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Ontogeny of interferon alpha secreting cells in the porcine fetal hematopoietic organs

Igor Šplíchal a,c, Michel Bonnneau b and Bernard Charley a,*

a Laboratoire de Virologie et d’Immunologie Moléculaires and b Centre de Recherche en Imagerie Interventionnelle, INRA, 78350 Jouy-en-Josas, France; c Institute of Microbiology, Department of Immunology and Gnotobiology, 54922 Nový Hrádek, Czech Republic

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1. Summary

We examined the ontogeny of IFN-α Secreting Cells (IFN-α SC) in different hematopoietic organs and blood of porcine fetuses at different stages of gestation. Cells were induced to produce IFN-α by incubation with the coronavirus TGEV and IFN-α SC were detected by ELISPOT. A striking finding was that IFN-α SC could be detected in the fetal liver as early as at 26 days of gestation, i.e., during the first quarter of gestation, a period at which T-cell markers could not be detected by flow cytometry. In addition, IFN-α SC could be detected in the cord blood, the spleen and the bone marrow of fetuses at later stages of gestation. These data indicate that IFN-α SC appear very early during the ontogeny of the immune system, long before the development of the specific immune system, and may therefore represent an early antiviral defence mechanism. IFN-α SC were found to be associated with hematopoietic organs, which argues for their hematopoietic lineage.

2. Introduction

Host defences against viral infections involve several mechanisms which include both the non-specific (or innate) and the specific immune systems. Interferons (IFN) are secreted proteins which represent one of the earliest non-specific host responses to viruses and can exert antiviral effects contributing to limit infection [1]. IFNs constitute a family of potent multifunctional cytokines among which IFN-alpha (IFN-α) are primarily produced by leukocytes following viral induction. Several leukocyte populations are able to secrete IFN-α depending on the virus used, and on whether the virus is infectious or not. Thus, whereas monocytes are most often associated with production of IFN-α in response to infectious viruses (such as Sendai virus) [2], a distinct population of non-phagocytic, non-adherent leukocytes is able to secrete IFN-α following brief exposure to non-infectious viral structures, such as inactivated virions or glutaraldehyde-fixed virus-infected cells [3,4,5]. These cells, which have tentatively been termed “natural interferon producing cells” (NIPC), were shown to be very infrequent among blood mononuclear cells and to exhibit unusual phenotypic features. Thus, in several animal species including man, NIPC were found to be negative for T- or B-cell markers, but positive for MHC class II antigens and for CD4 (reviewed in [5]). The question of the relationship of NIPC to dendritic cells was examined and discussed in several papers (reviewed in [6]), but remains a matter of controversy. Several reports, including our work, have suggested that viral glycoproteins might be essential in triggering IFN-α synthesis in NIPC [7–10]. Still it remains that little information is available regarding the biology of NIPC, their distribution, their origin, and their ontogeny during embryonic development.

The aim of the present investigation was to study the ontogeny of IFN-α producing cells in different hematopoietic organs of porcine fetuses. The main advantages of using the porcine species for studying the ontogeny of immune functions are: (1) a long term gestation (114 days), (2) multiparity and (3) the relatively large size of fetuses which allows easy and
quantitative sampling of cells from organs. In addition, the epitheliochorial type of placentation, which prevents transfer of immunoglobulins or antigens from the mother, and therefore avoids any immune activation of the fetus, qualifies the pig as a good model for developmental immunology [11,12].

We have previously shown that it was possible to characterize porcine IFN-α secreting cells (IFN-α SC) in adult blood leukocytes, in response to the coronavirus transmissible gastroenteritis virus (TGEV), by a solid phase enzyme-linked immunospot assay (ELISPOT) [13]. Porcine IFN-α SC were found to be positive for swine leukocyte antigen (SLA) class II, for CD4, but negative for CD2 and B-cell markers [13]. In addition, porcine IFN-α SC are non-adherent cells since their frequency was found to increase following depletion of adherent cells [13].

In the present report, IFN-α SC were characterized by ELISPOT in different organs of fetuses at different stages of gestation, and the nature of the mononuclear cells assayed was analyzed by flow cytometry.

