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Apoptotic DNA Alterations in Pig Leukocytes After Phagocytosis of Bacteria are Linked to Maturation of the Immune System

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Summary

The effect of phagocytosis of living bacteria on apoptotic DNA changes was examined in pig leukocytes in relation to immune system maturation. Blood samples of pigs (aged 6, 12 and 18 weeks) were cultivated with a suspension of bacterial cells *Salmonella typhimurium* LB 5000 at 37 °C. In the experimental groups, killed bacteria and microspheric particles were used to detect the influence of the phagocytic process. Phagocytic activity and index were determined in each sample by means of microspheric particles. The ability to kill engulfed microbes (bactericidal capacity) was estimated from the decrease in bacterial colony-forming units (CFU). Samples of cultured cells were taken for DNA analysis at given intervals. DNA ladder assay was used for qualitative apoptotic DNA break detection and the TUNEL AP test was employed for quantification of apoptosis. In 18-week-old animals, spontaneous DNA degradation was observed in the control group without phagocytosis after 8 h. In contrast, cells cultivated with microspheric particles or killed bacteria became apoptotic after 4 h. The rate of apoptotic DNA degradation was decreased in the group exposed to living bacteria. This prolonged survival of phagocytes was also detected in 12-week-old animals, but not at 6 weeks of age. These findings were supported by the ability of phagocytes in 6-week-old animals to engulf microbes, but their killing (bactericidal) ability was significantly decreased in comparison with other stages of immune system maturation. These results suggest that the process of phagocytosis itself is accompanied by activation of the apoptotic program in phagocytic cells of the pig immune system, but the presence of phagocyted living bacteria can delay this activation. The prolonged survival of short-lived cells was only observed in later phases of immune system maturation.

Key words
Apoptosis • Phagocytosis • Ontogenesis • Pig leukocytes

Introduction

Interest in bacterial pathogenesis has recently increased mainly because of antibiotic resistance, the lack of effective therapeutics and emergence of new pathogens (Finlay and Cossart 1997). Many host cell functions, including signal transduction pathways, cytoskeletal rearrangements and vacuolar trafficking are being exploited in the pathogenesis.

A major task that phagocyting blood cells have to fulfil in the organism is to participate in the non-specific immune response against bacterial infection. This role mainly involves phagocytosis followed by the killing of microorganisms. Phagocyting cells possess a variety of receptors and enzymatic mechanisms (Morel *et al.* 1991, Garland 1992) for this important function. Many cells of the hemopoietic system have a very short life-span, from days (polymorphonuclear granulocytes) to weeks (blood monocytes) and phagocyting cells are produced in high numbers during the whole life of the organism. A dynamic balance between cell production and elimination is essential for homeostasis of the organism (Squier *et al.* 1995). Under physiological conditions, phagocyting cells undergo programmed cell death through apoptosis (Fesus *et al.* 1991, Homburg and Roos 1996, Allen *et al.* 1997). This silent death of phagocytes avoids toxic damage of healthy tissue in contrast with phagocytes in inflammatory areas that die by necrosis (Fernandes and Cotter 1994, Haanen and Vermes 1995).

Apoptosis (Kerr *et al.* 1972), as a way of programmed cell death, represents a physiological genetically controlled process involved not only in tissue renewal but also in embryogenesis and in elimination of damaged and potentially dangerous cells. This process, in contrast to pathological necrosis, enables effective and rapid cell removal by monocytes and macrophages (Haslett *et al.* 1991, Savill 1997, Meszaros *et al.* 1999) without any inflammatory reaction. Programmed cell death (PCD) can be activated by many different extra- and intracellular factors. Information about the PCD influencing factors and the mechanisms of their function is important for developing targeted strategies to influence the apoptotic program.

The aim of this work was to observe how phagocytosis and the killing of bacterial cells of *Salmonella typhimurium* can influence the survival of pig leukocytes in relation to immune system maturation.

Methods

Blood samples

Blood samples (25 ml) were taken from 12 healthy pigs one hour prior to the start of the experiment in three developmental phases – aged 6, 12 and 18 weeks (Durok breed, 6 males, 6 females, Agricultural Farm, Nový Dvůr, Czech Republic). Blood samples were stabilized with heparin (12 IU/ml). Then the number of leukocytes was determined in each sample using a common Burker´s chamber.

