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Early Ontogeny of Monocytes and Macrophages in the Pig

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Summary

Prenatal development of cord blood monocytes and tissue macrophages was studied in pig foetuses by immunophenotyping and functional assays. The function of peripheral blood monocytes was compared in germ-free and conventional piglets. First macrophages were identified by electron microscopy in foetal liver on the 25th day of gestation. Monoclonal antibodies against porcine CD45 and SWC3 antigens were used for flow cytometric identification of myelomonocytic cells in cell suspensions prepared from the yolk sac, foetal liver, spleen and cord blood. Leukocytes expressing the common myelomonocytic antigen SWC3 were found in all organs studied since the earliest stages of development. Opsonized zymosan ingestion assay was used to determine the phagocytic capacity of foetal mononuclear phagocytes isolated from cord blood, liver and spleen. In the foetal liver, avid phagocytosis of apoptic cells had been found to occur before cells were able to ingest zymosan *in vitro*. The first cells capable of ingesting zymosan particles were found on the 40th day of gestation in umbilical blood and 17 days later in foetal spleen and liver. Their relative proportion increased with age. Cord blood monocytes and peripheral blood monocytes in germ-free piglets had low oxidatory burst activity as shown by iodonitrophenyl tetrazolium reduction assay. A remarkable increase of oxidatory burst activity was observed in conventional piglets, probably due to activation of immune mechanisms by the microflora colonizing gastrointestinal tract.

Key words

Macrophages – Ontogeny – Pig – Oxidatory burst – Flow cytometry – Phagocytosis

Introduction

The pig and its immune system has recently received a great deal of attention not only because of its importance as a food source but also as a potential donor for organ transplantation and a useful model in biomedical research. As most work on the porcine immune system has focused on lymphocytes, relatively little is known about cells involved in innate immunity. We have therefore studied the development of the monocyto-macrophage haematopoietic lineage during pre- and perinatal ontogeny.

During prenatal development, pluripotent haematopoietic stem cells (HSC) are generated independently at intra- and extra-embryonic sites (Moore and Metcalf 1970, Godin *et al.* 1995). HSC are generated from the mesoderm in these sites but they also have a self-repopulating capacity. Foetal precursors of macrophages were originally described by Cline and Moore (1972). Macrophages were found in the human yolk sac as early as in the 5th week of gestation (Fukuda 1973, Janossy *et al.* 1986) and, in mice, on the 9th day of intrauterine life (Takahashi *et al.* 1989). In pigs (full gestation 114 days), dawning

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haematopoiesis was described in the yolk sac where it starts on about the 16th day of gestation (DG16), reaches its maximum on DG20 and disappears 8 days later (Kovářů 1971). Stem cells and partially differentiated progenitors migrate among hepatic cords, where differentiation of macrophages and their precursors takes place (Enzan *et al.* 1983). Early haematopoietic activity can also be detected in the spleen and gradually in the bone marrow, which becomes the main haematopoietic centre in older foetuses and during whole postnatal life.

The ability of macrophages to ingest foreign material and to mount the oxidatory burst is closely associated with their defense role against invading microorganisms. Less mature cells of the myelomonocytic haematopoietic lineage were shown to have impaired functions (Hardy *et al.* 1973). The onset of oxidatory burst activity in pig phagocytes during their differentiation in the bone marrow has recently been reported (Summerfield and McCullough 1997).

After birth, gut microflora plays a certain role in the functional maturation of macrophages and monocytes. Germ-free (GF) mice were shown to have less effective macrophages as regards their ability to mount the killing machinery (Mitsuyama *et al.* 1986).

In this report, embryonic development of pig monocytes and macrophages was studied using immunophenotyping and functional assays.