3. Materials and Methods

3.1. Animals

Pregnant sows used in our experiments were housed in INRA animal facilities in Jouy-en-Josas. Five sows were halothane anaesthetized at the 26th, 34th, 44th, 63rd and 77th day of gestation. Hysterotomy was performed and fetuses (from 6 to 12 per sow, as indicated in the Results) were bled from umbilical cord vessels. After section of the umbilical cord, fetuses were removed aseptically from the uterus and kept on ice until removing the organs. Piglets at birth were obtained immediately after delivery, anaesthetized by halothane and sacrificed by cardiac puncture before organ removal.

3.2. Cell suspensions

Heparinized blood samples were diluted 1:4 in Minimum Essential Medium (MEM), then centrifuged in Ficoll density gradient (density 1.077, Eurobio, Paris). Mononuclear cells collected from the interface were washed twice, and the remaining erythrocytes were lysed by distilled water on ice for 20 sec followed by addition of PBS at ten times its usual concentration. Cells were washed twice and resuspended in RPMI 1640 with 10% FCS and antibodies. Cells from liver and spleen of younger fetuses were obtained by repeatedly aspirating these organs in MEME through needles with different diameters. For older fetuses, liver and spleen were minced in a tissue homogenizer. Cell suspensions were allowed to sediment for 10 min twice and cells in the supernatants were collected to remove debris of connective tissues. Cells were washed twice in MEME, erythrocytes were lysed as previously, and cell suspensions were prepared in RPMI 1640 medium.

The sternum and two femurs from each fetus were cut with sharp scissors into small pieces and bone marrow cells were obtained by homogenization as for other organs.

Cells in RPMI 1640 medium with 20% FCS and antibiotics were incubated for 90 min at 37°C at a concentration of 2–6 × 10^6/ml. Non-adherent cells were removed by shaking culture flasks. Cells were kept in the same medium overnight at 4°C, then resuspended in fresh RPMI 1640 medium with 10% FCS and antibiotics before enumeration of living nucleated cells by Trypan blue dye exclusion.

3.3. 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 [9].

3.4. IFN-α induction

Cells prepared from fetal hematopoietic organs were induced to produce IFN-α by incubation with TGEV in 96-well microplates: non-adherent cells were incubated at final concentrations of 1 to 10 × 10^6 cells per ml in a total volume of 2000 μl of RPMI 1640 plus 10% FCS, with 5 × 10^4 plaque forming units of TGEV. After 8 h at 37°C, the induced cells 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 cells were further incubated overnight at 37°C for IFN-α immunoassay.

3.5. ELISPOT (solid phase enzyme-linked immunospot) assay

The ELISPOT assay was performed as described previously [13]: nitrocellulose-bottomed 96-well filtration plates were coated with anti-porcine IFN-α monoclonal antibodies (mAb) "K9" [13,14]. The plates were then fixed with 0.05% glutaraldehyde and blocked with 3% glycine in PBS. TGEV-induced cells were added to the wells and incubated overnight at 37°C. Following extensive washing, the plates were then incubated with peroxydase-conjugated anti-porcine IFN-α mAb "F17" 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 macroscope. 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, with standard errors.

3.6. IFN-α immunoassay [15]

ELISA microtitration plates were coated overnight with anti-porcine IFN-α mAb K9 in 0.1 M bicarbonate buffer (pH 9.6); non-specific binding was then blocked by 0.5% BSA and 0.05% Tween in PBS. Serial log3 dilutions of cell culture supernatants were incubated at 37°C for 5 h, then peroxidase-conjugated F17 mAb was added for an overnight incubation at room temperature. After washing, the TMB substrate solution (Kirkegaard and Perry, Gaithersburg, MD, USA) was added for 15 min before the reaction was stopped by addition of 1 M H₃PO₄.

Absorbances were measured at 450 nm. In each assay, our internal standard of recombinant porcine IFN-γ [14] was included. This standard was calibrated on MDBK cells with the human international reference IFN B69/19 (NIH, Bethesda, MD, USA). 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 the IFN-α SC number per culture, as determined by ELISPOT.

