Samuel, 2001; Stark et al., 1998).

Interferons are believed to play a major role in the response against a number of retroviruses such as HIV (Shirazi and Pitha, 1992), HTLV (Feng et al., 2003), BLV (Kiermer et al., 1998) or MuLV (Aboud et al., 1981; Gerlach et al., 2006). In some of these retroviral infections, IFNs may act at the initial stages of the replication cycle (Kiermer et al.,

1998; Shirazi and Pitha, 1992), or at the final stages of the cycle (Aboud et al., 1981; Feng et al., 2003). IFNs- $\alpha$  and - $\omega$  have shown effectiveness in FeLV positive cats and are being proposed as treatment for these cats, either alone (de Mari et al., 2004; Riondato et al., 2003) or in combination with AZT (Hoover et al., 1990; Zeidner et al., 1990). However, it is not known whether it acts by improving the immune response as immunomodulator or it has a direct effect on the expression of the virus.

The aim of the present study was to determine whether Type I interferons, which are used in feline practice, have an effect on FeLV expression by evaluating parameters such as the presence of the capsid (CA) protein p27, and reverse transcriptase activity (RT) in the supernatant of persistently FeLV-infected cells treated with interferon. The induction of apoptosis in those cells was also evaluated.

## **2. Materials and Methods**

### *2.1. Interferons.*

Tenfold serial dilutions, prepared directly in culture media, of the following types of commercial IFNs were used: 1) recombinant human IFN $\alpha$ (2a), rHuIFN $\alpha$ (2a), which is commercialized for therapeutic use under the name Roferon-A® (Roche Diagnostics, Mannheim, Germany), at a final concentration between 30 and 300,000 IU/ml; 2) recombinant human IFN $\alpha$ (A/D), rHuIFN $\alpha$ (A/D), (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration between 0.4 and 400 IU/ml; and 3) recombinant feline IFN $\omega$ , rFeIFN $\omega$ , commercialized as Virbagen Omega® (Virbac, Carros, France) at a final concentration between 20 and 200,000 U/ml.

### *2.2. Cells and assay conditions.*

FL74 (Feline T-lymphoblastoid CRL8012), chronically-infected with FeLV (kindly provided by Dr. M. Horzinek, University of Utrecht, Netherlands) was used to determine the effect of IFNs on FeLV-infected cells. The human histiocytic lymphoma U937 (ATCC CRL-1593.2) was used as control to analyse the effect of IFNs on non-infected cells. The cells were incubated at 37°C in 5% CO<sub>2</sub> and >95% humidity in RPMI 1640 to which 2mM L-glutamine (Sigma-Aldrich), 100 µg/ml penicillin-streptomycin (BioMedia, Boussens, France), and 10% heat inactivated foetal calf serum (FCS, BioMedia) were added. Cell viability was estimated with Trypan Blue exclusion dye prior to the assay.

For the assays, 24-well plates (Iwaki Glass Co., Funahashi, Japan) were used, in which 1 ml containing  $2 \times 10^5$  cells were plated in each well and treated with the above mentioned final concentrations of IFN for 24h, 48h and 72h. Non-treated cells cultured in the same conditions as IFN-treated ones were used as controls. Only cells with viability greater than 96% were used in the assays. Assays were run in triplicate and repeated three times.

After 24, 48 and 72 h of incubation, plates were spun at 2000 rpm (450xg) for 5 min (Laborzentrifugen 3K10, Sigma) and 800 µl of cell-free supernatant was removed for the analysis of p27(CA) and RT activity.

### *2.3. Detection of p27(CA) and RT activity.*

The FL74 and U937 cell-free supernatants of both IFN-treated and non-treated cultures were diluted 1:4 in 0.9% NaCl and analysed with an ELISA-based test (PetChek FeLV<sup>®</sup>, Idexx Laboratories, Inc., Westbrook, ME) following the manufacturer's instructions. The concentration of p27 was quantified by comparing it to a standard curve plotted with 0.001-10 µg/µl of purified p27 (USBiological, Swampscott, MA) (Tejerizo et al, 2005).

RT activity in cell-free supernatants was quantified with an ELISA-based test (C-type-RT<sup>™</sup> Activity Assay, Cavid Tech Uppsala, Sweden) (Ekstrand et al., 1996). In

addition of manufacturer's controls, the supernatant of the non-infected cell line, U937, was used as a negative control. Both tests were read in an ELISA reader (Tecan Spectra-Fluor A-5082, Germany) at 405 nm.

#### *2.4. Detection of apoptosis by flow cytometry*

Treated and non-treated U937 and FL74 cells were washed twice with PBS. The cells were resuspended and stained with Annexin-V Fluos (Annex, Roche Diagnostics,) and propidium iodide (Sigma-Aldrich) following a protocol described previously (Tejerizo et al., 2005). The cells were analyzed by flow cytometry (FACScalibur, BD Biosciences, San Diego, CA, USA) to determine the viable non-apoptotic (Annex-/PI-), early apoptotic (Annex+/PI-), and late apoptotic and necrotic cells (Annex+/PI+) fractions, in the "Luis Bru" Electronic Microscopy Centre of the Complutense University (Madrid-Spain).