Materials and Methods

Animals

Foetuses of miniature pigs bred in our laboratory were obtained under halothane-oxygen anaesthesia as described elsewhere (Řeháková *et al.* 1996). Early foetuses were delivered at various intervals between DG20 and DG60 and older ones in 5 days intervals until the end of gestation. GF piglets were delivered by hysterectomy and hysteretomy, reared in glass-fibre sterile isolators and fed an autoclaved milk diet (Mandel 1996). The GF state was controlled twice a week by aerobic and anaerobic cultivation of rectal swabs. Peripheral blood from GF and CV piglets was collected when the animals were two weeks old.

Electron microscopy

Tissue samples were fixed in 1% paraformaldehyde and 1% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2), postfixed in 1% osmium tetroxide and embedded in polyester Vestopal W resin (Serva, Heidelberg, Germany). Ultrathin sections stained with uranylacetate and lead citrate were observed in a Tesla BS 500 transmission electron microscope (Brno, Czech Republic).

Isolation of cells for flow cytometry

Cord blood was obtained by heparinized glass capillary from punctured cord vein. Foetal liver and spleen were dissected, gently teased with pincettes and glass pipettes. The yolk sac was digested by collagenase V treatment (100 U/ml in the RPMI 1640 medium supplemented with 2 % foetal calf serum (FCS); all reagents from Sigma).

Pieces of tissue were removed by filtration through a fine nylon mesh (Nybolt, Zurich, Switzerland), cells were washed three times in phosphate buffered saline (PBS). Erythrocytes and their precursors were removed from whole blood or pelleted cells by hypotonic lysis with water for 30 s followed by osmotic pressure reconstitution with two-fold concentration of PBS. Finally, the cells were washed twice in ice-cold PBS-NPS (10 % normal pig serum and 0.1 % NaN_3 in PBS) and kept on ice until staining.

Monoclonal antibodies

The following primary immunoreagents were used. The K252.1E4 (IgG1) monoclonal antibody directed against CD45 (LCA – leukocyte common antigen), a kind gift from Dr. K. Haverson and Dr. C. Stokes (Haverson *et al.* 1994), and the 74-22-15 (IgG2b) monoclonal antibody (kindly provided by Dr. Joan Lunney), recognizing the SWC3 antigen, restricted to the myelomonocytic haematopoietic lineage in pigs (Lunney 1993).

Flow cytometry

Cell suspensions were set to the density of $5x10^6$ cells/ml and stained for flow cytometry (FCM) using a standard protocol (Cukrowska *et al.* 1996). Briefly, ice-cold PBS-NPS was used as the labelling and washing buffer. 100 ml of the cell suspension ($5x10^5$ cells) were incubated with primary monoclonal antibody at the optimal dilution for 30 min, washed three times and stained for 30 min using FITC-labelled anti-mouse IgG1 and PE-labelled anti-mouse IgG2b goat polyclonal antisera (Southern Biotechnology Associates). After three washings, the cells were ready for FCM measurement on the FACSort flow cytometer (Becton-Dickinson, BD). Dead and damaged cells were excluded from acquisition by propidium iodide fluorescence. Data analysis was performed using the PC-lysys software, version 1.0 (BD).

Isolation of adherent spleen and liver cells and peripheral blood mononuclear cells for phagocytosis and oxidatory burst assay

Foetal liver and spleen tissue were gently disintegrated using pincettes. After filtration through a fine nylon mesh (Nybolt, Zurich, Switzerland), the suspensions were washed three times in ice-cold RPMI 1640 medium with 2% FCS and cells were allowed to

adhere to Falcon tissue culture dishes coated with FCS for 90 min at 37 °C, 5 % CO_2 (Kumagai *et al.* 1979). Non-adherent cells were removed by washing with cold RPMI 1640 medium. The adherent fraction was scraped with a rubber policeman. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll-Paque centrifugation followed by washing in RPMI 1640 medium with 2 % FCS.