3.7. Flow cytometry analysis

The following mouse monoclonal antibodies directed against pig leukocyte cell surface markers were used as hybridoma cell supernatants: MSA4 (anti-CD2, Ig2a), 10.2H2 (anti-CD4, IgG2b), K252.1E4 (anti-CD45, IgG1), 74-22-15 (anti-monocyte/granulocyte, IgG2b), MSA3 (anti-SLA classII DR, IgG2a), as reviewed in [16]. As a conjugate, polyclonal goat anti-mouse IgG F(ab’) FITC-labelled antibodies (Silenus, Eurobio, France) were used.

Total blood, or cells prepared as described, were kept overnight at 4°C. After erythrocytes lysis by NH₄Cl buffer with EDTA, cells were washed in PBS and centrifuged in Ficoll. After two washes in cold PBS, cell concentrations were adjusted to 5–10 x 10⁶ per ml. At all stages of gestation and for each organ studied, two independent pools of cells were analysed by flow cytometry. 50 μl aliquots of cell suspensions were distributed into 96-well U bottom plates, to which 100 μl of cold PBS with 5% inactivated ovine serum (IOS) were added. Plates were centrifuged 5 min at 300 g at 4°C. Supernatants were flicked out and cells were resuspended before addition of optimally diluted hybridoma supernatants in 50 μl of cold PBS with 5% IOS and 0.1% NaN₃. After 45 min on ice with gentle agitation cells were washed four times, then incubated with FITC-labeled goat anti-mouse Ig conjugate, diluted 1:750. After washes, cells were fixed with 0.5% paraformaldehyde. Samples were kept in a cold and dark room until analysis on FACSscan (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). A total number of 15 000–50 000 events were counted. Fetal cells were electronically gated in a similar way as adult porcine blood mononuclear cells, to exclude contaminating cell debris. A CD45 positive area on FSC and SSC dot plot was gated, and among CD45 positive cells, percentages of fluorescence positive cells with different leukocyte surface markers were evaluated by using the software Lysys II, version 1.1 from Becton Dickinson. As internal positive controls for mAb staining of fetal cells, adult porcine blood leucocytes were used and analysed in each experiment. Results were expressed as means of two cell samples.

4. Results

4.1. Detection of IFN-α secreting cells in fetal hematopoietic organs by ELISPOT

Cells obtained by Ficoll gradient from fetal and newborn piglet blood or by mincing of hematopoietic organs (liver, spleen and bone marrow) were depleted of plastic-adherent cells before being induced to produce IFN-α by incubation with TGEV.

At early stages of gestation, it was not possible to obtain enough cells from each organ studied to allow them to be used. Thus, liver cell preparations from twelve fetuses at 26 days of gestation had to be pooled into 3 samples in order to obtain a sufficient number of cells to be assayed. Accordingly, blood cells from several 34- or 44-day old fetuses were also pooled before being tested. From 63 days of gestation until birth, individual cell preparations from blood, liver, spleen and bone marrow were assayed (Tables 1-4).

In fetal blood cell preparations, no IFN-α SC, detectable by spots, could be observed in the pooled samples from 34-day old fetuses (Table 1), and very low IFN-α titers (4 units per 10⁵ cells) were detected. On the contrary, IFN-α SC were detectable at 44 days of gestation (Table 1), then the IFN-α SC frequency increased, except at birth, when no spots were obtained although the number of living cells assayed was higher than for fetuses.

From the fetal liver, a very striking finding was that IFN-α SC were detected as early as 26 days of gestation (Table 2), then in the following samples, with again
TABLE 1
IFN-α SECRETING CELL FREQUENCY AND IFN-α YIELD PER CELL, IN FETAL BLOOD CELLS OBTAINED AT DIFFERENT STAGES OF GESTATION

Results expressed as means, with standard errors in brackets.

| Day of gestation | IFN-α SC frequency | IFN yield |
|------------------|--------------------|-----------|
| (number of samples) | (spots per 10^5 cells) | (units per IFN-α SC) |
| 34th (2) * | 0 | 0 |
| 44th (4) b | 7.6 (8.7) | 0.5 (0.5) |
| 63rd (6) c | 51.6 (28.8) | 0.7 (0.3) |
| 77th (7) c | 16.7 (7.8) | 2.4 (0.7) |
| newborn (5) c | 0 | 0 |

* 2 pooled cell suspensions from 12 fetuses.
b 4 pooled cell suspensions from 8 fetuses.
c samples from individual fetuses.