#### *2.5. Statistical analysis*

ANOVA and Duncan test of the statistic application SPSS 14.0 with a confidence interval of at least a 95% ( $\alpha$ : 0.05) was used to determine the significance of data. Inhibition concentration 50 ( $IC_{50}$ ) was determined by adjusting the data from the slopes obtained from the dilutions to a line and determining the point corresponding to 50% effect.

### **3. Results**

The IFNs had no effect on the parameters analysed in the U937 cell line (results not shown), and the p27 or RT values detected in this cell line were negligible. Contrarily, a dose dependent effect on RT activity and on apoptosis and viability was observed in the



infected cell line (FL74), which was more marked at longer times (Fig.1), and became evident and significant ( $p<0.05$ ) after 48-72 h treatment with the three types of IFN.

### *3.1. Effect on the expression of protein p27(CA) and on reverse transcriptase activity*

The amount of p27 detected in the supernatants of FL74 cell cultures treated with the three IFNs was constant during all assays, regardless of the type and concentration of IFN, and the length of time of the treatment (Fig.1).

The RT activity was measured on cell-free supernatants to evaluate the presence of free viral infectious particles. The levels of RT in the supernatants from FL74 markedly decreased with the IFN treatment as compared to controls. Statistically significant differences were observed at concentrations of  $\geq 20$  U/ml of rFeIFN $\omega$ ,  $\geq 4$  IU/ml of rHuIFN $\alpha$ (A/D) ( $p<0.01$ ) or  $\geq 30$  IU/ml of rHuIFN $\alpha$ (2a) ( $p<0.05$ ) (Fig.1). In rFeIFN $\omega$  and rHuIFN- $\alpha$ (A/D) differences were significant at 24, 48 and 72 h, but in rHuIFN $\alpha$ (2a) they were only significant at 48 and 72 h. According to the IC<sub>50</sub> (Table 1), rHuIFN $\alpha$ (A/D) was estimated to be the IFN which produced the strongest inhibition of RT, while rHuIFN $\alpha$ (2a) was at least 300 less effective.

### *3.2. Effect on cellular viability and programmed cell death*

In U937 viability ranged between 80-96%, and apoptosis between 3-7% (data not shown). On the other hand, FeLV-infected cells treated with IFN showed statistically significant differences, both in viability and apoptosis (Fig.1). Apoptosis and cell mortality increased in cells treated with  $\geq 2000$  U rFeIFN $\omega$ /ml for 48h, or  $\geq 20$  U/ml during 72h ( $p<0.05$ ), or with  $\geq 4$  IU rHuIFN $\alpha$ (A/D)/ml after 48 and 72h of treatment ( $p<0.05$ ). rHuIFN $\alpha$ (2a) was not as effective as the other IFNs tested, as 300,000 IU/ml did not reduce cell viability to 50% at any of the time periods assayed. Viability decreased significantly

when cells were incubated with concentrations  $\geq 3000$  IU/ml rHuIFN $\alpha(2a)$  during 48h, or 300 IU/ml during 72h ( $p < 0.05$ ), but apoptosis was not significantly affected. The  $IC_{50}$  was highest for rHuIFN $\alpha(A/D)$  and lowest for rHuIFN $\alpha(2a)$  (Table 1).

#### 4. Discussion

Type I IFNs have proved useful in the treatment of retrovirosis. They inhibit the replication of HIV-1 (Agy et al., 1995; Shirazi and Pitha, 1992), produce apoptosis in HTLV-I infected cells (Feng et al., 2003), reduce viral load in MuLV (Aboud et al., 1981; Gerlach et al., 2006), and reduce viral load and proviral DNA in MVV (Juste et al., 1996). When treating FIV or FeLV-infected cats, the haematological and clinical chemistry parameters, clinical signs, and survival indexes analysed have generally improved (de Mari et al., 2004), as well as plasma viremia and proviral load (Pedretti et al., 2006). However, to the best of our knowledge, no studies to compare the effect of Type-I IFNs on virological or cellular parameters in FeLV infection have been undertaken.

For this study we used three types of IFN. Although IFN- $\alpha$  subtype proteins from the same species are extremely homologous, differences in biological potency are often observed (Belardelli et al., 2002). Roferon® is a commercial product of rHuIFN $\alpha(2a)$  available for therapy, which is usually administered orally in cats. As it is of human origin and might not have an effect on feline cells, it was compared to rHuIFN- $\alpha(A/D)$ , considered “Universal Interferon”, a hybrid constructed from human IFN- $\alpha A$  and IFN- $\alpha D$ , that crosses the species barrier (Belardelli et al., 2002). The third IFN used, rFeIFN $\omega$ , is of feline origin and has been proposed to diminish the possible problems of intolerance and appearance of antibodies against human interferon which may appear in cats (Zeidner et al., 1990).

