APOPTOSIS ACTIVATION IN PIG LEUKOCYTES AFTER PHAGOCYTOSIS OF *Salmonella typhimurium*

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Abstract

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In this study we examined the effect of phagocytosis of living bacteria on apoptotic changes of DNA in pig leukocytes. The aim of this work was to observe how phagocytosis and killing of microorganisms can influence the survival of pig leukocytes.

Blood samples were cultivated with suspension of *Salmonella typhimurium* LB 5000 up to 48 h at a temperature of 37 °C. In the experimental groups, killed bacteria and microspheric particles, respectively, were used to determine the influence of the phagocytic process itself. In given intervals, samples of cultivated cells were taken for DNA analysis. DNA ladder assay was used for qualitative apoptotic DNA breaks detection by gel electrophoresis and TUNEL AP test for quantification of apoptotic changes. Cell morphology was observed after May-Grünwald Giemsa-staining under light microscope.

Spontaneous DNA degradation in the control group was observed after 8 h. In contrast, cells cultivated with microspheric particles or killed bacteria became apoptotic as soon as after 4 h. already. In the group exposed to living bacteria the apoptotic DNA degradation was slowed down and detected after 48 h mainly, while the other groups showed features of necrosis.

These results suggest that the process of phagocytosis itself activates the apoptotic programme in phagocytic cells of the pig immune system but the presence of phagocyted living bacteria can delay this activation.

Apoptosis, phagocytosis, pig leukocytes, Salmonella typhimurium LB 5000

Apoptotis - a way of programmed cell death - is a physiological genetically controlled process.

Apoptosis (Kerr et al. 1972) is involved in embryogenesis, tissue renewal as well as in damaged and potencially dangerous cells liquidation. In contrast to pathological necrosis, apoptosis enables effective elimination of cells without inflammatory reaction due to keeping cytoplasmic membrane integrity. Apoptotic debris (bodies) are recognised by the phagocytic system of the organism and eliminated. Programmed cell death (PCD) can be activated by different extra- and intracellular factors. Knowledge of these PCD influencing factors and their mechanisms is extremely important for targeted modulation of PCD.

Participation in non-specific immune response of the organism is the major task of phagocyting blood cells. Polymorphonuclear granulocytes (PMN) are mainly responsible for elimination of pathogens (Sládek et Ryšánek 1999). This role involves phagocytosis and killing of microorganisms. For this purpose, the phagocyting cells possess a variety of receptors and enzymatic mechanisms enabling them to engulf and kill microbes (Morel et al. 1991; Lawrence 1992). After their mission, PMN undergo apoptotic process and are eliminated from blood circulation by the monocytes/macrophages system (Fesus et al. 1991; Allen et al. 1997; Meszaros et al. 1999).

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In this work we tested the influence of phagocytosis of *Salmonella typhimurium* LB 5000 on survival and apoptotis activation in pig leukocytes *in vitro*.

Materials and Methods

Bacterial suspension

Laboratory strain of *Salmonella typhimurium* LB 5000 was grown overnight in LB medium (10 g Bacto trypton, 5 g Bacto yeast extract, 5 g NaCl in 1000 ml H_2O) and then washed twice with PBS buffer (phosphate-buffer saline: 100 g NaCl, 2.5 g KCl, 14.4 g $NaHPO_4$, 2.5 g KH_2PO_4 in 1000 ml H_2O). The final density of cells was 10^9/ml PBS. Suspension with killed bacteria was obtained after heating the culture 30 min/ 72 °C.

Samples

Blood samples (25 ml) were taken from 12 healthy 4-month-old pigs on empty stomach (Duroc breed, 6 males, 6 females, Agriculture Farm, Nové Dvory) one hour prior to the experiment. Blood samples were stabilized with heparin (12 i. u. /ml) and cultivated *in vitro* (37 °C) for 48 h.

Microspheric particles

Microspheric particles (diameter 1,5 μm) obtained from Artim, Czech Republic, were resuspended in adequate volume of PBS and used for phagocytic index and activity demonstration.

Phagocytosis of bacteria

Blood samples divided into four groups were incubated (37 °C) in Falcon tubes up to 48 h. Simultaneously, phagocytic index and activity were tested using standard method (MSHP kit, RK 031, Artim, Praha, Czech Republic). The experiment was started with addition of bacterial cells and MSHP, respectively. In given intervals (1, 4, 8, 12, 24, 48 h after start of experiment), samples for DNA analysis were taken.