Bacterial suspension

Salmonella typhimurium LB 5000 (Bullas and Ryu 1983) bacterial cells were used throughout this study. One colony of the bacterial strain was grown overnight in an LB medium (10 g Bacto tryptone, 5 g Bacto yeast extract, 5 g NaCl in 1000 ml H_2O) and then washed twice with a PBS buffer (phosphate-buffer saline: 100 g NaCl, 2.5 g KCl, 14.4 g Na_2HPO_4, 2.5 g KH_2PO_4 in 1000 ml H_2O). The final density of the cells was $1x10^9$ CFU/ml PBS. A suspension of killed bacteria was obtained after heating the cell culture in the LB medium for 30 min at 72 °C and then washed twice in the PBS buffer.

Microspheric particles (MSHP)

Microspheric particles (diameter 1.5 μm) from an MSHP kit, Rk 031 (Artim, Czech Republic) were resuspended in an adequate volume of PBS according to the manufacturer´s instructions.

Phagocytosis of bacteria and microspheric particles

Blood samples were divided into four groups and mixed with living or killed bacteria, and with microspheric particles, respectively (1 ml of blood + 0.1 ml of bacteria or particles, in PBS and PBS alone), and incubated at 37 °C. After 1 h of cultivation, the phagocytic activity (% of phagocyting cells out of all potential phagocytes) and the phagocytic index (the number of engulfed particles in one phagocyting cell) were measured using the method with microspheric particles (Větvička *et al.* 1982). Two hundred cells were evaluated in each sample.

Experimental design

The experiment was started by addition of bacterial cells (living or killed) and MSHP (control of phagocytosis) and PBS alone, respectively, to the blood samples. At given intervals (1, 4, 8, 12, 24, 48 h after start of the experiment), samples were taken for DNA analysis and at 1, 2 and 3 h for bactericidal capacity evaluation. To avoid bacterial cell division during prolonged cultivation, ampicillin (100 μg/ml) was added to all samples.

Estimation of bactericidal capacity of phagocytes

The ability of phagocytic leukocytes to kill living bacteria cells *Salmonella typhimurium* LB 5000 was measured according to the decrease of the number of *Salmonella* colony-forming units. Briefly, the phagocytic cells were incubated for 1, 2 and 3 h with living bacteria

(phagocyting cells : bacterial cells = 1 : 100). Samples were taken and washed from free bacteria (twice in PBS). The sediment was resuspended in 1 ml of ice-cold distilled water to lyse phagocytic cells. These lysates were diluted in LB medium and plated on Petri dishes with LB agar. Colonies were counted after 24 h incubation at 37 °C. The decrease in the percentage of killed microorganisms after 2 and 3 h was calculated (100 % – number of colonies after one hour).

DNA ladder assay

To determine apoptotic DNA fragmentation in blood cells, sedimented blood samples were digested in a lysis buffer (50 mM Tris, 10 mM NaCl, 100 mM EDTA, pH 8, 1 % SDS, 100 μg proteinase K in 1 ml H_2O) at the given time intervals. The lysates were stored at 4 °C until DNA electrophoresis on 1.8 % agarose. Neutral agarose gel electrophoresis was carried out (using MiroGene Comp. equipment) in the TBE buffer (5.4 g Tris base, 2.75 g boric acid, 0.5 M EDTA, pH 8 in 1000 ml H_2O) at 1.5 V/cm overnight. DNA in the gel was stained with ethidium bromide (1 μg/ml), visualized in a UV transilluminator, and photodocumented (CD 34 Polaroid camera, TT 667 film).

LM-PCR

Ligation-mediated polymerase chain reaction (Staley *et al.* 1997) was used to visualize apoptotic DNA fragments undetectable by common agarose gel electrophoresis. 100 ng of ligated DNA was used in 30 PCR cycles following the manufacturer´s instructions (Clon Tech, USA).

TUNEL AP test

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (In Situ Cell Death Detection Kit, AP) was used to detect apoptosis in individual cells. TUNEL test was carried out following the manufacturer´s instructions (Boehringer-Mannheim, Germany). Apoptotic cells (red) were counted under a light microscope after fluorescence signal conversion using antibody with conjugated alkaline phosphatase and the substrate for alkaline phosphatase (Fast Red, Sigma-Aldrich).

ANNEXIN - V- ALEXA 568

Detection of translocated phosphatidylserine was used to show membrane changes of apoptotic blood cells. Apoptotic cells were labeled and evaluated under fluorescence microscopy according to the manufacturer´s instructions (Boehringer-Mannheim).

Cell morphology

The cells were stained with May-Grünwald-Giemsa and examined by oil immersion light microscopy at a magnification of x1000 to evaluate the morphological changes characteristic for apoptosis.

