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Frequency of interferon-alpha-secreting blood leukocytes in irradiated and bone-marrow-grafted pigs

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Summary — The effects of irradiation were studied on porcine interferon-alpha (IFN- α) secreting cells (IFN- α SC). IFN- α SC were characterized by an ELISPOT assay on non-adherent PBMC following incubation with the transmissible gastroenteritis coronavirus. *In vitro* irradiation of PBMC was followed by a decrease in the number of IFN- α SC while IFN- γ production and cell viability were not affected. These data indicate that porcine IFN- α SC are relatively radiosensitive. Indeed, the frequency of blood IFN- α SC decreased markedly and rapidly after *in vivo* whole body or partial lymphoid irradiation. In addition, within several days of compatible bone-marrow engraftment in the irradiated animals, the number of blood IFN- α SC returned to normal values. These data demonstrate that circulating porcine IFN- α SC are derived from bone-marrow progenitors.

interferon-alpha / leukocyte / bone marrow / irradiation / transmissible gastroenteritis virus / porcine

Résumé — **Fréquence des leucocytes sécréteurs d'interféron alpha chez le porc après irradiation et greffe médullaire.** Nous avons étudié la radiosensibilité des cellules sécrétrices d'interféron alpha (IFN- α) dans l'espèce porcine. Les cellules sécrétrices d'IFN- α ont été étudiées par la technique ELISPOT, à partir de cellules sanguines mononucléées non adhérentes incubées en présence du coronavirus de la gastro-entérite transmissible. L'irradiation *in vitro* des cellules mononucléées du sang s'est traduite par une diminution du nombre des cellules sécrétrices d'IFN- α , sans affecter la production d'IFN- γ ni la viabilité cellulaire. Ceci montre que les cellules sécrétrices d'IFN- α du porc sont relativement radiosensibles. De fait, leur fréquence dans le sang d'animaux soumis à une irradiation corporelle totale ou à une irradiation lymphoïde partielle s'en trouve rapidement et très fortement diminuée. De plus, quelques jours après transfert d'une moelle osseuse compatible à des porcs irradiés,

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le nombre des cellules sécrétrices d'IFN- α redevient normal. Ces résultats montrent donc que les cellules sécrétrices d'IFN- α présentes dans la circulation sanguine du porc proviennent de précurseurs médullaires.

interféron alpha / leucocyte / moelle osseuse / irradiation / virus de la gastro-entérite transmissible / porc

INTRODUCTION

Interferon-alpha (IFN- α) are leukocyte-secreted proteins and are one of the earliest host responses to viral infections. *Via* their antiviral properties, they help limit viral spread (De Maeyer-Guignard, 1993). Distinct leukocyte cell populations can secrete IFN- α mainly depending on the nature of the IFN-inducing viral structure. Thus, macrophages are usually responsible for producing IFN- α in response to infectious viruses, but a distinct population of low-density, non-phagocytic, non-adherent mononuclear cells can also secrete IFN- α when exposed to non-infectious viral structures, such as inactivated virions or glutaraldehyde-fixed virus-infected cells (reviewed by Charley and Laude, 1992). This cell population is often referred to as 'natural interferon-producing cells' (NIPC). This is rare among blood mononuclear cells and exhibits unusual phenotypic features. Human NIPC do not express surface antigens specific for T- or B-cells, but are positive for MHC class II antigens and CD4 (reviewed by Fitzgerald-Bocarsly, 1993). NIPC may be circulating blood dendritic cells (Ferbas *et al*, 1994). In addition, human NIPC showed some similarities with the early progenitors of myeloid and lymphoid cells in flow cytometry analysis, but they lacked the stem cell-associated CD markers (Sandberg *et al*, 1991).

In the porcine species, IFN- α -producing cells were produced in response to *in vitro* induction by the coronavirus transmissible gastroenteritis (TGEV) or the pseudorabies (PR, or Aujeszky disease) viruses and were characterized in blood leukocytes by a solid-

phase enzyme-linked immunospot assay (ELISPOT) as well as by *in situ* hybridization (Nowacki and Charley, 1993; Artursson *et al*, 1992). TGEV-induced IFN- α -producing leukocytes were also characterized in porcine gut-associated lymphoid tissue (Naidoo and Derbyshire, 1992). Porcine blood IFN- α -secreting cells (IFN- α SC) were shown to share several features with their human counterparts since they are low-density, non-phagocytic, non-T, non-B, but CD4⁺ and SLA-class II⁺ cells (Nowacki and Charley, 1993; recently reviewed by La Bonnardière *et al*, 1994).