TABLE 2
IFN-α SC FREQUENCY AND IFN-α YIELD PER CELL IN FETAL LIVER CELLS

Results expressed as means, with standard errors in brackets.

| Day of gestation | IFN-α SC frequency | IFN yield |
|------------------|--------------------|-----------|
| (number of samples) | (spots per 10^5 cells) | (units per IFN-α SC) |
| 26th (3) a | 6.6 (4.5) | 1.1 (0.5) |
| 34th (7) b | 7.2 (4.9) | 0.9 (0.6) |
| 44th (8) b | 17.5 (8.7) | 1.1 (0.7) |
| 63rd (6) b | 171.5 (45.1) | 1.3 (0.3) |
| 77th (7) b | 41.5 (16.3) | 5.3 (4.5) |
| newborn (5) b | 0 | 0 |

* 3 pooled cell suspensions from 12 fetuses.
b samples from individual fetuses.

TABLE 3
IFN-α SC FREQUENCY AND IFN-α YIELD PER CELL IN FETAL SPLEEN CELLS

Results expressed as means, with standard errors in brackets.

| Day of gestation | IFN-α SC frequency | IFN yield |
|------------------|--------------------|-----------|
| (number of samples) | (spots per 10^5 cells) | (units per IFN-α SC) |
| 63rd (6) a | 123.9 (57.9) | 1.7 (0.6) |
| 77th (7) a | 36.6 (25.9) | 1.8 (0.4) |
| newborn (5) a | 17.9 (10.1) | 1.7 (0.9) |

* samples from individual fetuses.

TABLE 4
IFN-α SC FREQUENCY AND IFN-α YIELD PER CELL IN FETAL BONE-MARROW CELLS

Results expressed as means, with standard errors in brackets.

| Day of gestation | IFN-α SC frequency | IFN yield |
|------------------|--------------------|-----------|
| (number of samples) | (spots per 10^5 cells) | (units per IFN-α SC) |
| 63rd (6) a | 141.9 (98.7) | 1.8 (0.4) |
| 77th (7) a | 37.8 (10.9) | 3.6 (0.7) |
| newborn (5) a | 22.6 (12.2) | 3.1 (1.4) |

* samples from individual fetuses.

TABLE 5
FLOW CYTOMETRY ANALYSIS OF FETAL AND NEWBORN BLOOD CELL PREPARATIONS. (% POSITIVE CELLS WITH DIFFERENT LEUKOCYTE CELL SURFACE MARKERS)

Results are expressed as mean percentages from 2 samples.

| Day of gestation | CD45 Mono/ granulo | CD2 | CD4 | SLA class II |
|------------------|--------------------|-----|-----|-----------|
| 34th | 3 | – | – | 0 | 0 |
| 44th | 4 | 0 | 0 | 0 | 1 |
| 63rd | 7 | 1 | – | 0 | 3 |
| 77th | 8 | 2 | 2 | 3 | 5 |
| newborn | 16 | 2 | 11 | 9 | 3 |

– = not tested.
a results expressed as % of CD45 + cells.

the exception of birth when no spots, nor IFN-α in supernatants, were obtained.

Spleen cells could only be assayed at 63 days of gestation: the results in Table 3 show the presence of IFN-α SC in fetal spleen, with a decrease of their frequency at birth. A similar situation was obtained for bone marrow cells (Table 4).

4.2. IFN-α yield per IFN-α secreting cell

IFN-α yield per IFN-α SC was estimated from IFN-α titers and numbers of spots, as described in Materials and Methods. As shown in Tables 1 to 4, the IFN-α yield per cell did not change markedly during gestation in the different organs studied (around 1 to 1.5 unit per IFN-α SC) with the exception of blood and liver cells at birth for which no IFN-α or spot was detected.