Group 1 - negative control, only adequate volume of PBS was added, group 2 - control of phagocytosis with microspheric particles in PBS, group 3 was cultivated with killed bacteria in PBS, and experimental group 4 with suspension of living bacteria *S. typhimurium* LB 5000 in PBS.

Bactericidal capacity of phagocytes

The ability of phagocytic leukocytes to kill living bacteria cells *S. typhimurium* LB 5000 was measured by the standard CFU (colony forming units) method. Briefly, phagocytic cells were incubated for 1, 2 and 3 h with bacteria. Then the samples were washed from free bacteria (twice in PBS) and 900 μl of ice-cold distilled water was added to the suspensions to lyse phagocytic cells. These lysates were diluted in LB medium and placed onto LB agar plates. Colonies were counted after incubation for 24 h at 37 °C and the percentage of killed microorganisms was calculated.

DNA Ladder Assay

To determine apoptotic DNA fragments (Wyllie et al. 1980 Hale et al. 1996) blood samples were digested in lysis buffer (50 mM Tris - pH 8, 100 mM NaCl, 100 mM EDTA - pH 8, 1% SDS, 100 μg proteinase K in 1ml H_2O) immediately. Lysates were stored at 4 °C until DNA electrophoresis (1.8% agarose). Neutral gel electrophoresis was proceeded (using MiroGene Comp. equipment) in TBE buffer (5.4 g Tris base, 2.75 g boric acid, 0.5 M EDTA - pH 8 in 1000 ml H_2O). DNA in the gel was stained by ethidiumbromide (1 μg/ml) and photodocumented.

TUNEL AP test

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (Gavrieli et al. 1992) was used for proving apoptosis in individual cells. TUNEL test was proceeded following producer's directions (Boehringer-Mannheim). After conversion using substrate for alkaline phosphatase (Fast Red, Sigma-Aldrich) cells were counted under light microscope.

Cell morphology

To evaluate morphological changes characteristic for apoptosis, cells were stained with May-Grünwald-Giemsa and examined by oil immersion under light microscope at a magnification of × 1000.

Results and Discussion

The aim of this study was to observe interaction between prokaryotic and eukaryotic cell during phagocytic process and its influence on apoptosis activation. DNA Ladder Assay was used for qualitative detection of apoptotic DNA changes, TUNEL AP for quantification of apoptotic cells (red - cells with DNA breaks - Fig. 2). The number of apoptotic cells was statistically tested using Student's *t*-test (Benedík et Dušek 1993).

Blood samples from 12 healthy adult pigs with physiological value of leukocytes (18-21G/l) were examined. Using microspheric particles, phagocytic index (5.3-7.5) and

phagocytic activity (80-95%) of granulocytes were evaluated. Our observation was focused on granulocytes, with examination of apoptotic morphology in monocytes and lymphocytes. To determine whether apoptosis is induced spontaneously or in consequence of phagocytosis, we used a negative untreated control. To distinguish the influence of phagocytic process itself from factors of killed and living bacteria, respectively, we cultivated one group with microspheric particles. All samples were cultivated up to 48 h, and to avoid bacterial cell division during cultivation, ampicilin (100 ng/ml) was added. Bactericidal capacity of phagocytes was evaluated after separation of free bacteria, the decrease in number of living bacteria cells inside phagocytes was more than 99%/2 h.

Analyzed by light microscopy, monocytes exhibited altered morphology (disintegration of nuclei) after 4 h of cultivation in the group with living bacteria, in the control group after 12 h. In contrast, lymphocytes did not show any morphological features of apoptotic cells in the course of this experiment. Because of small percentage of monocytes in total leukocytes, no apoptotic ladder was observed by gel electrophoresis in the group with living bacteria after 4 h of cultivation. On the other hand, in granulocytes in groups engulfed particles and killed bacterial cells, DNA fragmentation was proved by gel electrophoresis after 4 hours while control group was negative (Fig. 3). Significant (*t*-test, $P < 0.01$) smaller amount of apoptotic cells in the control and group with living bacteria in comparison with other groups was confirmed by TUNEL AP test (Fig. 1) after 4 h cultivation. In contrast, apoptotic DNA ladder in analysed DNA taken after 48 h, was proved only in the group with living bacteria. The other groups showed features of necrosis (Fig. 4).

