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Contribution of molecular biology to the study of the porcine interferon system

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ABSTRACT

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We have performed molecular studies on the pig interferon (IFN) system (i) to analyse the role played by endogenous IFN in neonatal viral enteritis such as transmissible gastroenteritis and possibly to obtain, via recombinant DNA technology, a new anti-infectious and immunomodulatory agent in this species, (ii) to characterize the structure and biological functions of the IFN-like antiviral activity produced by the porcine embryo at the time of implantation in the uterus.

By probing porcine genomic libraries with human and porcine IFN-α probes to isolate related genes, we have shown that the porcine IFN-α multigene family included, like several other mammalian species, two subfamilies of related but distinct genes. Class I subfamily contains at least 11 loci, located on chromosome no. 1, among which nine have been cloned and two (potentially functional) sequenced. Class II subfamily, which is specifically expressed by the embryo of ruminants before implantation, contains at least seven loci among which six have been cloned.

One of the sequenced class I loci: PoIFN-α1 encodes a 189 amino acids (AA) preprotein. After removal of the sequence encoding the putative signal peptide (23 N-terminal AA) this gene was inserted into an Escherichia coli bicistronic expression vector allowing intracellular synthesis of mature porcine IFN-α1 (methionyl IFN-α1). Expression of the recombinant protein was optimized by insertion of a seven base pairs long random synthetic sequence in the intercistronic region, followed by cloning in E. coli and immunodetection of clones expressing high amounts of recombinant protein.

The E. coli strain obtained produced high levels of a 18 000 Da protein exhibiting the same in vitro overall biological properties as leucocyte derived porcine IFN (LeuIFN). However, it had a stronger antiviral effect on porcine cells than LeuIFN. After immunoaffinity purification to a specific activity of 5-10×10⁷ International Units (IU)/mg of protein, pharmacokinetic and pharmacological studies

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were realized to determine the in vivo half life of this rIFN-α in the pig. These experiments revealed no major toxic effects in newborn (given \(5 \times 10^6\) IU/kg) or adult \((1 \times 10^6\) IU/kg) pigs. A significant pyrogenic effect (+1.5°C) was noted only in the adults.

INTRODUCTION

Many in vivo experiments in laboratory animals have shown that interferons (IFNs) are implicated in viral and other infectious disease pathogenesis and recovery (Stewart, 1979). The fact that the effect of endogenous and exogenous IFN in experimentally induced viral diseases is very often beneficial led to the idea that IFNs can be considered as natural non-specific antiviral agents (Toy, 1983). These facts, with the recent advent of recombinant DNA technology, have prompted the use of IFNs as new anti-infectious agents with a broad antiviral spectrum and immunomodulatory properties in man (Oldham, 1985) and more recently in domestic animals, as demonstrated by several groups in the bovine species (Babiuk et al., 1985; Schwers et al., 1985; Werenne et al., 1985; Bielefeldt-Ohmann et al., 1987).

In addition, several teams of reproductive physiologists have recently shown that a protein named Trophoblastin or OTP-1 (for Trophoblast Protein 1), expressed at a high level in utero during the early stages of pregnancy by the trophoderm of the preimplantation embryo in sheep, has antiviral activity and is an authentic IFN-α (Imakawa et al., 1987; Charpigny et al., 1988). Several experimental data suggest that this protein might be a signal for the maternal recognition of pregnancy by prolonging the lifespan of the corpus luteum in the ovary (Bazer et al., 1986; Vallet et al., 1988). An immunomodulatory effect contributing to maintenance of the fetal allograft is also possible.

These pathogenic and physiological aspects of the IFN system can be developed in the porcine species. First, previous work in our laboratory showed that, during the experimental infection of newborn piglets by the transmissible gastroenteritis coronavirus (which induces acute and often fatal diarrhoea), high levels of IFN-α were produced early after infection in the intestinal tract, lungs, serum and urine, before the onset of clinical signs (La Bonnardière and Laude, 1981). We believed that it could be of great interest to investigate the pathogenetic significance of this IFN-α response using cloned recombinant IFN-α subspecies and monoclonal antibodies. Recombinant IFN-α could also represent a new therapeutic and/or prophylactic agent against infectious disease in this species.