Phagocytosis assay

Twenty-five milligrams of zymosan A (Sigma) in 10 ml saline were heated (90 °C, 30 min), opsonized with normal human serum and labelled with fluorescein isothiocyanate (FITC, Serva) according to Binns *et al.* (1981). Small aliquots of 10^8 particles/ml were kept frozen at −20 °C and thawed just before use. Ficoll purified PBMC were resuspended in RPMI 1640 medium supplemented with 5 % FCS at the density of $1x10^6$ cells per ml. $1x10^6$ cells were incubated with 10^7 FITC-labelled zymosan particles for 90 min at 37 °C and 5 % CO_2 in a humidified atmosphere, washed and observed in a Leitz Orthoplan fluorescence microscope. The relative number of cells containing zymosan particles was counted.

INT assay

The reduction of 3-(4-iodophenyl)-2-(4-nitrophenyl)-5(phenyl)-tetrazolium chloride (INT, Lachema, Czech Republic) to insoluble blue formazan was used to detect respiratory burst activity (Procházková and John 1986). $2x10^6$ cells were incubated for 45 min at 37 °C in Hanks' balanced salt solution (pH 7.2) containing 0.1 % INT with or without 1 % starch (amylum oryzae) in activated and control samples, respectively. The reaction was terminated by cooling the incubation tubes in ice-cold water. After centrifugation, 3 ml of acetone were added to the pellet to dissolve reduced INT. Optical density at 485 nm was read using absorption spectrophotometer Specord M 40 (Carl Zeiss, Jena, Germany). All experiments were run in triplicate. The oxidatory burst index (I) was calculated as the ratio of absorbance of starch-stimulated and control samples.