Because the precise nature of IFN- α SC is still poorly defined, the present study was undertaken to evaluate the possible bone-marrow origin of TGEV-induced porcine IFN- α SC. The frequency of IFN- α SC was assessed by ELISPOT in blood leukocytes from irradiated and bone-marrow-grafted pigs, using previously described protocols (Vaiman *et al*, 1975). We found that IFN- α SC rapidly disappeared in the circulating blood of irradiated animals and that they reappeared in bone-marrow-reconstituted pigs, which supports the idea that they originate in bone marrow.

MATERIALS AND METHODS

Animals

Large White breed piglets from several litters were used. Some of them belonged to a herd carrying a gene defect for the von Willebrand factor. Donors and recipients of bone-marrow graft

were compatible for the major histocompatibility complex.

Virus

The high-passage Purdue-115 strain of TGEV was used as a virus source. The procedures for virus propagation in the pig kidney cell line PD5, virus purification, UV inactivation and titration of infectivity in the swine testis (ST) cell line have been reported previously (Laude *et al*, 1986; Charley *et al*, 1994).

PBMC and bone-marrow cells

Non-adherent porcine peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood by Ficoll density centrifugation on MSL[®] (density 1.077, Eurobio, Paris) followed by adherent cell depletion on tissue culture flasks as described previously (Nowacki and Charley, 1993). PBMC were suspended in RPMI-1640 medium supplemented with 10% foetal calf serum (RPMI-10% FCS) and antibiotics and kept overnight at 4°C before use. Sternal bone marrow was surgically collected from anaesthetized pigs. Bone-marrow cells were prepared by centrifugation on MSL, and adherent cell depletion was performed as described above.

Irradiation and bone-marrow grafts

PBMC were irradiated for increasing lengths of time resulting in increasing irradiation doses, as indicated in the *Results*, using cobalt-60 sources. After 48 h in culture, the percentage of dead cells was estimated by trypan blue dye. Non-anaesthetized starved pigs were irradiated in a sling using symmetrically opposed cobalt-60 sources. Whole body irradiation (WBI) was carried out with 2 opposed sources at a dose rate of 9.2 rad/min (midline-tissue absorbed dose) (Vaiman *et al*, 1975).

The partial lymphoid irradiation (PLI) procedure has been described previously (Vaiman *et al*, 1981). Briefly, the pigs were irradiated by 6 sources at an average midline-tissue dose of 47 rad/min. The head, the upper part of the neck and parts of the thorax were protected with lead. The shielded region received less than 5% of the total dose. Two different PLI protocols were tested, as shown in table I: 3 x 3 Gy for 5 d; or 5 x 2.5 Gy for 10 d. WBI was performed with 8 or 8.75 Gy (table I).

The bone-marrow cells were injected 1 or 3 d after irradiation, as stated in the *Results*, at a dose of 2.5–5 x 10⁸ cells per gram body weight. The preparation of bone-marrow cells, injections and care of the recipients has been described previously (Vaiman *et al*, 1975).

Heparinized blood was serially collected from each animal. Giemsa-stained blood smears and total white blood cell counts (Unopette, Becton

Table I. *In vivo* experimental design.

<i>Irradiation</i>	<i>Animal number</i>	<i>Bone-marrow transfer</i>	<i>Number of Co-60 sources</i>	<i>Dose rate (rad/min)</i>	<i>Doses (Gy)</i>	<i>Schedule (d)</i>
PLI	39910	–	6	46	3 x 3	3
PLI	39912	–	6	46	3 x 3	3
PLI	15055	+	6	49.35	5 x 2.5	5
WBI	25061	+	2	9	1 x 8.75	1
WBI	15048	+	2	9	1 x 8	1

Dickinson, Rutherford, NJ, USA) were performed in order to determine the number of lymphocytes/ml blood. IFN- α SC number/ml was deduced from the number of IFN- α SC per 10^5 lymphocytes, determined as follows.

IFN- α induction

PBMC or bone-marrow cells were induced to produce IFN- α by incubation with TGEV in 96-well microplates. Non-adherent cells were incubated at final concentrations of $1-5 \times 10^6$ cells per ml in a total volume of 200 μ l RPMI 1640 plus 10% FCS, with TGEV. The virus used was either a crude, UV-inactivated viral preparation at 0.1 plaque-forming units per cell, or a purified, inactivated preparation at 0.6 μ g/ml, as indicated in the *Results*. After 8 h at 37°C, the induced cells were resuspended and 100 μ l cells from each well was transferred to nitrocellulose-bottomed microplates for the ELISPOT assay (see below). The other 100 μ l induced cells were further incubated overnight at 37°C for IFN- α immunoassay.