Second, we have detected in the uterine flushings of pregnant sows, during the implantation period (between the 12th and 20th day of pregnancy) the presence of an IFN-like antiviral activity (maximum 9000 IU/ml at day 16) which could be, as reported for ruminants, an IFN-α (La Bonnardière, unpublished results). Molecular characterization of this IFN, cloning of the genes encoding it and evaluation of its biological importance for the maintenance
of pregnancy could be of interest in a species in which prolificacy is an important economic factor.

For these reasons, we have undertaken the molecular cloning of porcine IFN-α genes and the subsequent production of an *E. coli*-derived recombinant porcine IFN-α.

STRUCTURE OF THE PORCINE IFN-α MULTIGENE FAMILY

In the human, bovine, equine and probably ovine species, the INF-α multigene family is divided into two related but distinct subfamilies referred as class I and class II (Capon et al., 1985; Hauptmann and Swetly, 1985; Velan et al., 1985; Himmler et al., 1986). Both families contain virus inducible genes, but in ruminants class II members appear to be specifically expressed by the trophoblastic part of the embryo before implantation in the uterus (Imakawa et al., 1987; Imakawa et al., 1989; Charlier et al., 1989).

We have also characterized these two subfamilies in the porcine genome (Fig. 1). Using a human class I IFN-α probe, we isolated from a porcine library nine distinct clones containing 10 porcine IFN-α loci (Fig. 2) among which three were sequenced and denoted PoIFN-α1, PoIFN-α2 and ψPoIFN-αII1 (Lefèvre and La Bonnardière, 1986). PoIFN-α1 and PoIFN-α2 are intronless, potentially functional, virus inducible, class I IFN-α genes that can encode 189 and 181 AA long preproteins respectively with a putative signal peptide of 23 AA so that the mature proteins are probably 166 (α1) and 158 (α2) AA long. An N-glycosylation site is present at position 78-80 only in IFN-α1 mature protein (not in α2). Sequence homologies of these two genes to human IFN-α1 gene is 78.5% for nucleotides and 64% for AA.

Sequence analysis of the third locus ψPoIFN-αII1 suggested that it was a class II IFN-α pseudogene which had evolved from an ancestral gene encoding, like previously described mammalian class II IFN-α genes, a 172 AA preprotein with a putative signal peptide of 23 AA. Several deletions and insertions occurred in its sequence so that it cannot encode a functional polypeptide. In order to isolate potentially functional class II genes, ψPoIFN-αII1 was used as a probe to screen another porcine genomic library. Six genomic clones were isolated containing five new porcine IFN-αII loci for which sequencing is in progress (Fig. 2).

Although the actual size of the porcine IFN-α multigene family is not yet known, we can estimate that class I subfamily contains at least 11 loci among which nine are cloned and two (potentially functional) sequenced. This subfamily was localized by in situ hybridization on the long arm of chromosome no. 1 (Yerle et al., 1986). Class II subfamily contains at least seven loci among which six have been cloned and one pseudogene sequenced. As ψPoIFN-αII1 is linked to a class I locus (Fig. 2), class II loci are probably also located on chromosome no. 1.
Fig. 1. Southern blot analysis of porcine class I and class II IFN-α subfamilies. Porcine genomic DNA was hydrolyzed with the five restriction enzymes shown and hybridized successively under stringent conditions with a porcine class I probe (PoIFN-α1, lane α₁) and a porcine class II probe (υPoIFN-αII1, lane αII). This figure clearly shows that each probe hybridizes with a different set of DNA fragments revealing porcine class I and class II subfamilies with different sizes. Size markers are indicated in kilobases at the left of the figure.

