Immunomodulatory effects in vivo of recombinant porcine interferon gamma on leukocyte functions of immunosuppressed pigs

D Saulnier, S Martinod, B Charley

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Original article

Immunomodulatory effects *in vivo* of recombinant porcine interferon gamma on leukocyte functions of immunosuppressed pigs

D Saulnier [1], S Martinod [2], B Charley [1*]

[1] *INRA, Laboratoire de Virologie et d'Immunologie Moléculaires, Centre de Recherches de Jouy-en-Josas, Domaine de Vilvert, 78352 Jouy-en-Josas Cedex, France;*
[2] *Ciba-Geigy SA, CRA, 1566 Saint-Aubin FR, Switzerland*

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Summary – Immunological parameters of porcine peripheral blood mononuclear cells after *in vivo* injections of recombinant porcine interferon gamma (rPoIFNγ) were studied in pigs immunosuppressed by dexamethasone (6 mg/kg body weight in a single injection). A 2-d period of rPoIFNγ injected alone and intramuscularly at a dose of 1 μg/kg body weight increased interleukin 1 (IL1) production ($P < 0.05$). Recombinant porcine IFNγ also reversed the immunosuppressive effects of dexamethasone on : i), lymphocyte responsiveness to mitogens : PHA ($P < 0.03$), ConA ($P < 0.053$); ii), IL1 production; and iii), IL2 production ($P < 0.05$). However, rPoIFNγ had no effect on neutrophilia induced by dexamethasone. These data show that rPoIFNγ modulates leukocyte functions of pigs *in vivo*.

interferon gamma / swine/interleukin / lymphocyte / immunosuppression

Résumé – Effets immunomodulateurs *in vivo* de l'interféron gamma recombinant porcin (rPoIFNγ) sur les fonctions leucocytaires de porcs immunodéprimés. *Plusieurs paramètres immunologiques des cellules mononucléées du sang périphérique du porc ont été étudiés à la suite de l'injection* in vivo *d'interféron gamma recombinant porcin à des porcs immunodéprimés par la dexaméthasone (6 mg/kg de poids vif en une seule injection). L'IFNγ recombinant porcin administré seul et par voie intramusculaire, pendant 2 j, à la dose de 1 μg/kg de poids vif a augmenté la production d'interleukine 1 (IL1), (P < 0,05). Il a pu également limiter les effets immunosuppresseurs de la dexaméthasone :*
— sur la réponse lymphocytaire aux mitogènes : PHA (P < 0,03), ConA (P < 0,053),
— sur la production d'IL1,
— sur la production d'IL2 (P < 0,05).
Néanmoins, l'IFNγ recombinant porcin n'a pas eu d'effet sur la neutrophilie induite par la dexaméthasone. Ces résultats montrent que l'IFN recombinant porcin est capable de moduler, in vivo, *plusieurs fonctions leucocytaires chez le porc.*

interféron gamma / porc / interleukine / lymphocyte / immunosuppression

* Correspondence and reprints

INTRODUCTION

Interferon gamma (IFNγ) is a lymphokine produced by activated T lymphocytes following stimulation by specific antigens (during an immune response) or T-cell mitogens such as phytohemagglutinin (PHA) or concanavalin A (ConA) (Ronnblom *et al*, 1982). In addition to its antiviral activity, IFNγ can exert a number of immunomodulatory effects such as enhancement of natural killer (NK) cell and T-cell mediated cytotoxicity, and neutrophil activation (Trinchieri and Perussia, 1985).

In the porcine species, the gene coding for IFNγ has been cloned and recombinant porcine IFNγ (rPoIFNγ) has been shown to inhibit, *in vitro*, the replication of transmissible gastroenteritis virus, vesicular stomatitis virus (Charley *et al*, 1988) and African swine fever virus (Esparza *et al*, 1988). Little is known about the immunomodulatory effects of rPoIFNγ, contrary to the results obtained with recombinant bovine IFNγ (rBoIFNγ). However, Charley *et al* (1990) have shown that rPoIFNγ is able to activate porcine adherent mononuclear cells to secrete interleukin 1 (IL1) after stimulation with lipopolysaccharide (LPS). Furthermore, rPoIFNγ is able to increase production of TNFα by LPS-stimulated porcine macrophages (Dunham *et al*, 1990).

