Spontaneous and induced cytolysis of leukocytes from bovine mammary gland in the course of cultivation *in vitro* – the correlation with neutrophil granulocytes apoptosis

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ABSTRACT: The process of leukocyte cytolysis and the manifestations of apoptosis and secondary necrosis of neutrophil granulocytes (hereafter only "neutrophils") were studied on four virgin heifers after the induction of leukocyte influx into the mammary gland and after their lavage in *in vitro* conditions. Phosphate buffered saline, muramyl dipeptide and a lipopolysaccharide were used for influx induction. Cytolysis and apoptosis were induced with heat stress, ultraviolet irradiation and spontaneous aging for 24 hours. The cytolysis was detected indirectly by determining the lactate dehydrogenase activity in the cultivation medium after the enzyme was released through cell lysis. The neutrophil apoptosis was detected using flow cytometry and two staining methods (i) simultaneous staining with Annexin V labelled FITC and propidium iodide and (ii) with SYTO 13. It was found that leukocytes of the mammary gland of virgin heifers undergo spontaneous aging during *in vitro* incubation. The fraction of lysed leukocytes rose in the course of the *in vitro* incubation and reached 21% up to 34% after 4 hours and 73% up to 79% after 24 hours, depending on the inductor of influx used. From among them, phosphate buffered saline resulted in the lowest incidence of cytolysis, the lipopolysaccharide in the highest incidence. The differences in the effect of influx inductors on leukocyte cytolysis became manifest during the first 4 hours of incubation in particular; the differences between inductors became insignificant after 24 hours. Heat stress, unlike ultraviolet irradiation, resulted in a significant increase in the fraction of lysed leukocytes. Ultraviolet radiation induced neutrophil apoptosis in a dominant way, while the effect of influx inducers and/or of the staining method used for flow cytometry had no effect. Heat stress also induced neutrophil apoptosis but to a lower extent than ultraviolet irradiation. Spontaneous leukocyte aging during the *in vitro* incubation resulted in an increasing share of apoptotic neutrophils depending on the duration of incubation. An increase in the share of necrotic neutrophils was only significant after influx induction with the lipopolysaccharide, but not after induction with buffered saline. Highly significant correlation between the percentage representation of apoptotic neutrophils and the percentage proportion of lysed leukocytes was shown, both after influx induction with phosphate buffered saline, and with the lipopolysaccharide and after both staining techniques (r = 0.767; 0.932; 0.966; 0.922). Statistically significant correlation was demonstrated between the proportion of necrotic neutrophils and the share of lysed leukocytes only after influx induction with the lipopolysaccharide (r = 0.579; 0.765). After the influx induction with phosphate buffered saline and staining with Annexin V and propidium iodide, statistically significant negative correlation between the percentage share of necrotic neutrophils and the percentage of lysed leukocytes (r = –0.653) was demonstrated. Thus it means that situations can occur when the more leukocytes succumb to cytolysis, the smaller the share of necrotic neutrophils that can be detected with flow cytometry. One can state that the *in vitro* model of parallel quantitative determination of apoptosis and secondary neutrophil necrosis as well as of leukocyte cytolysis was verified.

Keywords: heifers; mammary gland; leukocyte cytolysis; neutrophils; apoptosis; necrosis

Supported by the Grant Agency of the Czech Republic (Grant No. 524/03/1531) and Ministry of Agriculture of the Czech Republic (Project No. MZE 0002716201).

Neutrophil granulocytes (hereinafter only "neutrophils") play an important role in antibacterial defence mechanisms. They migrate from the blood to damaged tissues early in the acute inflammatory response, where they eliminate pathogenic bacteria either within the cell following phagocytosis or outside the cell by releasing toxic mediators after their own cytolysis. The latter function is associated with more or less collateral damage (Simon, 2003).

Neutrophils can be considered as not only effector cells, but also as inflammation mediating cells. They release pro-inflammatory cytokines (Cassatella, 1995) and activate macrophages to secret pro-inflammatory mediators after macrophage phagocytosis of neutrophils undergoing necrosis. Moreover, macrophages produce anti-inflammatory mediators, phagocytosing apoptotic leukocytes (Savill and Fadok, 2000; Savill et al., 2002).

