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Age-related increase of porcine natural interferon α producing cell frequency and of interferon yield per cell

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ABSTRACT

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Porcine blood mononuclear cells (PBMC) were shown to secrete interferon α (IFN-α) after induction by a coronavirus, the transmissible gastroenteritis virus (TGEV). IFN-α producing cells, referred to as natural interferon α producing (NIP) cells, were detected by an ELISPOT assay using anti-porcine IFN-α monoclonal antibodies. The frequency of NIP cells among blood cells is low, at most 40-110 per 10^5 PBMC and each NIP cell was found to produce several units of IFN. We have shown that NIP cell frequency and IFN yield per cell gradually increased with the age of the donor animals, from the neonatal period to the adult age, with a significant increase around puberty. Our present results also indicate that NIP cells may be influenced by physiological and genetic factors; thus (1) NIP cell frequency and IFN yield per cell were decreased during lactation; (2) Chinese (Meishan) pigs were found to have higher NIP cell frequency and IFN yield per cell than European (Large White) animals.

ABBREVIATIONS

FCS, fetal calf serum; HSV, herpes simplex virus; LW, Large White; MS, Chinese Meishan; NIP, natural interferon α producing; PBMC, porcine blood mononuclear cells; TGEV, transmissible gastroenteritis virus.

INTRODUCTION

Peripheral blood mononuclear cells (PBMC) from several mammalian species can be induced in vitro to produce interferon α (IFN-α) following a brief exposure to non-infectious viral structures. Thus, inactivated virus par-

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particles or glutaraldehyde-fixed virus-infected cells can induce IFN-α synthesis in leucocytes (Lebon, 1985; Capobianchi et al., 1985). In addition, several reports suggest that glycosylated viral proteins may be the essential triggers for IFN-α-induction (Lebon, 1985; Charley et al., 1991; Laude et al., 1992). Human IFN-α-producing cells in response to herpes simplex virus (HSV) are very infrequent (1 per 1000 PBMC), but highly efficient, blood mononuclear cells, producing 1–2 IFN-α units per cell (Cederblad and Alm, 1990). These particular cells have been referred to as natural interferon α producing (NIP) cells (Ronnblom et al., 1983). The NIP cells, which circulate throughout the body and are able to produce high levels of IFN without being infected by the inducing virus, are likely major components of the early non-specific antiviral defense mechanisms.

In the porcine species, PBMC can be induced to secrete IFN-α by in vitro incubation with transmissible gastroenteritis virus (TGEV)- or pseudorabies virus-infected glutaraldehyde-fixed cell monolayers (Charley and Laude, 1988; Artursson et al., 1989). TGEV-induced porcine NIP cells appear to be non-adherent, non-phagocytic, non-T, non-B cells (Charley and Lavenant, 1990), but their frequency among PBMC has not yet been determined. In the present report, we have used anti-porcine IFN-α monoclonal antibodies in order to detect and to enumerate TGEV-induced porcine NIP cells by a solid-phase enzyme-linked immunospot (ELISPOT) assay as originally described by Ronnblom et al. (1988) and Cederblad and Alm (1990) for human NIP cells.

The biology of the NIP cell population remains largely unknown: no information is, for instance, available about the origin of this circulating cell population and few studies have been undertaken so far to investigate possible in vivo variations of the frequency or functions of NIP cells. Thus it has been only recently observed that the frequency of human NIP cells was lower in AIDS patients than in healthy control individuals, each cell producing a lower amount of IFN-α (Howell et al., 1991). The frequency of NIP cells was also shown to be lower in premature infants than in term infants or than in adults (Cederblad et al., 1990). Finally, it was suggested that stress such as transportation could impair porcine NIP cell functions (Artursson et al., 1989). The present study describes an age-related increase of porcine NIP cell frequency and of IFN yield per cell, from the neonatal period to the adult age. We also describe possible physiological and genetic influences on NIP cell functions.