4.3. Flow cytometry analysis

Flow cytometry analysis of fetal cells prepared in this study was performed with anti-porcine leukocyte

TABLE 6
FLOW CYTOMETRY ANALYSIS OF FETAL AND NEWBORN LIVER CELL PREPARATIONS. (% POSITIVE CELLS WITH DIFFERENT LEUKOCYTE CELL SURFACE MARKERS)

Results are expressed as mean percentages from 2 samples.

| Day of gestation | CD45 Mono/ granulo | CD2 | CD4 | SLA class II |
|------------------|--------------------|-----|-----|-----------|
| 26th | 32 | 28 | – | 0 | 4 |
| 34th | 24 | 19 | 1 | 0 | 1 |
| 44rd | 39 | 18 | 3 | 0 | 4 |
| 63rd | 58 | 35 | 3 | 4 | 11 |
| 77th | 48 | 29 | 9 | 7 | 10 |
| newborn | 74 | 44 | 28 | 17 | 10 |

– = not tested.
a results expressed as % of CD45 + cells.
TABLE 7
FLOW CYTOMETRY ANALYSIS OF FETAL AND NEWBORN SPLEEN CELL PREPARATIONS. (% POSITIVE CELLS WITH DIFFERENT LEUKOCYTE CELL SURFACE MARKERS)

| Day of gestation | CD45 | Mono/ granulo | CD2 a | CD4 a | SLA class II a |
|------------------|------|---------------|-------|-------|---------------|
| 63rd             | 57   | 12            | 2     | 29    |               |
| 77th             | 71   | 18            | 33    | 17    | 16            |
| newborn          | 96   | 13            | 70    | 40    | 12            |

Footnotes are the same as in Table 5.

TABLE 8
FLOW CYTOMETRY ANALYSIS OF FETAL AND NEWBORN BONE MARROW CELL PREPARATIONS. (% POSITIVE CELLS WITH DIFFERENT LEUKOCYTE CELL SURFACE MARKERS)

| Day of gestation | CD45 | Mono/ granulo | CD2 a | CD4 a | SLA class II a |
|------------------|------|---------------|-------|-------|---------------|
| 63rd             | 54   | 19            | 3     | 30    |               |
| 77th             | 76   | 27            | 25    | 4     | 40            |
| newborn          | 79   | 30            | 18    | 7     | 34            |

Footnotes are the same as in Table 5.

surface marker mAb. Cells expressing CD45, the pan leukocyte marker, were detected in all the preparations studied, whatever their organ of origin, and at all stages of gestation (Tables 5–8), including at very early periods such as 26 days of gestation in the liver (Table 6). This result shows that leukocytes were present in all the cell preparations from which IFN-α production was assayed. The percentage of CD45 positive cells increased during gestation, as shown for instance for fetal liver cells in Fig. 1. Among CD45 positive cells, monocytes or granulocytes, as detectable by 74-22-15 mAb, were present in all cell preparations (which were not depleted of adherent cells), at low percentages in the blood but at higher levels in the other organs. CD2 positive cells could be detected at low frequency in liver cells from 34 days of gestation (Table 6), and later on in blood, spleen and bone marrow (Tables 5, 7–8). No positive cells for CD4 could be detected in any organ before the 63rd day of gestation. SLA class II (DR) positive cells were present as early as 26 days of gestation in the fetal liver, but not before 34 days in the blood and at 63 days in spleen and bone marrow (Tables 5–8).

5. Discussion

In the present report, the ELISPOT assay was used for detecting IFN-α SC in different hematopoietic organs of porcine fetuses. The main result was that IFN-α SC could be detected at very early stages of gestation, in fetal hematopoietic organs, such as fetal liver at 26 days of gestation, a period when CD45 and SLA (MHC) class II positive cells were already present but long before the appearance of differentiated lymphocytes.