Statistical evaluation

Standard F- and t-tests and correlation analysis (Benedík and Dušek 1993) were used to evaluate the number of apoptotic cells.

Results

Oxidative stress is considered to be an important factor contributing to cell apoptosis (Morel *et al.* 1991), thus a negative, untreated control was used to determine spontaneously occurring apoptosis. To distinguish the influence of the phagocytic process itself on apoptosis activation, samples were cultivated with microspheric particles.

Number of leukocytes in the blood volume

The number of leukocytes (G/l) in the blood volume was within physiological limits for all tested samples (Reece 1998) – 6 weeks: 17.5±1.23, 12 weeks: 18.3±0.82, and 18 weeks: 19.8±1.04.

Phagocytic activity, index of monocytes and granulocytes

Phagocytosis measurements were carried out using whole blood samples. The phagocyting ability was assayed in monocytes and granulocytes using microspheric particles. The ability of granulocytes and monocytes to engulf microspheric particles was observed microscopically in all samples at all three developmental stages. Phagocytic activity of monocytes at 6 weeks of age was 95.9±3.91, at 12 weeks 91.9±8.54, and at 18 weeks 85.5±10.42. Phagocytic activity of granulocytes: 6 weeks: 85.7±2.43, 12 weeks 91.3±3.93, 18 weeks: 86.2±4.77. Phagocytic index of monocytes: 6 weeks: 18.14±0.67, 12 weeks: 17.9±1.2, 18 weeks: 18.5±0.98. Phagocytic index of granulocytes: 6 weeks: 6.6±0.28, 12 weeks: 6.2±0.53, 18 weeks: 6.4±0.73.

Bactericidal capacity of phagocytes

Bactericidal capacity was evaluated at three time intervals (after 1, 2 and 3 h from the beginning of phagocytosis) and compared at three developmental

stages. A significantly reduced (67 % and 59 %) bactericidal capacity was found in the phagocytes of 6-week-old pigs after 2 h and 3 h, respectively. At 12 and 18 weeks of age, pig phagocyting leukocytes were able to kill more than 99 % of engulfed bacteria, whereas most bacterial cells survived in phagocytes for more than 3 h in the 6-week-old animals.

Apoptotic DNA changes in leukocytes during phagocytosis

In six-week-old animals DNA fragmentation (evaluated using the DNA ladder assay) was detected in phagocyting groups after 4 h, where two fragments (1500 and 2400 bp) were observed. After DNA purification and application of LM-PCR the typical apoptotic DNA ladder was observed in all samples of the phagocyting groups. In the control group without phagocytosis, no DNA fragmentation was observed either using the DNA ladder assay or LM-PCR. The number of apoptotic cells was evaluated by the TUNEL AP test (Fig. 1) and was found to be significantly lower in the control group compared with the phagocyting groups in all the tested time intervals. No significant changes were found among the phagocyting groups (Table 1).

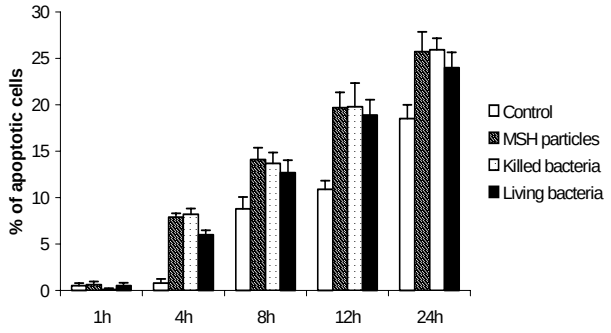

Fig. 1. *Quantification of apoptotic leukocytes in control and phagocyting groups using the TUNEL AP test. Blood samples were taken from pigs aged 6 weeks and analyzed at given intervals (1, 4, 8, 12 and 24 h after phagocytosis onset). All values are means* ± SD *of 12 animals.*

In twelve-week-old animals the typical DNA ladder pattern was observed in the groups with particles and bacteria after 4 h. After quantification of apoptotic cells by TUNEL AP (Fig. 2) significant differences were seen between the control group and the phagocyting groups after 4 and 8 h (Table 1). Comparable numbers of apoptotic cells were found in the control and the group with living bacteria after 12 h, while the number of apoptotic cells in other groups was significantly higher. A decrease in apoptotic cells was observed in the group with living bacteria after 24 h (by 1/3 as compared with other groups).