MATERIALS AND METHODS

Animals

European Large-White (LW) and Chinese Meishan (MS) pigs used in these experiments were obtained and maintained in INRA facilities (Jouy en Josas
and Le Magneraud). The experiment designed to evaluate age-related variations of NIP cells in LW pigs was performed with five groups of non-vaccinated animals, from 10 weeks old animals to adult sows, as indicated in the Results. The experiment designed to evaluate age-related variations of NIP cells in MS pigs was performed with four litters (19 females and five males) and 11 adult sows. Blood samples for IFN induction were collected from each animal every 3 months over a total period of 9 months. MS pigs were vaccinated twice against erysipela and parvovirus, at the age of 22 and 25 weeks.

Production of 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 and titration of infectivity in the swine testis ST cell line have been reported previously (Charley and Laude, 1988).

PBMC

Porcine peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood by Ficoll density centrifugation on MSL (density 1.077, Eurobio, Paris). PBMC were suspended in RPMI-1640 medium supplemented with 10% foetal calf serum and antibiotics. PBMC were then used directly in IFN induction or depleted of plastic-adherent cells as follows: PBMC were resuspended in RPMI 1640 medium supplemented with 20% foetal calf serum (FCS) at a concentration of 2–5×10^6 cells ml^{-1} and incubated for 90 min at 37°C in tissue culture flasks. ‘Plastic non-adherent cells’ were then collected by shaking the flasks.

IFN-α induction

PBMC were induced to produce IFN-α by incubation with TGEV in 96-well microplates: PBMC or plastic non-adherent cells (as stated in the Results) were incubated in duplicates at final concentrations of 4×10^6, 2×10^6 and 1×10^6 cells ml^{-1} in a total volume of 200 μl of RPMI 1640 plus 10% FCS containing 2×10^4 plaque forming units of TGEV. After 8 h at 37°C, the induced PBMCs were resuspended and 100 μl from each well were transferred to nitrocellulose-bottomed microplates for the ELISPOT assay (see below). The other 100 μl of induced PBMCs were further incubated overnight at 37°C for IFN bioassay.
ELISPOT (solid phase enzyme-linked immunospot) assay

The ELISPOT assay was performed as described by Cederblad and Alm (1990): nitrocellulose-bottomed 96-well filtration plates (ref. MAHAN 4550 from Millipore, Guyancourt, France) were coated with anti-porcine IFN-α monoclonal antibodies (MoAb 'C21' or 'K9' as stated in the Results) described by Lefèvre et al. (1990) and L'Haridon et al. (1991). The MoAbs were gel-filtration purified from ascitic fluid and used at a final concentration of 1.6 µg ml⁻¹ and 0.8 µg ml⁻¹ respectively, in PBS. The plates were then fixed with 0.05% glutaraldehyde and blocked with 3% glycine in PBS. TGEV-induced PBMCs were added to the wells and incubated overnight at 37°C. Following extensive washing, the plates were then incubated with peroxidase-conjugated anti-porcine IFN-α MoAb 'F17' (unless otherwise stated) at a final concentration of 2 µg ml⁻¹ for 1 h at 37°C in 5% BSA-0.05% Tween PBS. After washing, each well received 0.1 ml substrate solution consisting of diaminobenzidine with perhydrol. The plates were finally dried and spots were counted under a microscope. The frequency of NIP cells was calculated as the mean number of spots divided by the total PBMC number in each well.

IFN bioassay

Serial log₂ dilutions of supernatants from induced PBMC were assayed for IFN on bovine MDBK cells using vesicular stomatitis virus as a challenge (La Bonnardière and Laude, 1981). Our internal standard porcine IFN-α was included in each assay. This standard was calibrated on MDBK cells with the human international reference IFN B69/19 (NIH, Bethesda, MD). The estimated amount of IFN produced by each NIP cell was calculated from the titre of IFN (units) in induction culture supernatants and the NIP cell numbers per culture.