In this study immunomodulatory properties of rPoIFNγ were evaluated for their influence, *in vivo*, on lymphocyte blastogenesis and IL1 and IL2 production by peripheral blood mononuclear cells. Recombinant porcine IFNγ activity was evaluated in normal pigs to investigate any direct effects of the cytokine on immune parameters, and in dexamethasone-treated pigs in order to determine whether rPoIFNγ could reverse defective immune functions. Because mechanisms of glucocorticoid-induced immunosuppression are well characterized *in vitro* and *in vivo* (Mormède and More, 1980; Roth and Kaeberle, 1983; Westly and Kelley, 1984; Blecha and Baker, 1986; Klemcke *et al*, 1987; Frank and Griffin, 1989; Rafaî and Tuboly, 1989; Roth and Frank, 1989), we used dexamethasone to induce immunosuppression in pigs. Timing of dexamethasone administration and dosage were evaluated to obtain optimal immunosuppressive conditions. Our results show that a 2-d period of rPoIFNγ (1 μg/kg intramuscularly) treatment significantly increased IL1 production by untreated pigs and significantly reversed the immunosuppressive effects of a single intramuscular administration of dexamethasone on lymphocyte proliferative responses to PHA and on IL2 production. Recombinant porcine IFNγ also tended to enhance lymphocyte blastogenic responses to ConA and IL1 leukocyte production in dexamethasone-treated pigs.

MATERIALS AND METHODS

Animals and experimental design

Sixteen Large White pigs (4 pigs/group) were used. The animals weighed 13.9 ± 1.4 kg and were randomly assigned to each group. Dexamethasone-treated groups received 6 mg/kg of body weight (BW) of dexamethasone 21-phosphate (Sigma, St Louis, USA) by one intramuscular injection on d1. Animals treated by rPoIFNγ alone were given

1 μg/kg BW rPoIFNγ (1.12×10^8 U/mg protein, lot No PoG016144 supplied by Ciba-Geigy Inc, Basel, Switzerland) per animal by intramuscular injection on days 0 and 1. A group received rPoIFNγ alone on d0 and both dexamethasone and rPoIFNγ treatment on d1. Untreated animals received an equal volume of PBS (phosphate buffered saline) at the same time as other treatments. All injections were performed between 08.00 and 09.00 am. Animals were bled daily for 3 consecutive days (*ie* d0, 1 and 2).

Total and differential leukocyte cell counts

Total leukocyte counts were determined following red blood cells lysis and differential leukocyte counts were performed on May–Grünwald–Giemsa-stained blood films.

Preparation of porcine blood mononuclear cells

Peripheral blood mononuclear cells were separated from heparinized venous blood by a Ficoll density centrifugation method on MSL® ($d = 1.077$ from Eurobio, Les Ulis, France) (Charley *et al*, 1983).

Lymphocyte blastogenesis

Mononuclear cells were cultured in flat-bottom microtiter plates (Costar, Broadway, USA) with 6 replicates of 3×10^5 cells in RPMI-1640 complete medium per well for each sample. Mitogen-stimulated cultures received phytohemagglutinin (PHA-P from Difco, Detroit, USA) at a final concentration of 25 μg/ml, and concanavalin A (ConA from Miles, Yeda, Israel) and pokeweed mitogen (PWM from Gibco) at final concentrations of 12.5 μg/ml. Cultures were incubated for 2 d at 37 °C and pulsed overnight with 37 kBq/well of tritiated thymidine (TMM 48 C from CEA, Saclay, France). The incorporated radioactivity was collected on filter paper by an automated cell harvester. Results were expressed as the difference in counts per min (dcpm) between thymidine incorporation in mitogen-treated and untreated cultures.

IL1 and IL2 production

IL1 and IL2 were prepared from mononuclear cells by stimulation with *E coli* lipopolysaccharide (LPS) from Sigma at a final concentration of 20 μg/ml in the presence of indomethacin (1 mg/ml from Sigma) or with PHA-P at a final concentration of 50 μg/ml, respectively. Mononuclear cells were cultured at a cell concentration of 1.5×10^6 cells per ml in RPMI-1640 complete medium. Following 48 h of incubation at 37 °C, supernatants were collected and stored at −20 °C before titration (Charley *et al*, 1985; Cavaillon *et al*, 1989).