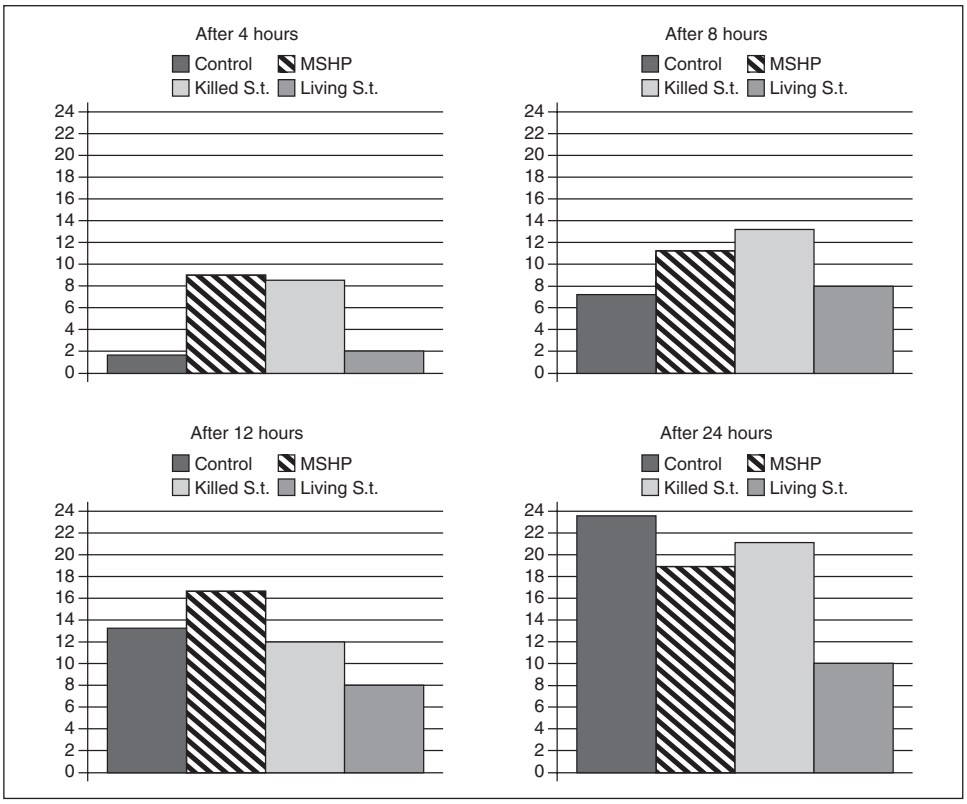

Fig. 1. Percentage of apoptotic cells in total leukocytes using TUNEL AP in given intervals.

Our results suggest that phagocytosis by itself activates the apoptotic programme in granulocytes of the pig immune system but the presence of phagocyted living *S. typhimurium* LB 5000 can slow down its activation. This prolonged survival of short-lived cells seems to be very important for the elimination of pathogens during non-specific immune response of the organism (Baran et al. 1996).

Apoptotické změny DNA v prasečích leukocytech po fagocytóze bakteriálních buněk *Salmonella typhimurium*

Pohlcení a následné usmrcení mikroorganismů jsou dvě důležité úlohy leukocytů při nespecifické imunitní odpovědi organismu.

Tato studie byla zaměřena na interakce prokaryotické a eukaryotické buňky při fagocytóze extracelulárních bakterií prasečími leukocyty, zejména vliv fagocytárního procesu na aktivaci apoptotického programu fagocytujících buněk. Cílem práce bylo sledování, jak může fagocytární proces a následná cidie mikroorganismů ovlivnit přežívání prasečích leukocytů.

Krevní vzorky ze 12 prasat (Duroc, věk 4 měsíce) byly inkubovány se suspenzí bakteriálních buněk *Salmonella typhimurium* LB 5000 po dobu 48 hodin při 37 °C. Souběžně byla kultivována kontrolní skupina, skupina s mikrosférickými partikulemi a s usmrcenými bakteriálními buňkami. V několika časových intervalech byly odebírány vzorky pro analýzu DNA. Průkaz apoptotického žebříčku po elektroforéze umožnil detekci apoptotických zlomů DNA a TUNEL AP test byl využit ke kvantifikaci apoptotických změn v jednotlivých buňkách. Buněčná morfologie byla sledována na krevních nátěrech barvených May-Grünwald-Giemsa.

Leukocyty ve vzorcích inkubovaných s usmrcenými bakteriemi a mikrosférickými částicemi vykazovaly apoptotické změny DNA po 4 hodinách kultivace. Spontánní apoptotický proces byl detekován v kontrolách po 8 hodinách kultivace. Ve skupině s živými bakteriálními buňkami byl po 48 hodinách pozorován odklad aktivace apoptotického programu, kdy ostatní skupiny vykazovaly známky nekrózy.