Fig. 2. Restriction maps of porcine cloned DNA fragments bearing class I and/or class II loci. The nine upper clones (λ1 to λ23) were isolated using a human IFN-α1 probe (Mantei et al., 1980) and the six lower clones (λII23 to λII11) were isolated using the porcine υPoIFN-αII1 probe. Boxes indicate regions of maximal length containing an IFN-α loci as determined by Southern blot analysis. Class I and class II loci are represented by black and grey boxes respectively. Arrows indicate the position and direction of transcription of sequenced genes. Note that some clones contain two distinct loci. Clones λ17 and λ28 contain a class I locus and a class II locus (υPoIFN-αII1) which is also present in clone υII10 (in the opposite orientation).
THE STUDY OF THE PORCINE IFN SYSTEM USING MOLECULAR BIOLOGY

\[
\begin{align*}
\lambda_1 & \hspace{1cm} \text{PolFN-\(\alpha_1\)} \\
\lambda_2 & \\
\lambda_5 & \hspace{1cm} \text{PolFN-\(\alpha_2\)} \\
\lambda_{15} & \hspace{1cm} \text{PolFN-\(\alpha_3\), } \varphi\text{PolFN-\(\alpha_{II1}\)} \\
\lambda_{17} & \hspace{1cm} \text{PolFN-\(\alpha_3\), } \varphi\text{PolFN-\(\alpha_{II1}\)} \\
\lambda_{28} & \\
\lambda_{18} & \\
\lambda_20 & \\
\lambda_{23} & \\
\lambda_{23} & \hspace{1cm} \varphi\text{PolFN-\(\alpha_{II1}\)} \\
\lambda_{II23} & \hspace{1cm} \varphi\text{PolFN-\(\alpha_{II1}\)} \\
\lambda_{II10} & \hspace{1cm} \varphi\text{PolFN-\(\alpha_{II1}\)} \\
\lambda_{II25} & \\
\lambda_{II17} & \\
\lambda_{II9} & \\
\lambda_{II11} & \\
\end{align*}
\]

\[0 \hspace{1cm} 5 \hspace{1cm} 10 \hspace{1cm} 15 \hspace{1cm} 20 \text{ kb}\]
PRODUCTION AND PURIFICATION OF AN E. COLI DERIVED RECOMBINANT PORCINE IFN-α (rIFN-α)

We chose to express PoIFN-α1 gene in E. coli because it was previously shown to encode a protein with IFN-α antiviral activity (Lefèvre and La Bonnardière, 1986). The gene introduced in E. coli plasmidic expression vectors was modified by deletion of its signal peptide coding sequence in order to obtain intracellular synthesis of mature IFN-α1 (methionyl IFN-α1, MetIFN-α1: the 166 AA mature protein with an additional N-terminal methionine). Construction of the vectors, using standard recombinant DNA techniques, will be described elsewhere. We designed an expression vector (Fig. 3) allowing transcription, from the lambda bacteriophage PR therminducible promotor, of a bicistronic mRNA in which the cistron encoding MetIFN-α1 was preceded by a short cistron encoding a very efficiently translated protein. To obtain a very efficient reinitiation of translation of the MetIFN-α1 cistron, the two cistrons were placed in a translational coupling configuration allowing their consecutive translation by the same bacterial ribosome: stop and start codons were placed so that the reading frames of the two cistrons slightly overlapped in different phases (Oppenheim and Yanofsky, 1980; Gold, 1988). An efficient Shine and Dalgarno (SD) sequence had to be placed in the 3' end of the first cistron to allow translation initiation of the second cistron. SD sequence (see Gold, 1988) is a short sequence showing complementarity to the 3' end of the E. coli 16 S ribosomal RNA and essential for translation initiation of a cistron located a few nucleotides downstream. However, as it was impossible to identify a priori the best fitted SD sequence allowing efficient translation of a given cistron, we chose to insert in vitro in the 3' end of the upstream cistron a random synthetic sequence (7 successive base pairs) instead of a given SD sequence. After E. coli transformation, clones expressing high amounts of MetIFN-α1 were selected by a semiquantitative immunodetection technique allowing rapid screening of a large number of clones (Fig. 3): ampicillin resistant clones were plated at high density, replicated on nitrocellulose filters and induced at 42°C for PR dependent expression. Immunodetection of the recombinant protein was performed on in situ lysed colonies using an anti-porcine IFN-α monoclonal antibody and bound antibody was revealed with 35S labeled protein A. The intensity of the autoradiographic spots was used to identify clones producing high amounts of recombinant protein.