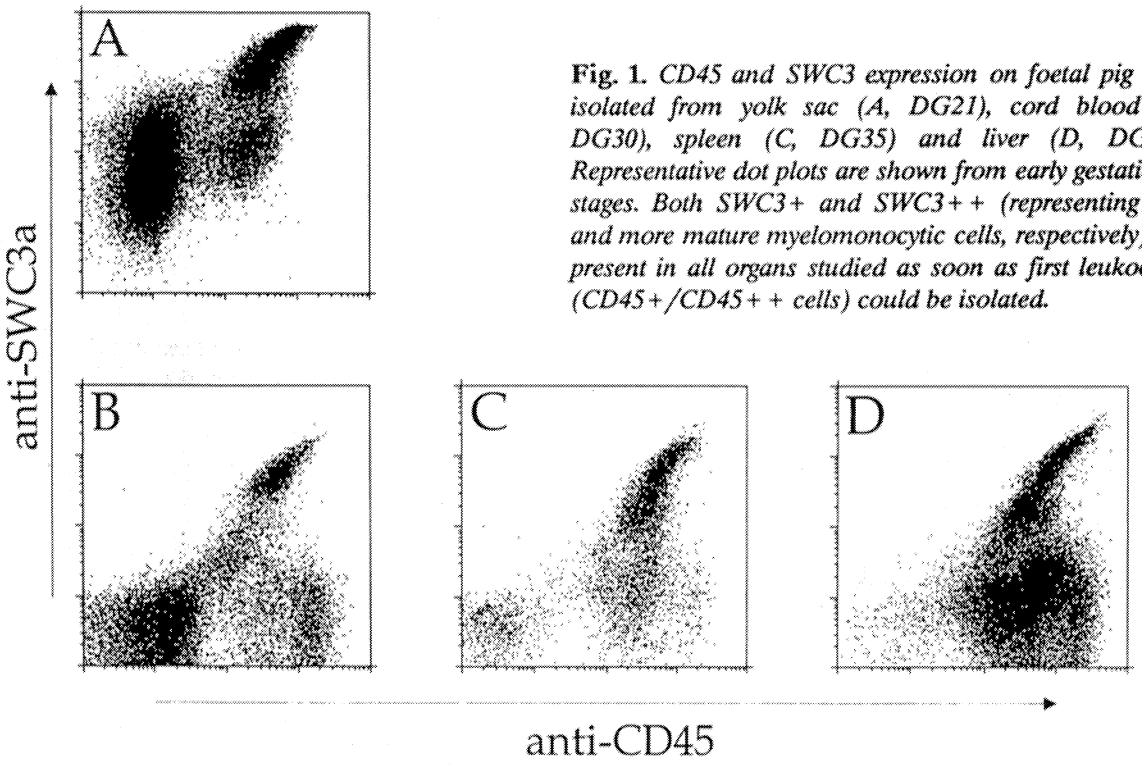

Fig. 1. *CD45 and SWC3 expression on foetal pig cells isolated from yolk sac (A, DG21), cord blood (B, DG30), spleen (C, DG35) and liver (D, DG21). Representative dot plots are shown from early gestational stages. Both SWC3+ and SWC3++ (representing less and more mature myelomonocytic cells, respectively) are present in all organs studied as soon as first leukocytes (CD45+/CD45++ cells) could be isolated.*

Results

Yolk sac

In pigs, as in other mammals, yolk sac is formed by a bilayer of the entoderm and mesoderm (Yamashita 1996). Using electron microscopy, we could not detect any cells with typical macrophage morphology in yolk sac mesenchymal blood islands which lay between these two epithelial layers. However, some mesenchymal cells contained an extensive lysosomal system (typical for primitive macrophages in mice)(Naito *et al.* 1996). FCM data showed that a prominent subset of leukocytes (CD45+ cells) can be found in yolk sac, most of them being positive for the common myelomonocytic antigen SWC3 (Fig. 1A). All leukocytes were large mononuclear cells as judged

from the forward and perpendicular light scatter (FSC/SSC) characteristics. We have never observed any yolk sac cells that were able to ingest opsonized zymosan particles *in vitro*.

Liver

Cells with macrophage morphology were detected first in erythroblast nests by electron microscopy on DG25 (Fig. 2). These cells endocytosed apoptic bodies and naked nuclei. Great sessile macrophages in liver sinusoids known as Kupffer cells were found first on DG40.

CD45+SWC3+ cells were isolated from foetal livers beginning on DG21 and their frequency gradually increased with foetal age (Fig. 1D).

No adherent cells capable of ingesting opsonized zymosan were present in the foetal liver at early stages of gestation. Only 1 % of adherent cells on DG57 contained ingested particles following incubation with zymosan. The highest proportion of phagocytes was detected in the adherent fraction in the last third of gestation; about 9% of cells ingested zymosan on DG80 and later until birth (Fig. 3).

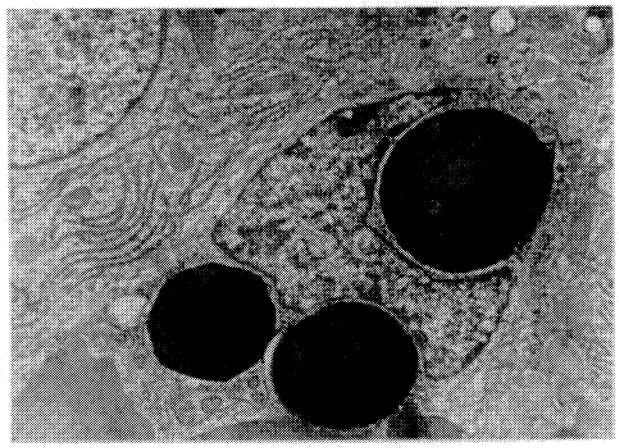

Fig. 2. *Early liver macrophage ingesting three erythroid cells. A porcine embryo on the 25th day of gestation (4000x).*