Naše výsledky naznačují, že fagocytární proces sám o sobě aktivuje apoptotický program ve fagocytujících buňkách imunitního systému prasečího organismu, ale přítomnost fagocytovaných živých bakteriálních buněk *S. typhimurium* může tuto aktivaci zpomalit.

Toto prodloužené přežívání jinak krátce žijících buněk se zdá být velmi důležité pro eliminaci patogenů během nespecifické imunitní odpovědi organismu.

Acknowledgements

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Plate I

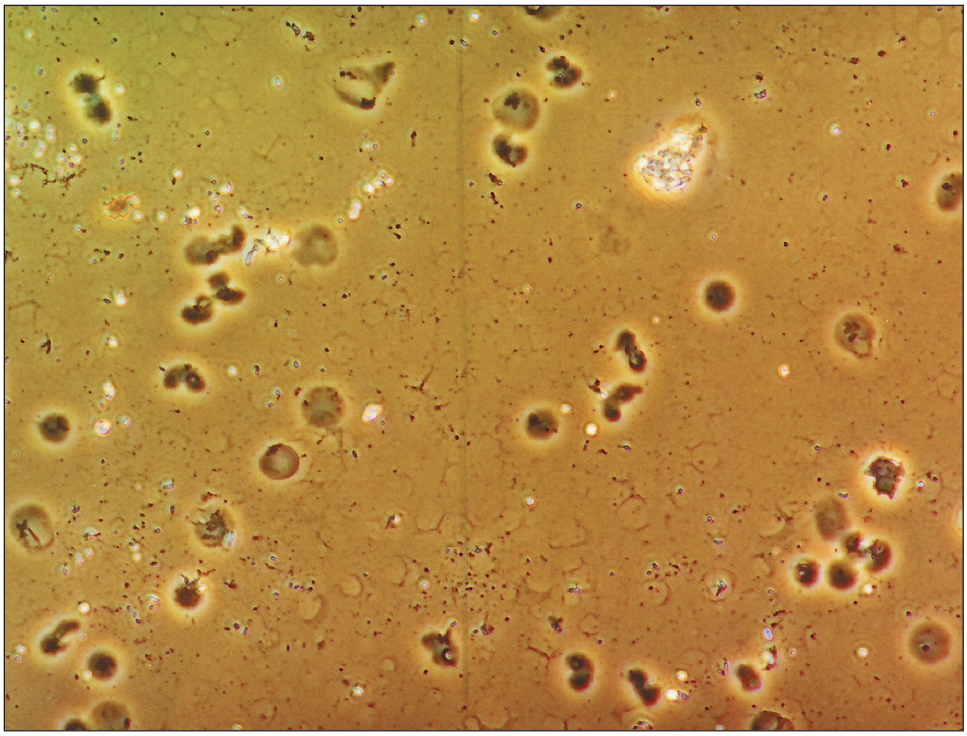

Fig. 2. TUNEL AP test proving apoptotic DNA fragmentation in leukocytes; a) Cell nuclei without fragmentation (see above), b) Cell nuclei with DNA fragmentation (red), (see below).