Apoptosis plays an important role in the elimination the neutrophils accumulated in inflammatory tissue without releasing hazardous intracellular contents. Rapid elimination of neutrophils by macrophages following bacterial neutralization is essential to minimizing inflammatory-derived injury to the host in the resolution of mammary gland inflammation (Paape et al., 2003).

The induction of neutrophil apoptosis during the resolution of the neutrophil inflammatory response can be mimicked by culturing the cells in the absence of sufficient concentration of survival factors (Simon, 2003). Neutrophils are predisposed to apoptosis (Raff, 1992) and they also undergo this kind of cell death during *in vitro* cultivation as a consequence of senescence (Savill et al., 1989; Payne et al., 1994). Most studies of this problem have been performed on purified blood neutrophils but only a few studies aimed to understand the process of neutrophil senescence and death after migration into the inflammatory focus (for details see Rysanek et al., 2005; Sladek and Rysanek, 2005). As far as we know, the senescence process of the bovine mammary gland neutrophils *in vitro* has not yet been studied with respect to the neutrophil cytolysis.

It has been well known that ultraviolet irradiation and heat shock induce programmed cell death in human neutrophils *in vitro* (Watson et al., 1996; Sweeney et al., 1997; Callahan et al., 1999; Zheng et al., 2004). The possibility of using physical agents as positive controls in *in vitro* experiments prompted us to study the effects of these agents on the leukocyte lysis and on the induction of neutrophil apoptosis and of their secondary necrosis under *in vitro* conditions.

Recently we have made morphological observations that leukocytes undergo exocytosis and spontaneous cytolysis after the phagocytosis of *Staphylococcus aureus* bacteria (Rysanek and Sladek, 2006). Therefore, and also because we planned *in vitro* studies targeted on interactions of neutrophils and macrophages with pathogenic bacteria, we decided to investigate the influence of selected biological agents, physical agents and natural neutrophil aging on leukocyte cytolysis and on the manifestations of neutrophil apoptosis and secondary necrosis during cultivation *in vitro*. The aim of this study was to elucidate the influence of certain leukocyte influx inducers on the life span of mammary gland neutrophils and on the moderation of leukocyte cytolysis in the course of *in vitro* cultivation. The effects of heat treatment and ultraviolet irradiation on the induction of neutrophil apoptosis, necrosis and leukocyte cytolysis as potential positive controls were studied. The correlations between percentages of neutrophil apoptosis, secondary necrosis and leukocyte cytolysis were also the matter of our interest.

MATERIAL AND METHODS

Animals and experimental design. Three experiments (at two-week intervals) were carried out involving one group of 4 clinically normal, Holstein × Bohemian Red Pied crossbred virgin heifers aged 14 to 15 months. The heifers were free of intramammary infection, as demonstrated by bacteriological examinations of mammary lavages collected before every replication of the experiments. The heifers were housed in a certificated experimental tie-stall, and fed a standard ration. The animals were cared for in compliance with the good care practice protocol.

Each of the experiments was performed using one of three different leukocyte influx inductors. The leukocyte viability, the expression of neutrophil apoptosis, secondary necrosis and cytolysis were studied in the course of 24 hours of *in vitro* cell cultivation.

Inducers of leukocytes migration. The three inducers used were:

(i) Sterile phosphate-buffered saline solution – PBS (0.01M; pH 7.4; NaCl 0.138M; KCl 0.0027M, prepared with apyrogenic water) – treatment dose: 20 ml/quarter;

(ii) Sterile solution of synthetic muramyl dipeptide analogue – MDP (MurNAc-L-Abu-D-IsoGln)

(Institute of Organic Chemistry and Biochemistry, Czech Academy of Science, Czech Republic) – treatment dose: 500 μg in 20 ml of PBS;

(iii) Sterile lipopolysacharide solution – LPS from *Escherichia coli* serotype 0128:B12, (Sigma, MO, USA) – treatment dose: 5 μg in 20 ml of PBS.

Induction of leukocyte migration and the cell suspension collection. Modified urethral catheters (AC53061H06, Porges, France) were inserted into the teat canal after thorough disinfection of the teat orifice with 70% ethanol. Through the catheter, each mammary quarter was rinsed with PBS, and the lavages were collected through the same catheter to a syringe as samples for bacteriological examination. The catheter was then used to administer the respective influx inducer. The same procedure was used 18 h later after the administration of inducers for the collection of samples of leukocyte suspensions used for the examination.