IL1 and IL2 assays

IL1 activity was tested by the ability of supernatants to induce the proliferation of mouse thymocytes stimulated with submitogenic doses of PHA-P (10 μg/ml) (Arenzana-Seisdedos *et al*, 1985). IL2 activity was determined by measuring its proliferative effects on the IL2-dependent, murine cytotoxic T cell line (CTL-FD) as described previously (Leclerc *et al*, 1984). Briefly, thymocytes (5×10^6/ml) and CTL-FD cells (1.25×10^6/ml) for the IL1 and IL2 assays respectively were incubated in 96-well tissue culture plates with serial 2-fold dilutions of supernatants. After 48 h for the IL2 assay and 72 h for the IL1 assay, cultures were pulsed overnight with 37 kBq tritiated thymidine and cells were collected on filter paper by a multiple sample harvester. IL1 and IL2 activity were obtained as cpm of tritiated thymidine incorporation. IL1 and IL2 activities were correlated to standard values and expressed as arbitrary units, through use of a computer-assisted logit analysis of the data (kindly provided by F Blecha).

Data analysis

Means and SEM were determined for each parameter of each group of pigs and statistical comparisons were performed

using an analysis of variance procedure (Student's *t*-test) blocked by day to determine the level of significance of any differences between two groups.

RESULTS

Dexamethasone-induced immunosuppression

Preliminary experiments were conducted to determine a dose of dexamethasone that depressed leukocyte functions in pigs. No significant effects of dexamethasone injected at doses of 100 μg/kg BW (2 daily injections over a 2-d period) or 1 mg/kg BW (one daily injection over a 2-d period) were observed on differential polymorphonuclear (PMN) counts, lymphocyte proliferative response and IL1 or IL2 production. In subsequent experiments with 3 and 9 mg/kg BW of dexamethasone, polymorphonuclear cell counts were increased and lymphocyte proliferative responses were decreased (data not shown). Therefore, 6 mg/kg BW of dexamethasone was the dosage used in the following experiment to induce immunosuppression.

Effects of rPoIFNγ and/or dexamethasone on total and differential polymorphonuclear cell counts

We did not observe any effects of either treatment on total leukocyte counts. Nevertheless, treatment with dexamethasone caused significant neutrophilia ($P < 0.05$) 3 h after its administration (table I, d1). Recombinant porcine IFNγ by itself had no effect on PMN counts. Moreover, no significant differences were observed between PMN counts from animals receiving combined rPoIFNγ plus dexamethasone treatments and PMN counts from dexamethasone-treated pigs (table I).

Effects of rPoIFNγ and/or dexamethasone on lymphocyte blastogenesis

Dexamethasone-treated animals had significantly decreased proliferative responses to ConA, PHA or PWM 3 h after dexamethasone injection (table II). Dexamethasone treatment also depressed the lymphocyte blastogenic response to PHA 24 h after glucocorticoid administration (data not shown). Administration of rPoIFNγ for

Table I. Polymorphonuclear cells counts ($\times 10^6$/ml) : influence of IFN γ, dexamethasone and combined treatments.

Days	Treatment groups			
	Untreated	rPoIFN γ	Dexamethasone	Dexamethasone and rPoIFN γ
0	4.45 ± 1.01	3.29 ± 1.01	6.02 ± 0.82	3.34 ± 0.82
1	5.57 ± 1.1	3.81 ± 0.74	8.9 ± 1.39[a]	10.47 ± 2.04[a]
2	5.26 ± 1.38	2.94 ± 1.02	5.63 ± 3.38	3.9 ± 1.84

Data are expressed as mean ± SEM ($n = 4$). [a] The indicated value is significantly different from that of the untreated group, $P < 0.03$.

Table II. Lymphocyte blastogenesis response 3 h after dexamethasone (6 mg/kg BW) administration following a 2 d period of rPoIFNγ treatment.