ELISPOT

The ELISPOT assay was performed as described previously (Nowacki and Charley, 1993). Nitrocellulose-bottomed 96-well filtration plates were coated with anti-porcine IFN- α monoclonal antibody (MAb) 'K9', and then fixed with glutaraldehyde and blocked with glycine, before the addition of TGEV-induced cells for an overnight incubation at 37°C. Following extensive washing, the plates were incubated with peroxidase-conjugated anti-porcine IFN- α MAb 'F17' for 1 h at 37°C before the addition of diaminobenzidine with perhydrol. Spots were counted using a magnifier. The frequency of IFN- α SC was calculated as the mean number of spots divided by the total cell number in each well. Results were expressed as means of spots per 10^5 cells and as IFN- α SC/ml blood, taking into account the number of lymphocytes/ml.

IFN- α immunoassay

A specific ELISA for porcine IFN- α was performed as described previously (De Arce *et al.*, 1992; Splichal *et al.*, 1994), by using MAb K9 for coating,

and peroxidase-conjugated F17 MAb as a second Ab. In each assay, our internal standard of recombinant porcine IFN- α was included. The estimated amount of IFN- α produced by each IFN- α SC was calculated from the titer of IFN- α (units) in induction culture supernatants as determined by ELISA, and IFN- α SC number per culture, as determined by ELISPOT.

IFN- γ induction and titration

PBMC were incubated in microtiter plates at 3×10^6 per ml with 50 μ g/ml PHA (ref HA 16 from Wellcome, Paris) for 48 h. IFN- γ in supernatants was titrated by a specific ELISA, using anti-porcine IFN- γ MAb G47 for coating, and a rabbit antiserum (No 652; Charley *et al.*, 1988) as a second Ab, as described previously (D'Andréa, 1995).

RESULTS

Effects of irradiation on IFN- α SC

In a preliminary experiment, PBMC were irradiated *in vitro* for varying lengths of time, in order to assess the radiosensitivity of TGEV-induced porcine IFN- α SC. The results in table II show that increasing doses of irradiation led to a reduction of IFN- α SC frequency, as determined by the ELISPOT assay, although the amount of IFN- α produced by each IFN- α SC (IFN- α yield) was not affected. The reduced IFN- α SC frequency may not be due to increased bulk cell death *in vitro* since the percentage of dead cells at 48 h did not vary with the irradiation doses (table II). Moreover, PHA-induced IFN- γ production was also unaltered following irradiation. These data indicate that porcine blood IFN- α SC are relatively more radiosensitive than other mononuclear cell populations such as IFN- γ -producing cells (presumably T-cells).

In subsequent experiments, the IFN- α SC frequency was studied in the blood of

Table II. Effects of *in vitro* PBMC irradiation on IFN- α and IFN- γ production.

	Irradiation dosage (rads)					
	0	50	100	200	400	800
IFN- α SC frequency (spots per 10^5 cells)	31	13.3	14.7	11.4	12	6
IFN- α yield (units per IFN- α SC)	3.3	6.2	3.5	3.4	2.4	5.4
IFN- γ yield (ng/ml)	11	10	13.8	13.5	14.8	11.1
% dead cells at 48 h	64	65	76	75	71	72

For IFN- α induction, PBMC were incubated with inactivated crude TGEV.

animals irradiated *in vivo*. Two animals were subjected to partial, non-lethal, irradiation PLI and the number of lymphocytes per millilitre blood, and the number of TGEV-induced IFN- α SC/ml blood as assessed by ELISPOT, were determined for 1 month after irradiation. Figure 1 shows that total lymphoid irradiation was followed by a dramatic and rapid reduction of circulating total lymphocyte and IFN- α SC numbers, the lowest values being obtained 2 d after the end of the irradiation procedure. Within 1 month of PLI, the lymphocytes and IFN- α SC counts had almost returned to normal values.