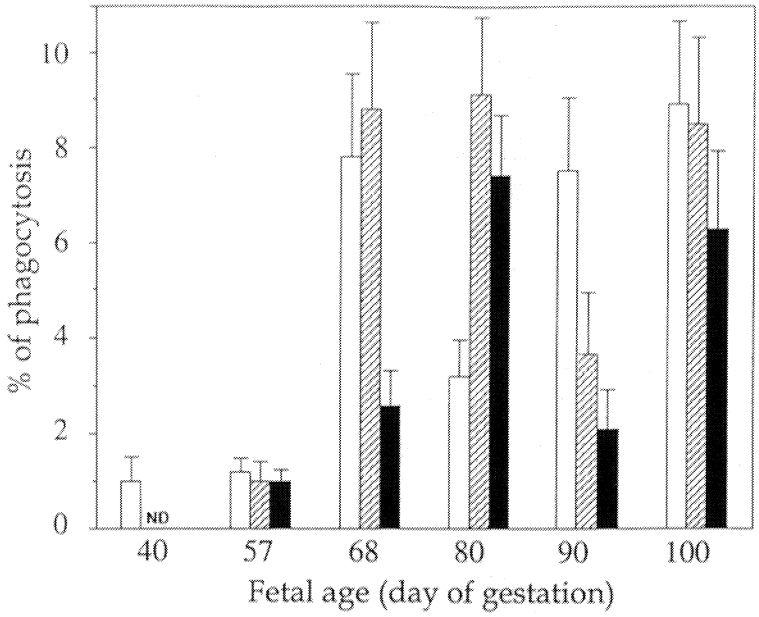

Fig. 3. *Phagocytic activity of cord blood monocytes (open bars) and adherent cells from foetal liver (hatched bars) and foetal spleen (black bars) at different developmental stages. The percentage of cells ingesting opsonized zymosan is shown. The earliest phagocytosis was observed in cord blood monocytes on DG40, first phagocytes in the liver and spleen were observed later (on DG57). N.D. – not detected.*

Spleen

The SWC3 molecule was first expressed in foetal spleen on DG35 (Fig. 1C). This organ contains numerous SWC3 positive cells since this developmental stage until DG56. Lymphocytes predominate in the spleen later, when it becomes a typical secondary lymphatic organ. For instance, 41±6 % of all cells and 25±4 % of mononuclear cells bear the SWC3 antigen on DG67. The first phagocytes ingesting opsonized zymosan particles appear in the spleen in the second half of gestation (Fig. 3). The maximal percentage of phagocytes among adherent cells was observed on about DG80 (9.1 %) and a slight decrease was observed thereafter (Fig. 3).

Cord and peripheral blood

Myelomonocytic leukocytes (CD45+, SWC3+) were found in the cord blood as early as DG30 (Fig. 1B). No mononuclear cells ingesting zymosan, however, were observed until DG40. The proportion of phagocytes gradually increased with foetal age until DG100, when relatively highest numbers of cells with ingested particles were observed (9% cells with phagocytic activity) (Fig. 3).

The oxidatory burst in peripheral blood mononuclear cells was tested in the second half of gestation, because it was impossible to isolate sufficient numbers of PBMC from early foetuses (Table 1). Foetal cells expressed weak production of reactive oxygen radicals (the value ranged between 1.02–1.17) and this low activity was comparable with 14-day-old GF animals (I = 1.15±0.08). On the contrary, age-matched CV piglets had almost a two-fold higher oxidatory burst index (I = 2.11±0.64).

Table 1. Oxidatory burst of cord and peripheral blood mononuclear cells quantified by INT reduction assay in pig foetuses (A) and 14-day-old piglets (B) germ-free (GF) or conventional (CV), respectively.

A) Pig foetuses

Day of gestation	A485 stimulated cells	A485 controls	Index
65	0.349±0.017	0.297±0.005	1.173±0.059
70	1.510±0.230	1.474±0.088	1.020±0.170
80	0.910±0.009	0.774±0.023	1.177±0.037
85	0.202±0.035	0.193±0.009	1.050±0.190
90	0.133±0.005	0.123±0.005	1.079±0.063
105	0.132±0.002	0.122±0.003	1.084±0.037
110	0.155±0.030	0.132±0.003	1.140±0.300

B) 14-day-old piglets

Pigs	A485 stimulated cells	A485 controls	Index
GF	0.166±0.006	0.144±0.009	1.150±0.080
CV	0.224±0.051	0.106±0.021	2.110±0.640

Data are means±S.D. of individual animals. A485 stimulated cells and A485 controls represent absorbance (read at 485 nm) of solubilized formazan in starch-activated and control samples, respectively. Index of oxidatory burst was calculated as the ratio of A485 stimulated cells/A485 controls. Samples were run in triplicates and at least 3 animals per group were tested.