The early presence of IFN-α SC during the first quarter of gestation is an unexpected finding. Few reports described the ontogeny of IFN-α production: leukocytes prepared from the cord blood of human fetuses at 1/3 of gestation were found to produce IFN following incubation with Sendai or rubella viruses [17], but the nature of the IFN was unknown. More recently, IFN-α SC were demonstrated by ELISPOT in the cord blood of preterm infants born between the 25th and 40th week of gestation [18], but our current study may be the first one which followed the development of IFN-α SC in several organs at all stages of gestation. Our results indicate therefore that IFN-α production appeared during the development of immune functions long before specific immune responses since pig fetuses were shown to be able to mount an antigen specific immune response only during the second half of gestation (around 50 days) [11]. In addition, the presence of IFN-α SC during fetal development may indicate that

Fig. 1. Flow cytometry analysis of fetal (26 and 63 days of gestation) and newborn liver cells incubated with anti-CD 45 mAb. Cells incubated only with goat anti-mouse Ig conjugate are shown as controls.
non-specific defenses are potentially able to protect the fetus from mother-derived viral infections.

In the present report, IFN-α SC were found primarily in the fetal liver, then in the fetal blood, spleen and bone-marrow. We know that the pig fetal liver is an hematopoietic organ from about 20 days of gestation when erythroid cells are present. Cells from the myeloid lineage appear at 25 days whereas lymphoid cells are found at 28 days of gestation [19]. Although the results from Sandberg et al. [20] have shown that human IFN-α SC did not express surface antigens of the myeloid and lymphoid lines of differentiation, our present data showing that IFN-α SC are present in hematopoietic organs are an argument in favor that they belong to an hematopoietic lineage.

IFN-α SC were detected at the end of gestation in all the organs tested; however, IFN-α SC were much less frequent or even not detectable at birth. This observation might be related to a cortisol mediated inhibition of IFN-α production since cortisol is present at high concentrations in the blood of piglets for a few hours after birth [21]. Accordingly, IFN-α SC were again normally detectable in newborn animals a few days after birth [22 and Splichal et al., unpublished data]. From the neonatal period to the adult age, the IFN-α SC and the IFN-α yield increased from 15 to 38 IFN-α SC per 10^5 cells, and from 2 to 6 units per cell, respectively [22].

Because IFN-α SC from adults were shown to express CD4 and MHC class II molecules [2,13], the flow cytometry analysis of the cells used in the present study was mainly focused on these two markers. Leukocytes, including monocytes and granulocytes, were found to be present, as judged by reactivity with relevant mAb (CD45 and 74-22-15, respectively), at 26 days of gestation in the liver and later on in the other organs tested, which confirmed previous observations [19,23]. The analysis of T-cell markers (CD2 and CD4) confirmed previous data [19,23] and showed that fetal IFN-α SC were present long before the onset of differentiated T-cell markers. In addition, these data indicate that fetal IFN-α SC, as opposed to adult IFN-α SC, may be negative for CD4 expression. Therefore, we suggest that CD4 surface expression is not a constant phenotypic characteristic of IFN-α SC, but may depend on their developmental stage. A markedly important feature of phenotypic analysis of porcine fetal cells is that SLA class II antigens are present from the early stages of development. A markedly important feature of IFN-α SC, as opposed to adult IFN-α SC, may be present long before the onset of differentiated T-cell markers. In addition, these data indicate that fetal IFN-α SC, as opposed to adult IFN-α SC, may be negative for CD4 expression. Therefore, we suggest that CD4 surface expression is not a constant phenotypic characteristic of IFN-α SC, but may depend on their developmental stage. A markedly important feature of phenotypic analysis of porcine fetal cells is that SLA class II antigens are present from the early stages of gestation. Thus, SLA class II positive cells were found in the present study at 26 days of gestation. Moreover, SLA class II positive cells have even been described in blood islands of the porcine yolk sac [24]. Because several authors have discussed the possible relationship of NIPC to dendritic cells [6], it is worth pointing out that HLA-DR^+ cells with dendritic morphology are also present in the human yolk sac [25]. Thus, our present data showing the early and concomitant presence of SLA class II positive cells and of IFN-α SC are compatible with the hypothesis that IFN-α SC may indeed be dendritic cells.

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