Mitogen	Treatment groups			
	Untreated	rPoIFNγ	Dexamethasone	Dexamethasone and rPoIFNγ
None (cpm)	1 772 ± 284	1 214 ± 190	712 ± 101[a]	961 ± 87[a]
PHA (dcpm)	123 604 ± 4 448[b]	151 274 ± 6 881[a,b]	8 846 ± 7 253[a]	109 923 ± 5 332[c]
ConA (dcpm)	117 020 ± 6 794[b]	153 301 ± 4 324[a,b]	59 599 ± 8 678[a]	81 613 ± 8 569[a]
PWM (dcpm)	82 235 ± 2 018[b]	93 854 ± 12 853[b]	54 416 ± 6 903[a]	41 904 ± 7 762[a]

Data are expressed as mean ± SEM (n = 4). [a] The indicated value is significantly different from that of the untreated group, $P < 0.05$. [b] The indicated value is significantly different from that of the dexamethasone-treated group, $P < 0.05$. [c] The indicated value is significantly different from that of the dexamethasone-treated group, $P < 0.03$.

2d significantly enhanced the lymphocyte blastogenic responsiveness to PHA ($P < 0.03$) in dexamethasone-treated animals and also tended to enhance their responsiveness to ConA ($P < 0.056$) as measured 3 h after dexamethasone injection. However, these effects were no longer observed after 24 h (data not shown). Although the data shown in table II indicate significant differences between the lymphocyte proliferative responses to PHA and ConA of rPoIFNγ-treated and untreated animals, similar differences were already observed between the same 2 experimental groups before rPoIFNγ administration (data not shown). Such differences cannot therefore be related to rPoIFNγ treatment.

Effects of rPoIFNγ and/or dexamethasone on IL1 and IL2 production

Dexamethasone significantly inhibited IL2 production ($P < 0.05$) 3 h (data not shown) and 24 h after injection, but had no significant effect on IL1 production (table III). Recombinant porcine IFNγ significantly enhanced IL1 production ($P < 0.05$) 3 h (data not shown) and 24 h after injection (table III) in comparison with untreated pigs. Nevertheless, rPoIFNγ has no significant effect on IL2 production.

Administration of rPoIFNγ significantly reversed the immuno-suppressive effects of dexamethasone on IL2 production only 24 h after injection

Table III. IL1 and IL2 production 24 h after dexamethasone (6 mg/kg BW) administration following a 2-d period of rPoIFNγ treament.

IL production	Treatment groups			
	Untreated	rPoIFNγ	Dexamethasone	Dexamethasone and rPoIFNγ
IL1	0.408 ± 0.199	1.54 ± 0.439[a,b]	0.158 ± 0.059	0.953 ± 0.312
IL2	1.378 ± 0.793[b]	0.793 ± 0.2	0.135 ± 0.135[a]	1.208 ± 0.3[b]

Data are expressed as mean ± SEM (n = 4) of IL arbitrary units. [a] The indicated value is significantly different from that of the untreated group, $P < 0.05$. [b] The indicated value is significantly different from that of the dexamethasone-treated group, $P < 0.05$.

(table III). It tended to have the same effects on IL1 production, but differences between IL1 production from dexamethasone-treated animals and animals treated both with dexamethasone and rPoIFNγ were not significant.

DISCUSSION

The *in vivo* immunomodulatory effects study of rPoIFNγ on leukocyte functions in normal pigs showed an increased production of IL1. In immunosuppressed pigs, rPoIFNγ significantly reversed the immunosuppressive effects of dexamethasone on i), lymphocyte responsiveness to PHA; and ii), IL2 production.

Considering the *in vivo* effects of IFNγ treatment in normal animals, our results indicate that rPoIFNγ injected alone only affects IL1 production, which is significantly increased 3 and 24 h after treatment, whereas other immune parameters studied remained unchanged. In the bovine species, Roth and Frank (1989) observed that a dose of 0.5 mg per animal of rBoIFNγ induced relatively minor changes in total and differential leukocyte cell counts. Moreover, they showed that rBoIFNγ had no effect on lymphocyte blastogenic responses to mitogens (PHA, ConA, PWM). In addition, although *in vitro* studies demonstrated that IL1 could increase IL2 production (Dinarello, 1987), we did not observe a concomitant enhancement of IL1 and IL2 production in IFNγ-treated animals (table III).