The cell-free supernatants from cell cultures were used to quantify two viral parameters which provide different information. The protein p27 is commonly used to detect viremia in infected cats. It is produced in excess by infected cells, and it may be secreted into the extracellular environment not bound to infectious particles (Jarrett, 1999). Thus, it was used to determine viral expression. The results of its quantification suggested that the synthesis of viral proteins, at least p27, was not affected by the treatment with interferon. This is in agreement with that reported for HTLV-I (Feng et al., 2003) and for Mo-MuLV (Aboud et al., 1981).

IFNs act through an IFN-stimulated response element (ISRE) in the promoter region of IFN stimulated genes. We explored the sequence of FeLV-A Rickard Strain (FeLV GenBank Accession Number AF052723) to determine the presence of such an element. The sequence GGTTCATTTTCG, matching the consensus NAGTTTCNNTTTCNC/T (Williams, 1991), was found at nucleotide 798, in a region just upstream from that which encodes the Gag-Pol precursor polyprotein gPr80; however, it should not affect viral protein expression as it is located in an area irrelevant for initiation of transcription and not in the LTR regulatory region.

Unlike p27, RT in the supernatant of infected cells is generally used to estimate the number of infectious viral particles (Niermann and Buehring, 1997). In the experiments presented here, the level of RT markedly decreased with time and at all concentrations of IFN used, regardless of the class. This decrease was not considered an artefact of the technique as the supernatants of cells treated for 24h did not show a significantly different RT than the non-treated ones.

Data from several studies with retroviruses have shown a decrease in the amount of viral particles released from infected cells treated with IFN, but not viral protein synthesis: Mo-MuLV, (Aboud et al., 1981), HIV-1 (Shirazi and Pitha, 1992), and HTLV-I (Feng et al.,

2003). The mechanism for this decreased amount of virions is not well understood and it has been proposed that IFN may interfere with the processing of viral proteins and their assembly into virions (Aboud et al., 1981), specifically at the level of Gag association with lipid rafts (Feng et al., 2003). However, it might also be related to the integrity of the plasma membrane, which would be affected at a later stage of virogenesis avoiding budding from the cell. To test this possibility, we evaluated the development of apoptosis and cellular necrosis. Our results showed that IFNs induced a decrease in viability of FeLV-infected cells. Cells appeared to die rather by apoptosis (Annex+/PI- cells) than by necrosis (Annex±/PI+ cells). It may be that IFN affects lymphocytes directly; however, the viability of the non-infected cell line (U937) did not appear to be affected by any concentration of IFN. Thus, it appeared that the intracellular and/or the slight changes in the cell membrane derived from the synergic action of viral infection and IFN could lead to a reduced release of viral particles (evaluated indirectly by the RT activity). This would limit the spread of the infection to other cells, and to the selective death of infective cells.

According to the  $IC_{50}$ , rHuIFN $\alpha$ (A/D) appeared to be the most effective, around 3-20 times more potent than rFeIFN $\omega$ , and 300-1000 times more than rHuIFN $\alpha$ (2a), in inhibiting RT. Though IFN- $\alpha$  subtypes have very similar protein structures, differences in biological potency are observed frequently. Both IFN- $\alpha$  used are of human origin, but rHuIFN $\alpha$ (A/D) is considered “Universal Interferon”, and it may have a higher effect on cells than rHuIFN $\alpha$ (2a) (Belardelli et al., 2002). According to our data, IFNs had more effect on the release of infectious particles (as measured by RT), than on cellular viability.

In conclusion, all three IFNs tested affect FeLV cycle at the post-transcriptional level, as in other retroviruses, the protein synthesis is not altered. This entails little or no release of infectious virions. In addition, it appears that IFN may act synergically with the virus, enhancing apoptosis development in treated infected cells.

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**Table 1.** Inhibition concentration 50 (IC<sub>50</sub>) for reverse transcriptase activity (RT) and viability.

	Reverse transcriptase activity		Viability	
	48 h	72 h	48 h	72 h
rFeIFN $\omega$ (U)	10.11	15.02	6093.85	124.77
rHuIFN $\alpha$ (A/D) (IU)	3.12	0.73	146.72	14.09
rHuIFN $\alpha$ (2a) (IU)	943.43	868.04	>10 <sup>7</sup>	> 10 <sup>6</sup>

## FIGURE LEGENDS

**Figure 1.** Effect of increasing concentrations of type I interferons on cell viability, cell death and apoptosis, reverse transcriptase activity (RT) and presence of p27 protein in the persistently FeLV-infected FL74 cells. Cells were incubated in the presence of the indicated concentrations of rFeIFN $\omega$ , rHuIFN $\alpha$ (2a), and rHu IFN $\alpha$ (A/D), for 24h, 48h and 72h. Non-treated cells cultured in the same conditions as IFN-treated ones were used as controls. Cell viability, death and apoptosis are expressed as percentage; RT and p27 as absorbance lecture registered after analysing the cell-free supernatans with ELISA based tests. Data represent the mean of each assay determined in triplicate. nt: not treated.