Three animals were irradiated either by an 8 Gy unique dose (WBI: fig 2) or by 5 successive doses of 2.5 Gy (PLI: fig 3). They were then injected with compatible bone-marrow cells. Figure 2 shows that an 8 Gy irradiation was followed by a rapid (within 2 d) and almost total (3 log reduction) disappearance of circulating IFN- α SC. Coincidentally, the circulating lymphocyte numbers also dropped, although less extensively (around 1 log reduction) than IFN- α SC. Five successive irradiation doses of 2.5 Gy each induced a similar, although less rapid, cell disappearance (fig 3). The reappearance

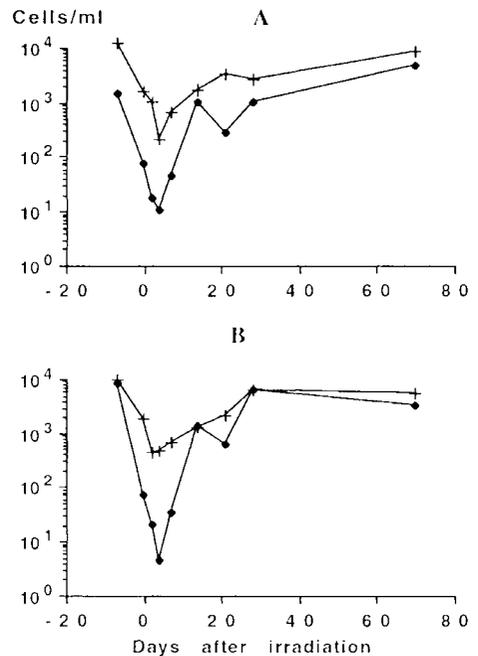


Fig 1. Effect of whole body irradiation (WBI) on IFN- α SC. Two pigs (**A**: No 39910; **B**: No 39912) were irradiated with 3 daily doses of 3 Gy each, at days -5, -2 and 0. IFN- α induction was performed by incubating PBMC with 0.6 μ g/ml inactivated purified virus. (—+— lympho ($\times 10^{-3}$); —◆— IFN- α SC).

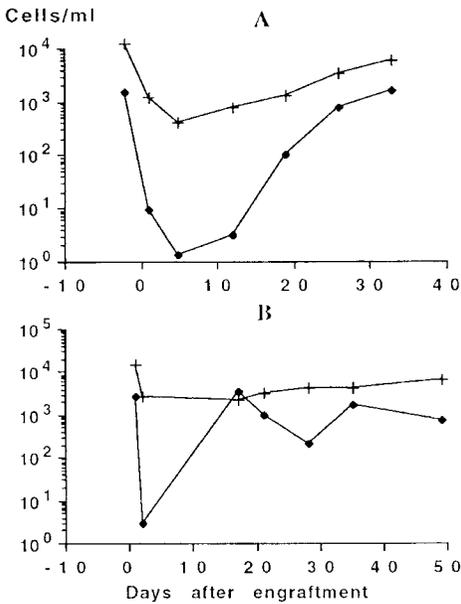


Fig 2. WBI and bone-marrow engraftment effects on IFN- α SC. In **A** one pig (No 25061) was submitted to 8.75 Gy 1 d before iv injection of bone-marrow cells. IFN- α induction was performed by incubating PBMC with 0.6 μ g/ml inactivated purified virus. In **B**, 1 pig (No 15048) was submitted to 8 Gy 2 d before iv injection of bone-marrow cells. IFN- α induction was performed by incubating PBMC with crude virus. (—+— lympho ($\times 10^{-3}$); —◆— IFN- α SC).

ance of blood IFN- α SC was observed within several days (from 5–12 d depending upon the irradiation protocol) after the bone-marrow cell injection (figs 2 and 3). Figure 2 shows that IFN- α SC counts returned to normal values within 15–25 d. In contrast, the lymphocyte counts returned to normal values after a longer period of time (fig 2). The data shown in figure 3 are incomplete due to the death of an animal from an acute respiratory infection. These data indicate that the population of circulating IFN- α SC was dramatically affected by lethal irradiation, but was reconstituted within several days following a bone-marrow transfer.

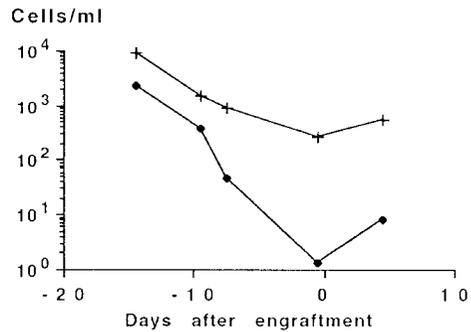


Fig 3. PLI and bone-marrow engraftment effects on IFN- α SC. One pig (No 15055) was submitted to 5 doses of 2.5 Gy each, at days -13, -10, -8, -6 and -3 before iv injection of bone-marrow cells. IFN- α induction was performed by incubating PBMC with crude virus. (—+— lympho ($\times 10^{-3}$); —◆— IFN- α SC).