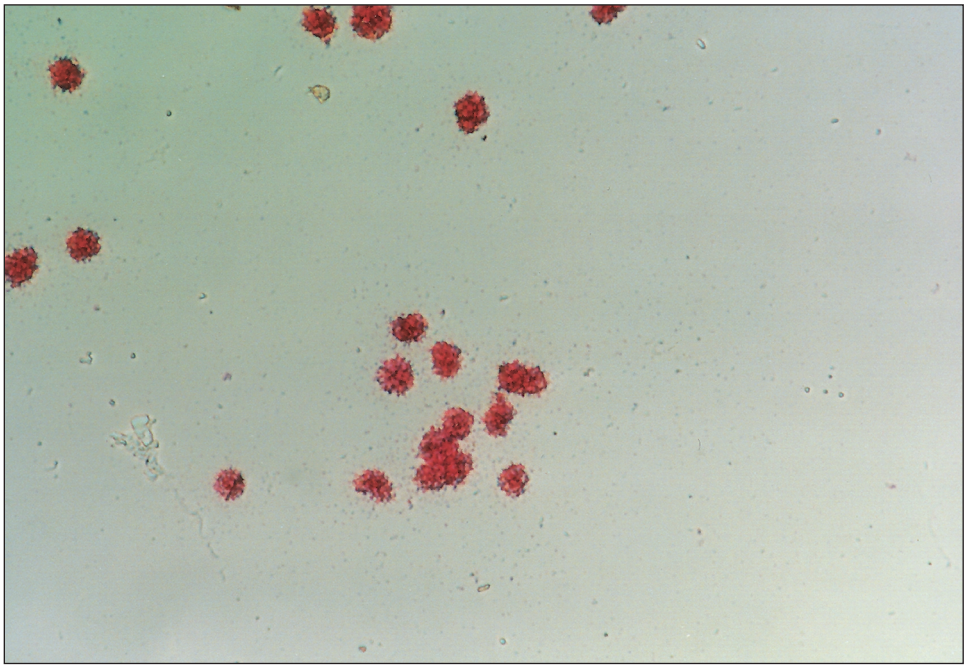

Plate II

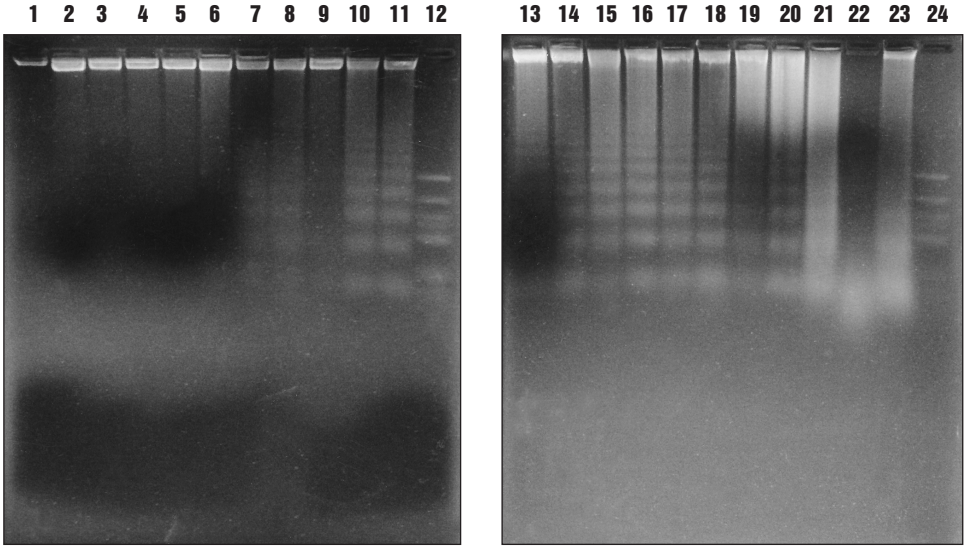

Fig. 3. DNA ladder assay.
Comparison of all groups (A-control, B-group with MSHP, C-group with killed bacteria, D-group with living bacteria *Salmonella typhimurium* LB 5000) at intervals of 0, 1, 4, 8 and 12 h after the beginning of the experiment. Runs: 1- 0 h, 2- A/1h, 3- B/1h, 4- C/1h, 5- D/1h, 6- A/4h, 7- B/4h, 8- C/4h, 9- D/4h, 10- A/8h, 11- B/8h, 12- DNA marker (970-155 bp), 13- C/8h, 14- D/8h, 15- A/12h, 16- B/12h, 17- C/12h, 18- D/12h, 19- A/24h, 20- B/24h, 21- C/24h, 22- D/24h, 23- A/48h, 24- DNA marker (970-155bp).

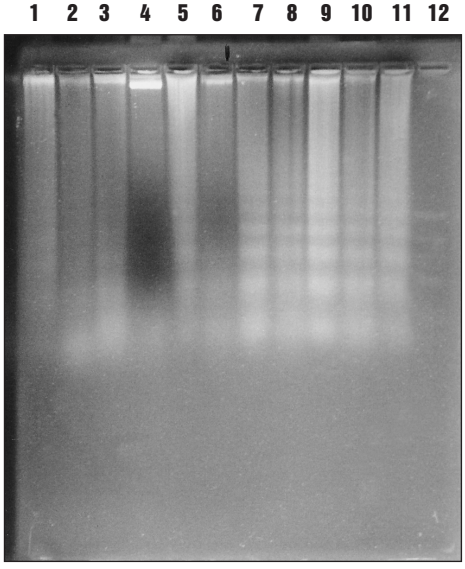

Fig. 4. DNA ladder assay.
Control (runs 1-6) and group with living bacteria (runs 7-11) after 48 h of cultivation.
Run 12- DNA marker (970-155bp).