Discussion

In the rapidly growing foetal body, where contact with microorganisms occurs only accidentally, the main role of phagocytes is to remove damaged and superfluous cells to maintain homeostasis and participate in tissue remodelling. In prenatal ontogeny of mammals, macrophages, as the ultimate differentiated stage of monocyto-macrophage haematopoietic lineage, are supposed to be the most effective and heterogeneous immune cell population that can be found dissipated throughout the body. During early ontogeny in mice, primitive macrophages develop, proliferate, and differentiate into foetal macrophages before monocytes appear in the foetal haematopoietic tissues (Naito *et al.* 1990). The precise mechanism of this early monocyte-independent differentiation remains unclear. In contrast to this, it has been postulated that the majority of macrophages originates from blood monocytes and their bone marrow precursors (Van Furth *et al.* 1972).

Although several monoclonal antibodies restricted to macrophages are known in other species (e.g. in rats, Yamashita *et al.* 1990), no specific monocyte- or macrophage-specific monoclonal antibodies have been available in pigs so far. We used the 74-22-15 monoclonal antibody (Lunney 1993) to identify myelomonocytic cells in foetal blood and haematopoietic organs. In pigs, the earliest haematopoiesis was observed by cytological screening in the yolk sac on DG16 (Kovářů 1971). We found no phagocytic activity of cells isolated from the yolk sac, although a prominent subset of myelomonocytic

(CD45+SWC3+) cells could be detected here by flow cytometry.

Yolk sac-derived stem cells migrate to and seed foetal liver between DG20 and DG28. We observed the first CD45+SWC3+ cells in the foetal liver on DG21; early phagocytosis was proved by electron microscopy to occur in cells engulfed by macrophages on DG25. Fully functional liver macrophages were observed by the end of the first half of gestation (DG57). A relative proportion of cells capable of ingesting zymosan particles increased until DG80 and only marginal changes were observed in older foetuses. Similarly to pigs, foetal liver monocytic cells were found to occur in mice at mid-gestation (Naito *et al.* 1990). Foetal spleen in pigs, especially in younger foetuses, is a haematopoietic centre with a huge myelopoietic activity (Fig. 1C, Řeháková *et al.* – in preparation). The first zymosan-ingesting phagocytes were found in the spleen on DG57 and their relative proportion among plastic-adherent cells increased until DG80. A transient decrease in the frequency of phagocytic cells was observed in foetal liver and spleen on DG90. We cannot exclude, however, that this was partially due to a lower yield of adherent cells during the isolation procedure. Absolutely the first phagocytes capable of ingesting foreign material were found among cord blood PBMC; the first phagocytosis *in vitro* was detected on DG40. Similarly to the liver and spleen, their relative number increased with age.

Finally, blood monocytes were functionally assessed by their capacity to generate the oxidative burst. Monocytes isolated from foetal blood and from peripheral blood in germ-free animals were shown to be a rather inefficient source of oxidative burst metabolites. In conventional piglets, on the other hand, peripheral blood monocytes had almost two-fold higher capacity to synthesize reactive oxygen metabolites. A similar situation was described by others in germ-free and conventional mice (Mitsuyama *et al.* 1986). Irrespective of partial functional immaturity as regards reactive oxygen metabolite synthesis, phagocytes in germ-free piglets are as efficient as those in conventional piglets in their ability to clear gram-negative bacteria and endotoxin from the circulation (Miler *et al.* 1968, Tlaskalová-Hogenová *et al.* 1980). This corresponds to the gradually increasing phagocytic activity with foetal age, which reaches its plateau by the end of gestation. Both phagocyte functions – ingestion of foreign material and intracellular killing – thus appear to be differentially regulated during ontogeny, the former being independent of the presence of microorganisms while the latter attains full activity upon microbial colonization.

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