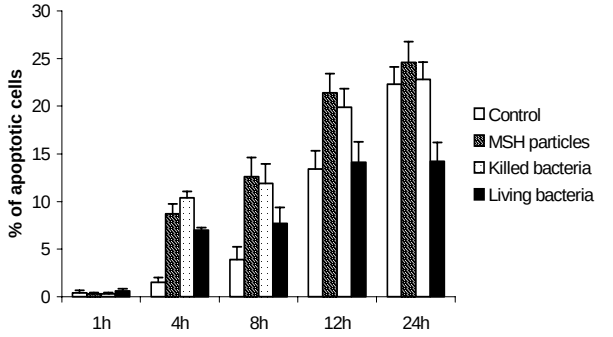

Fig. 2. *Quantification of apoptotic leukocytes in control and phagocyting groups using the TUNEL AP test. Blood samples were taken from pigs aged 12 weeks and analyzed at given intervals (1, 4, 8, 12 and 24 h after phagocytosis onset). All values are means* ± SD *of 12 animals.*

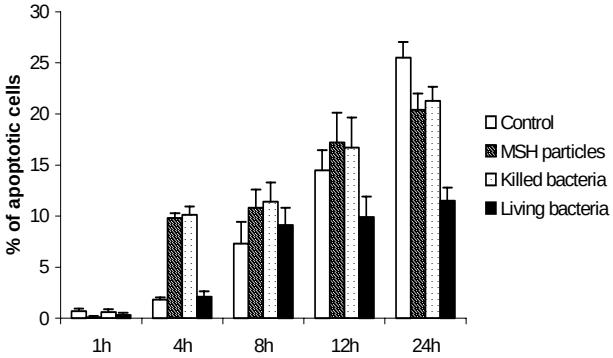

Fig. 3. *Quantification of apoptotic leukocytes in control and phagocyting groups using the TUNEL AP test. Blood samples were taken from pigs aged 18 weeks and analyzed at given intervals (1, 4, 8, 12 and 24 h after phagocytosis onset). All values are means* ± SD *of 12 animals.*

In eighteen-week-old pigs the typical apoptotic DNA ladder was observed in groups with particles and killed bacteria after 4 h, whereas DNA fragmentation was observed in the control and the group of phagocyting living bacteria after 8 h. A significantly lower number of apoptotic cells was seen in the group with living bacteria in comparison with the other phagocyting groups or the control after (Fig. 3, Table 1).

Table 1. Statistical evaluation of differences in the percentage of apoptotic cells among tested groups using T-test (* P<0.025, ** P<0.005). *Pigs aged 6 weeks (depicted in Fig. 1) are always shown in the first row, 12-week-old ones (Fig. 2) in the second row and 18-week-old pigs (Fig. 3) in the third row. A – control group, B – group with MSHP, C – group with killed bacteria, D – group with live bacteria. Index 0 represents 1 h, index 1...4 h, index 2...8 h, index 3...12 h, and index 4 represents 24 h after phagocytosis onset.*

	B0	C0	D0
A0	0.217 0.403 2.132*	1.271 0.403 0.295	0.000 0.599 1.000
B0		1.265 0.000 1.414	0.271 1.033 0.853
C0			1.271 1.033 0.590

	B1	C1	D1
A1	12.442** 6.123** 15.528**	10.009** 10.612** 9.583**	10.811** 7.634** 0.572
B1		0.253 1.409 0.347	3.960** 1.523 10.776
C1			3.163** 4.334** 8.012**

	B2	C2	D2
A2	8.330** 10.257** 3.725**	7.923** 9.210** 4.242**	5.119** 5.621** 1.966
B2		0.756 0.742 0.676	1.948 5.814** 2.032
C2			1.372 4.845** 2.655*

	B3	C3	D3
A3	13.021** 8.089** 2.202*	9.309** 6.750** 1.872	9.482** 0.825 4.917**
B3		0.164 1.524 0.291	0.760 7.861** 5.980**
C3			0.770 6.431** 5.698**

	B4	C4	D4
A4	7.798** 2.348* 3.226*	10.759** 0.605 1.987	6.306** 9.295** 9.120**
B4		0.213 1.807 0.648	1.615 10.796** 12.910**
C4			2.237* 9.967** 6.856**

Apoptotic DNA changes in leukocytes linked to immune system maturation

A DNA ladder was demonstrated in 6- and 12-week-old animals as early as after 4 h of cultivation, whereas, no apoptotic DNA cleavage pattern was observed in 18-week-old animals until 8 h of cultivation (not shown). These findings were supported by statistical evaluation after apoptotic cell quantification using the TUNEL AP test. As shown in Figures 1-3, the number of apoptotic cells was comparable in all evaluated intervals in the groups with particles and killed bacteria only. The typical apoptotic ladder was seen in the control group without phagocytosis after 8 h of cultivation at all three developmental stages.