Using this method, a clone (pRND2D-231) was obtained producing after thermoinduction large amounts of 18 000 Da protein with antiviral activity (400 000 IU/ml of bacterial culture) corresponding to MetIFN-α1. The selected sequence present in pRND2D-231 was shown to contain the 5'-GGAG-3' tetrancleotide which is reminiscent of a Shine and Dalgarno sequence (Fig. 3). Making the expression of MetIFN-α1 constitutive (by deleting the
Fig. 3. Strategy used to optimized the production of rIFN-α1 in E. coli with a bicistronic expression vector. See section II for detailed explanations. The upper part of the figure shows the structure of the plasmid vectors with the two consecutive cistrons croβgal' and MetIFN-α1 placed under the transcriptional control of the PR promotor. These plasmids also contain the thermosensitive repressor gene cll857 and the ampicillin resistance gene (AmpR). The intercistronic region constructed in vitro using synthetic oligonucleotides and designed to promote a translation coupling phenomenon is shown in detail. The presence or the absence of the boxed GC base pair (bp) allowed translation termination of the first cistron to occur at two different stop codons (boxed). The lower figure shows the structure of the selected clone pRND2D-231.

thermosensitive repressor gene cll857 in pRND2D-231) increased IFN production at least three times.

Monoclonal antibodies were obtained against this rIFN-α and used to purify it to homogeneity from bacterial lysate by immunoaffinity (Fig. 4). Its specific activity was estimated to 5-10×10^7 IU/mg protein.
Fig. 4. Purification of recombinant porcine IFN-α1. Denaturant polyacrylamide gel electrophoresis of a crude extract of bacteria harbouring pRND2N-231 plasmid (left lane) and monoclonal antibody purified mature porcine IFN-α1 (right lane). The gel was developed by Coomassie brilliant blue staining. Molecular weight markers locations are indicated on the left.

BIOLOGICAL PROPERTIES OF RECOMBINANT PORCINE IFN-α1

The properties of the recombinant molecule were investigated in vitro and in vivo, by comparison, whenever possible, with influenza virus induced leucocyte IFN (LeuIFN).

In tissue culture

Antiviral effect. We compared the antiviral spectrum of both kinds of porcine IFN's on a panel of 5 cell lines belonging to four different species (Table 1). It appeared that, like LeuIFN, rIFN-α exhibits a broad spectrum of antiviral
TABLE 1

Antiviral activity (IU/ml) in cells from different species

| Species   | Cell line | Leu IFN | rIFN-α1 |
|-----------|-----------|---------|---------|
| Bovine    | MDBK      | 3000    | 3000    |
| Human     | WISK      | 330     | 330     |
| Murine    | L 929     | 440     | 250     |
| Porcine   | PD 5⁴     | 330     | 2200    |
|           | ST 83²    | 110     | 660     |

¹Pig kidney cells (established cell line).
²Pig testicle cells (established cell line).

TABLE 2

Neutralization of antiviral activity (IU/ml) by an anti-human IFN-α serum

| IFN       | No serum | Dilution of serum |
|-----------|----------|-------------------|
|           |          | 10⁻⁵  | 10⁻⁴  | 10⁻³  | 10⁻²  |
| Leu IFN   | 6800     | 6800  | 1300  | 84    | 4     |
| rIFN-α1   | 6800     | 5200  | 2300  | 110   | 5     |

activity (10% activity on mouse cells as compared to MDBK cells). But interestingly, rIFN-α1 was six times more active than its natural counterpart on homologous, porcine cells. This suggests that IFN-α1 is not a major component, if any, of the virus-induced LeuIFN.

Cytocidal effect. We previously showed that IFNs from several species exerted a direct toxic effect on primary cultures of pig kidney epithelial cells (Laude and La Bonnardière, 1984). We assayed rIFN-α1 on these cells and found that it induced the same kind of cytocidal effect, but with less efficiency: 10 times more antiviral units were needed to induce cell death, than with LeuIFN (unpublished results). This result suggests the existence of two different pathways for antiviral/cytocidal effects.

Antigenicity. Different authors have shown a high antigenic homology between human and porcine IFN-α (Soloviev et al., 1982; La Bonnardière et al., 1986; Piasecki, 1988). It was of interest to verify if rIFN-α1 was similar to LeuIFN in this respect. Anti-Human IFN-α sheep globulins neutralized both species of porcine IFN to the same extent (Table 2). Recent results obtained with monoclonal antibodies directed to rIFN-α1 confirm this close antigenic similarity between the cloned species and the natural IFN.