RESULTS

ELISPOT assay to characterize porcine NIP cells

Different anti-porcine IFN-α monoclonal antibody (MoAbs) were tested to set up a sensitive ELISPOT assay able to detect TGEV-induced NIP cells. The following MoAbs were assayed on solid phase: C21, F17, F20, M7, E23 or K9 (Lefèvre et al., 1990; L'Haridon et al., 1991). The Abs were combined with three different peroxidase-conjugated Abs: F17, K9 or M7. Four combinations of two Abs were found to allow the detection of porcine NIP cells as brown spots on nitrocellulose: either C21-F17 (1st and 2nd Ab) or K9-F17 or F17-K9 or C21-M7. Among those, the best results were obtained with K9 or C21 MoAb on solid phase with F17 as peroxidase-conjugate, K9-F17 giv-
ing the most pronounced and visible spots. When PBMC were TGEV-induced as described in Materials and Methods, and transferred onto nitrocellulose-bottomed plates at different times (from 1 h to 26 h) after induction, the number of positive spots peaked at 8 h and then declined (not shown). In all successive experiments, PBMC were therefore transferred for ELISPOT assay after 8 h of TGEV-induction. Under such conditions porcine NIP cells appear to be infrequent cells among PBMC since the mean frequency of NIP cells per 10^5 PBMC obtained from 29 Meishan (MS) pigs was 71 ± 30 as determined with K9-F17 MoAbs and 63.7 ± 29.8 when C21-F17 Abs were used.

**NIP cell frequency and IFN yield in animals of different ages**

Table 1 shows that the frequencies of NIP cells were lower in PBMC collected from young Large-White (LW) animals (10 weeks old) than in PBMC obtained from adult sows (P< 0.01, by Student’s t-test). From the number of NIP cells per culture, as determined by the ELISPOT assay, and the amount of IFN present in culture supernatant, as determined by the antiviral bioassay, it is possible to estimate the amount of IFN produced by each NIP cell. Results in Table 1 show that the IFN yield (units per NIP cell) was also lower

| PBMC source          | Frequency (NIP cells per 10^5) | IFN yield (units per NIP cell) |
|----------------------|-------------------------------|-------------------------------|
|                      | PBMC^1                         | non-adherent cells^2          | PBMC                        | non-adherent cells |
| **Young animals**    |                               |                               |                             |                   |
| (n=5; 10 weeks old)  | 7.7±5.9                        | 15.6±8.2*                     | 1.5±1.1                     | 1.9±0.8           |
| (n=10; 14 weeks old) | 4.2±2.4                        | 8.3±4*                        | 1.3±0.8                     | 1.1±0.6           |
| (n=17; 16 weeks old) | 6.3±3.6                        | 8.5±3.4                       | 0.6±0.2                     | 0.7±0.4           |
| (n=17; 20 weeks old) | 15.5±5.9                       | 16.9±5.3                      | 1.8±0.9                     | 2.1±1.3           |
| **Adult sows**       |                               |                               |                             |                   |
| (n=10)               | 19.1±5.8                       | 37.8±13.5**                   | 5.5±3                       | 6.1±2.9           |

^1PBMC or plastic non-adherent cells (2×10^6 m^-1) from LW pigs were induced for 8 h with TGEV. The NIP cell frequency was subsequently determined by the ELISPOT assay, using C21 MoAb on solid phase and F17 MoAb as peroxidase-conjugate. The amount of IFN (units) per NIP cell was calculated from the amount of IFN in culture supernatants at 24 h and the NIP cell number per culture.

^2Significance levels as determined by the Student t test: non-adherent cells versus PBMC, *P<0.05; **P<0.01.
in young animals than in sow PBMC cultures (significantly different values obtained on 10 weeks old animals and on adult sows: \( P < 0.01 \)). In addition, the NIP cell frequency was lower among PBMC as compared with plastic non-adherent cells (Table 1).