Marked differences in apoptosis activation were observed in the blood samples of three tested developmental stages in the group with living bacteria (Figs. 1-3). There was a comparable number of apoptotic cells in 6-week-old animals in the groups with phagocytosed MSH particles and killed bacteria as well as in the group with living bacterial cells (Fig. 1). However, a significant decrease in apoptosis activation was demonstrated in 12-week-old animals with living bacteria (8 h, 24 h) (Fig. 2), compared with groups with MSH particles and killed bacteria. The most significant differences in apoptosis activation were found among the

groups of 18-week-old animals. The number of apoptotic cells in the group with living bacteria was comparable with the control group after 4 h of cultivation (Fig. 3). A significant decrease in apoptosis activation was seen in all tested intervals as compared to the groups with particles and killed bacteria (Table 1). No significant differences in the number of apoptotic cells were found between the sexes.

Discussion

The influence of phagocytosis of intracellular *Salmonella typhimurium* LB 5000 on survival and apoptosis activation in phagocyting pig peripheral blood cells was investigated in relation to postnatal immune system maturation. Three developmental stages were chosen (6, 12 and 18 weeks) and for each stage 12 blood samples (6 males, 6 females) from healthy pigs were tested. The selection of those stages was based on previous data showing maturation of immunological defense mechanisms during neonatal and the early postnatal period, using such parameters as: membrane localized enzymes (ATPase and gama-glutamyltrans-peptidase) of the effector cells and fluidity of their plasmatic membranes (Kovářů *et al.* 1992), lysosomal enzymes and bactericidal factors in pig leukocytes (Ferenčík *et al.* 1979), phagocytic activity development – clearance of blood in the prenatal and postnatal period (Dlabač *et al.* 1970), development of Gα subunits of heterotrimeric GTP binding proteins in the immune system (Kovářů *et al.* 2001) and physiological development of immunocompetence (Tlaskalová-Hogenová *et al.* 1994, Kovářů *et al.* 1999a,b). In a previous study (Matalová *et al.* 2000), we demonstrated different apoptosis activation after phagocytosis of living bacteria in adult pig blood cells compared with killed bacteria and microspheric particles. Here we focused on evaluation of apoptosis activation of phagocytes in relation to pig immune system maturation.

It has been reported that apoptosis in polymorphonuclear granulocytes (PMN) can be accelerated after phagocytosis of erythrocytes coated with antigen (Gamberale *et al.* 1998) or with leukotoxins, e. g. from *Pasteurella haemolytica* (Stevens and Czuprynski 1996). In some instances, apoptosis can also be suppressed (Cox *et al.* 1992, Baran *et al.* 1996, Watson *et al.* 1997, Dunican *et al.* 2000). Inhibition of apoptosis in neutrophil granulocytes can be achieved by at least two mechanisms: increased expression of survival factors or disruption of the death signal. Among the bacterial factors, inhibitory effects of lipopolysaccharides on spontaneous PMN apoptosis have been reported (Haslett *et al.* 1991, Colotta *et al.* 1992, Yamamoto *et al.* 1993, Watson *et al.* 1997) *in vivo* and *in vitro* as well as on radiation-induced PMN apoptosis *in vitro* (Sweeney *et al.* 1997).

PMN survive better when exposed to cytokines or bacterial products inducing their production (Colotta *et al.* 1992, Lee *et al.* 1993), probably by affecting tyrosine phosphorylation (Yousefi *et al.* 1994), which plays a major role in receptor-mediated transmembrane signal transduction. Furthermore, increasing concentrations of NO exert a protective action on mitochondria which prevents the onset of apoptosis (Beltran *et al.* 2000). However, the influence of cytokines on PMN survival cannot be excluded in this study as whole blood samples were used. However, the conditions were the same in all four groups examined. Although monocyte apoptosis was not excluded, our observations were focused on PMN, because the number of monocytes is minimal compared with that of PMN.

As was reported previously (Kovářů *et al.* 1995, Kovářů and Kovářů 1997, Kovářů *et al.* 1999a,b) some defense mechanisms of the pig immune system mature during early postnatal development. The results of this study suggest that the establishment of a fully functional bactericidal ability of phagocytes and their prolonged survival after engulfing living bacteria depends on maturation of the immune system.

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