We concluded that rIFN-α1 should represent a good substitute of natural
IFN for subsequent in vivo studies. Whether due to its unique primary AA sequence or to the absence of N-glycosylation, rIFN-α1 is indeed more efficient than LeuIFN at triggering an antiviral state in porcine cells.

**In vivo**

In order to evaluate some important pharmacological properties of rIFN-α1, two series of experiments were conducted in non-infected pigs. It was not possible to compare rIFN-α1 with LeuIFN, as the latter was not available in sufficient quantity and purity.

In newborn pigs, doses of $1 \times 10^6$ units of rIFN-α1 were given intramuscularly (i.m.) and temperature and weight gain were measured for each animal; some pharmacological data were also recorded and calculated. In summary the rIFN-α1 did not lead to any weight loss in 5-day-old piglets, including those which received five doses of $10^6$ units. In two experiments, no significant hyperthermia was noted. The main pharmacokinetic parameters, measured in the blood after i.m. injection, were as follows: time of maximum concentration ($T_{max}$) < 1 h, maximum serum concentration ($C_{max}$) = 1700 IU/ml, apparent elimination half-life ($T_{1/2}$) = 210 ± 40 min., measured between 9 and 24 h (Fig. 5B). The same results hold true for one day-old piglets. Therefore we concluded that rIFN-α1 exert no detectable toxic effect in the

![Fig. 5. Pharmacokinetics of rIFN-α1 in Large White pigs. After one i.m. injection in the hind leg, IFN was measured in the arterial blood collected by aortic puncture. Antiviral assay was performed in bovine MDBK cells as previously described (La Bonnardiére and Laude, 1981). Each curve corresponds to an individual pig.](image)

(A) Adult pigs (70 to 80 kg each), given $10^6$ IU/kg (◇, □) and $2 \times 10^5$ IU/kg (■, ◇).

(B) Five-day-old piglets, given $10^6$ IU/kg.
newborn. This is important if treatment or prevention of neonatal viral infections is contemplated using this recombinant molecule.

Four adult Large White pigs weighing on average 80 kg, were injected i.m. with the same preparation of purified rIFN-α1. Two received $10^6$ IU/kg of eight, two received $5 \times 10^5$ IU/kg. The kinetics of antiviral activity was followed in the blood after aortic puncture (Figure 5A). There were two differences from the results with newborn animals: first, whereas the parameters $C_{\text{max}}$ and $T_{\text{max}}$ were roughly identical for the young and the adults, elimination half-life was higher in the adults: apparent $T_{1/2} = 280$, and $470 \pm 90$ min respectively for the doses of $10^6$ and $2 \times 10^5$ IU, in the time-interval of 8 h to 24 h after injection. Second, there was a significant hyperthermia observed in the two individuals given the highest dose, followed at 32 h by a significant hypothermia. This transient fever, also recognized in human patients administered high IFN dosage, was not observed with the lower dose. The pigs, when observed for several weeks afterwards, did not show any problems.

CONCLUSIONS

This work illustrates a possible contribution of molecular biology to the study of the structure, biological and pathogenetic role of cytokines in domestic animals, and to their potential use as anti-infectious agents.

We have shown that the porcine IFN-α multigene family contained, like several other mammalian species, class I and class II subfamilies of intronless genes encoding several IFN-α subspecies.

One of the class I subspecies was expressed at high levels in *E. coli* and several neutralizing and non-neutralizing monoclonal antibodies were obtained against this molecule allowing its purification to homogeneity. The in vivo preliminary data we obtained will be used to test the effect of rIFN-α1 on the outcome of experimental viral disease of the pig. Particular attention will be paid to those diseases occurring at critical periods in the pig's lifetime.

We are now trying and characterize the physicochemical and biological properties of the IFN-like antiviral activity detected in uterine flushings at the time of implantation. Hybridization analysis of mRNA from porcine embryos will soon reveal if this IFN-like antiviral activity is a class II IFN-α gene product like the previously described ruminant embryonic IFNs.

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