Age-related variations in IFN-\( \alpha \) production were also studied on MS pigs from the age of 3 weeks after birth until the age of 40 weeks, and on adult MS sows. Figure 1 shows that the frequencies of NIP cells were rather constant during several weeks after birth and then gradually increased to the age of 40 weeks, at which the frequency was about the same as the one observed in adult sows in gestation (110 ± 66 NIP cells per \( 10^5 \) PBMC). The NIP cell frequency was significantly higher (\( P < 0.01 \), by Student's \( t \) test) after 20 weeks of age than at 3 weeks of age. A peak of NIP cell frequency was observed at Weeks 23 and 26 of age, the last value (155 ± 91) being significantly higher (\( P < 0.05 \)) than the mean frequencies observed at Week 20 (37 ± 10) and Week 31 of age (91 ± 40). This latter peak could be partly explained by the fact that the animals were vaccinated against erysipelas and parvovirus at the age of 22 and 25 weeks. Indeed, we have observed in a preliminary experiment a low but significant transient increase of NIP cell frequency only 3 weeks after injection of anti-erysipela and anti-parvovirus combined vaccines (data not shown).

The capacity of each NIP cell to secrete IFN was also found to vary with the age of the animals: Fig. 2 shows that the IFN yield per cell increased about

![Fig. 1. Frequency of TGEV-induced porcine NIP cells per \( 10^5 \) PBMC obtained from MS pigs of different age (from 3 to 40 weeks) and from MS sows either during gestation (indicated as ‘G’) or lactation (indicated as ‘L’). NIP cells were characterized by an ELISPOT assay in which C21 and F17 MoAbs were used.](image)
NATURAL INTERFERON α PRODUCING CELLS

Fig. 2. IFN yield (units per NIP cell) in TGEV-induced PBMC cultures obtained from 3- to 40-weeks old MS pigs and from MS sows (see legend for Fig. 1).

six times from the age of 3 weeks to the age of 40 weeks, at which the obtained value was identical to the one determined for adult sows in gestation. The IFN yield per cell was significantly \((P<0.05)\) higher after 17 weeks of age as compared with the yield at 3 weeks.

We have also observed that both the NIP cell frequency and the IFN yield per cell were lower \((P<0.02)\) in lactating sows than in pregnant sows (Figs. 1 and 2).

Comparison of NIP cells in European LW and Chinese MS pigs

We have presently observed that MS pigs, older than 16 weeks of age, have a higher proportion of NIP cells in blood mononuclear cells (Figs. 1 and 2), each NIP cell producing higher amounts of IFN, than LW pigs (Table 1). Thus, the NIP cell frequency was 80.9 \(\pm\) 45.1 per 10\(^3\) PBMC in adult MS sows \((n=11,\) from data in Fig. 1) and 19.1 \(\pm\) 5.8 in adult LW sows \((n=10,\) Table 1, \(P<0.01\) by \(t\) test). The mean IFN yield per cell was 16.2 \(\pm\) 7.4 for adult MS pigs \((n=11,\) from data in Fig. 2) and 5.5 \(\pm\) 3 for adult LW animals \((n=10,\) Table 1, \(P<0.001)\). Similar differences were observed between MS and LW animals at 16 or 20 weeks of age: thus, NIP cell frequencies were 16.2 \(\pm\) 8.7 and 6.3 \(\pm\) 3.6 \((P<0.05)\) and IFN yields were 6.4 \(\pm\) 3.1 and 0.6 \(\pm\) 0.2 \((P<0.001)\) for 16 week-old MS and LW pigs, respectively (Figs. 1 and 2, Table 1).