The observed effects of rPoIFNγ in dexamethasone-treated animals included a significant, short-term enhancement of lymphocyte proliferative responses to PHA (3 h after injection; table II). Interleukin-2 production was significantly increased only 24 h after treatment (table III). Recombinant porcine IFNγ also tended to increase lymphocyte blastogenic response to ConA 3 h after injection. The mitogens PHA and ConA are considered as being T-lymphocyte specific, whereas PWM stimulates both B and T pig lymphocytes (Binns, 1982). The fact that rPoIFNγ reduces dexamethasone-induced suppression of lymphocyte blastogenesis in response to PHA and ConA without a significant effect on the lymphocyte blastogenic response to PWM indicates that rPoIFNγ could primarily affect porcine T lymphocytes. Several mechanisms could account for such effects : i), IFNγ could increase IL2 production, which in turn would favor T cell proliferation; ii), alternatively, IFNγ could act on IL1 production, leading itself to a greater T cell growth; and III), finally, IFNγ could also directly affect the T-cells responsiveness to mitogens or to interleukins. Our results suggest that rPoIFNγ can influence T-cell responsiveness by indirect effects at the level of IL1 and IL2 production. Thus, 24 h after the 2 rPoIFNγ injections in dexamethasone-treated pigs, IL2 production was significantly increased and IL1 yield was elevated, although not significantly (table III). Recombinant porcine IFNγ could also cause a redistribution of peripheral blood leukocyte populations : modifications in ratios of monocytes to lymphocytes or in ratios between different T-cell subsets could explain

the observed alterations in lymphocyte responsiveness to mitogens. A redistribution of circulating T-cell subsets in various lymphoid organs was achieved by treating pigs with high doses of corticoids (Salmon, 1983).

In order to evaluate rPoIFNγ in immunosuppressed animals, we had to develop a model of corticoid-induced immunosuppression in pigs. One injection of dexamethasone at a dose of 6 mg/kg BW significantly inhibited lymphocyte blastogenic responses to PHA, ConA, PWM and synthesis of IL2 by lymphocytes. Such a reduction in mitogenesis and IL2 production may be explained by lysis of lymphoid cells. Indeed, it has been demonstrated in the porcine species that pharmacological concentrations of cortisol caused, *in vitro*, significant but minimal cell death as compared to results in other species (Westly and Kelley, 1984). However, in the present experiments, cell concentrations were determined on the basis of viable cells; it appears that the pig is much more resistant to immunosuppression by corticoids than cattle (Roth and Flaming, 1990). Indeed, dexamethasone administered to pigs intramuscularly at 2.0 mg/kg BW did not consistently alter lymphocyte and neutrophil functions which are inhibited in cattle given 0.04 mg/kg of dexamethasone intramuscularly. In addition to the possible lytic effects of pharmacological doses of dexamethasone on lymphoid cells immunosuppression might also be due to functional alterations of leukocytes.

In conclusion, our results show that rPoIFNγ modulates several lymphocyte functions of pigs *in vivo*. In similar experiments conducted in the bovine species, Roth and Frank (1989) did not observe significant effects of rBoIFNγ on lymphocyte functions of dexamethasone-treated animals, although neutrophil functions were enhanced. Our data support the idea that rPoIFNγ could also be used in other situations such as immunosuppression induced by infectious diseases. Thus recombinant porcine IFNγ was shown to protect piglets against *Actinobacillus pleuropneumoniae*-induced pneumonia *in vivo* (Bielefeld-Ohmann and Martinod, 1990). Taken together, these data suggest that IFNγ immunotherapy might be more effective for pigs than for cattle in which IFNγ treatments were previously evaluated (Bilefeld-Ohmann and Babiuk, 1986; Chiang *et al*, 1990).

Current and future research on immunomodulators may provide a mechanism to overcome the immunosuppressive effects of stress and viral infections which are considered to be important components in the pathogenesis of many infectious disease syndromes that affect domestic food producing animals.

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