IFN- α SC in bone-marrow

The presence of IFN- α SC among bone-marrow cells was determined from bone-marrow collected by surgery, in order to reduce blood contamination in the samples. In 5 independent experiments, the IFN- α SC frequency in bone-marrow mononuclear cells was 11.4 ± 2 per 10^5 cells. In comparison, the value obtained with PBMC from the same animals was 21 ± 14 per 10^5 cells. Taking into account the concentration of red blood cells in the bone-marrow cell preparations, the presence of PBMC in these bone-marrow preparations was estimated to vary from 0.06 to 0.4% total cells. These data indicated, therefore, that IFN- α SC present in porcine bone-marrow cells do not reflect blood contamination of the samples.

DISCUSSION

The present study was undertaken to evaluate the possible bone-marrow origin of TGEV-induced IFN- α SC in the porcine species. Enumeration of IFN- α SC was per-

formed with a specific ELISPOT technique. *In vitro* exposure of PBMC to increasing irradiation doses (table II) showed that IFN- α SC was relatively radiosensitive (but not some step in the mechanism for IFN- α induction by TGEV since the IFN- α yield per cell remained constant). Under similar conditions, cell viability and cell ability to secrete IFN- γ , a feature of T-cells (La Bonnardière *et al*, 1994), were not altered. In a study on herpes-simplex-virus-induced IFN- α production, Howell *et al* (1993) also found that exposure of human PBMC to irradiation doses up to 100 Gy reduced IFN- α , but relatively less than the reduction of natural killer (NK) activity. Taken together, these data provided experimental evidence that IFN- α SC differ from T-cells and from NK cells, confirming previous phenotyping studies (reviewed by Fitzgerald-Bocarsly, 1993 and Charley and Laude, 1992).

Because of the IFN- α SC radiosensitivity, it was feasible to conduct irradiation experiments *in vivo*, in order to determine the *in vivo* behaviour of circulating IFN- α SC in irradiated pigs. After a non-lethal partial irradiation (PLI), IFN- α SC frequency was greatly and rapidly reduced but, similar to the frequency of blood lymphocytes, it returned to normal values within several weeks. Because PLI was previously shown to affect circulating leukocytes but ensured the recovery of a functional haematopoietic bone-marrow (Vaiman *et al*, 1981), our data suggest that newly developing blood IFN- α SC after PLI were derived from bone-marrow progenitor cells. To address this point, pigs were irradiated (8 Gy, as previously published, Vaiman *et al*, 1975) and reconstituted with compatible bone-marrow cells. The WBI-depleting effect, due partly to bone-marrow destruction (Vaiman *et al*, 1975), and the successful reconstitution effect of bone-marrow engraftment were demonstrated by the modifications in lymphocyte counts (figs 2 and 3). Under such conditions, IFN- α SC counts were markedly

decreased following irradiation, in fact much more than were lymphocyte counts. This result is compatible with the relatively higher radiosensitivity of IFN- α SC compared with other lymphocyte subpopulations (see table II). Within several days following bone-marrow engraftment, IFN- α SC counts returned to normal values, even more rapidly than total lymphocytes (fig 2). These data strongly suggest that circulating IFN- α SC originate from bone-marrow progenitors. However, the presence of a significant proportion of IFN- α SC in pig bone-marrow cells, for which blood contamination may not be accounted, might have explained the reconstitution observed following bone-marrow *iv* injection. Such an explanation remains unlikely because the transfer of already functional IFN- α SC along with bone-marrow would have immediately reconstituted a circulating IFN- α SC population. IFN- α SC reconstitution, however, took place several days after the transfer, which suggests the production of newly differentiated cells from progenitors. Although human IFN- α SC (or NIP cells) were shown to lack stem cell-associated CD markers (Sandberg *et al*, 1991), our present data supported the hypothesis that circulating IFN- α SC were derived from haematopoietic progenitors, and therefore constituted a distinct, although atypical, leukocyte population. These results confirmed recent data from our laboratory showing that IFN- α SC were present in early haematopoietic organs of pig foetuses (Splichal *et al*, 1994). Our present study in pigs extended the results obtained in mice injected with Sindbis or Newcastle disease virus, showing that bone-marrow transplantation restored serum interferon production in lethally irradiated animals (De Maeyer *et al*, 1967). The likely haematopoietic origin of IFN- α SC implies that the production of this cell population might be regulated by haematopoietic factors. It was indeed shown that IFN- α gene expression in human NIP cells relies on the presence of Interleukin-3 and GM-CSF (Cederblad and

Alm, 1991). It will be important to determine to what extent viruses affecting lymphohaematopoietic tissues will alter the IFN- α SC population.

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