DISCUSSION

In the present report, porcine PBMC were induced by the porcine coronavirus TGEV and NIP cells were subsequently detected by an ELISPOT assay
with specific anti-pig IFN-α MoAbs. Our results indicate that the frequency of TGEV-induced NIP cells is very low among blood mononuclear cells: at most 20–40 per 10⁵ cells from European LW adult pigs (Table 1) and 100–110 per 10⁵ cells from Chinese MS adult animals (Fig. 1). In the human species, the frequency of HSV-induced NIP cells, as assessed by ELISPOT assay, by in situ hybridization with IFN-α specific probes or by immunochemistry with specific antibodies, was also shown to be low: 2–55 per 10⁴ PBMC (Cederblad and Alm, 1990; Feldman and Fitzgerald-Bocarsly, 1990). The nature of this infrequent cell population remains partially unknown: thus, human NIP cells were shown to lack specific cell surface markers for B-, T-, NK- cells or monocytes but to express low levels of the CD4 molecule (Sandberg et al., 1989, 1990; Feldman and Fitzgerald-Bocarsly, 1990). TGEV-induced porcine NIP cells were also described as non-T non-B cells expressing the CD4 surface marker (Charley and Lavenant, 1990). It is likely therefore that a similar infrequent blood cell population is responsible for IFN-α production in several animal species following induction by different RNA or DNA viruses. We also show in Table 1 that porcine NIP cells are plastic non-adherent cells. Furthermore, removal of plastic-adherent mononuclear cells increased markedly the NIP cell frequency, which suggest that adherent PBMC could exert inhibitory effects on NIP cells. In addition, our present data indicate that each individual TGEV-induced porcine NIP cell was able to secrete several units of IFN (Table 1 and Fig. 2), as shown for HSV-induced human NIP cells by Cederblad and Alm (1990).

We have observed that the IFN-α response of PBMC from adults was markedly higher compared with that of PBMC from young animals, both in terms of NIP cell frequency and of amount of IFN produced per cell (Table 1, Figs. 1 and 2). Moreover, we have described age-related gradual increases for both parameters, with a significant enhancement of IFN yield and NIP cell frequency beginning between 17 and 20 weeks of age for MS and LW pigs. Since LW pigs were not vaccinated and MS animals received the initial vaccine injection at the age of 22 weeks, the gradual increases observed cannot be explained by possible effects of vaccination. Interestingly, it is known that sexual maturity takes place at 3 months in MS pigs (Legault and Caritez, 1983). Our present data therefore suggest possible influences of sexual maturation on IFN-α production, especially around puberty. Age-dependent modifications of NIP cell numbers have been described in the human species: Cederblad et al. (1990) demonstrated that the frequency of HSV-induced NIP cells was very low in premature infants and significantly higher in term infants and in adults. Several other immune functions are known to vary with the age: thus, in the porcine species, the immune system is immature at birth and will acquire fully effective functions within several weeks after birth (reviewed in Blecha and Charley, 1990). It is possible to speculate that a lower number of circulating NIP cells, as evidenced in this study, could contribute
to the higher susceptibility of young animals to viral infections. However, in the case of the TGEV experimental infection, young pigs were shown to be able to produce high amounts of IFN-α in vivo (La Bonnardiére and Laude, 1981).

Our data reveal, in addition, marked differences in IFN-α production by PBMC from sows either during gestation or lactation (Figs. 1 and 2): NIP cell frequency and IFN yield per cell were decreased during lactation. Pregnancy and lactation are known to have an effect on several immune parameters (Lloyd, 1983). The mechanisms behind decreases in immune functions during lactation are not known. However, hormonal and physiological changes that occur at parturition and during lactation are factors to be considered in changes of NIP cell number and functions.

Our data also clearly demonstrate that TGEV-induced IFN-α production in vitro was higher in PBMC cultures from MS pigs than from LW animals, both in terms of NIP cell frequency and IFN yield per cell. Such breed differences may reflect host genotype influences on IFN-α production as already described in the mouse (De Maeyer and De Maeyer-Guignard, 1988). In addition, it would obviously be interesting to know if MS pigs are more resistant to viral infections than LW animals.

Although the nature of virus-induced NIP cells is still largely unknown, our present data indicate that several factors, such as age, physiological status or host genotype may influence this cell population. Further studies on the modulation of NIP cells in vivo should increase our knowledge on the biology and the significance of this possibly unique cell entity in the blood circulation.

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