Cell processing and *in vitro* cultivation. The lavages obtained before induction were examined bacteriologically by cultivation on blood agar plates (5% washed sheep erythrocytes) and aerobic incubation at 37°C for 24 hours. No bacterial infections were detected in the mammary gland quarters examined in the experiments. (The results of cell suspension samples with bacterial contamination were excluded from statistical processing.) The cell lavages were centrifuged at 4°C and 200 × g for 10 min and washed with PBS. The resulting pellets were resuspended in the RPMI-1640 medium (Sigma, MO, USA) and adjusted to the final dilution of 1.5×10^6 neutrophils per 1 ml using a haemocytometer and appropriate dilution. The usually used supplementation of the RPMI 1640 medium with foetal bovine serum (5% vol/vol) was replaced by bovine serum albumin supplementation (1% w/v) RPMI 1640 in order to reduce the background lactate dehydrogenase activity (LDH), as recommended by the manufacturer.

Adjusted suspensions were inserted into 6 × 4 microplates (Costar® 24 Well Clear Flat Bottom Ultra Low Attachment Microplates, Headquarters, NY, USA) and cultivated for 24 h with experimental time points: 0; 4 h and 24 h at 37°C in a 5% CO_2 atmosphere in a moist chamber.

Separate microplates were used for physical inducers of cell death – heat treatment (HT) (immersion incubation at 60°C in water bath for 30 min); ultraviolet irradiation (UT) with a germicide lamp (30 W) for 30 min at the distance of 50 cm. The microplates with cell suspensions were cultivated for 30 min at 37°C in a 5% CO_2 atmosphere in a moist chamber.

After the cultivation, the microplates were centrifuged at 250 × g for 10 minutes. The supernatants were then carefully removed and transferred (100 μl/well) to a 96-well flat-bottom microtiter plate (MTP) (GAMA, Ceske Budejovice, Czech Republic). To determine the LDH activity in these supernatants, 100 μl of reaction mixture was added to each well and the MTPs were incubated for 30 min at laboratory temperature, protected from light.

Total cell counts and cell viability. The trypan blue dye exclusion test and a haemocytometer were used. In brief: After thorough resuspension of cells adhering in microplate-wells, 100 μl of cell suspension were treated with 20 μl of trypan blue stain at each experimental time interval. Cell counts and the percentage of dead leukocytes were determined by counting the cells in 20 large squares in the haemocytometer. The percentages of trypan blue positive cells amounted to 2.5 to 7.5% in control fresh cell suspensions.

Microscopic determination of differential leukocyte counts – the percentage of neutrophils. Immediately after the cultivation (30 min), cells adhering to the slides were stained by the Pappenheim method. To view the preparations, the JENAMED 250 CF light microscope with a 100× oil-immersion plan achromate objective (Carl Zeis, Germany) was used. Differential cell counts were determined by evaluating at least 200 cells. The percentage of neutrophils after influx induction by PBS, MDP and LPS were (mean ± SD) 78.80 ± 6.30; 82.15 ± 6.51 and 89.45 ± 5.34, respectively.

The quantification of the mammary leukocyte cytolysis. The Cytotoxicity Detection Kit (LDH) (Roche Diagnostic GmbH, Panzberg, Germany) was used. The colorimetric assay for the quantification of cell death and cell lysis, based on the measurement of lactate dehydrogenase activity released from the cytosol of damaged cells into the supernatant was used. The increase in the level of enzyme activity in the supernatant directly correlates with the amount of formazan formed during an enzymatic test in which LDH catalyses the conversion of lactate to pyruvate and, in the second step, to a tetrazolium salt which is reduced to formazan. The formazan dye formed is water-soluble and shows a broad absorption maximum at about 500 nm whereas the tetrazolium salt shows no significant absorption at these wavelengths.

The microtiter plate reader, the Sunrise photometer (Tecan Group Ltd., Männedorf, Switzerland), and the wavelength of 492 nm (reference wavelength of 630 nm) were used.

The high control (HC) and low (LC) control were used in accordance with the manufacturer's recommendation. The maximum amount of releasable LDH enzyme activity (HC = 100%) was determined by lysing fresh cells with Triton X-100 (final concentration of 1% v/v). The background LDH activity (LC) was determined in supernatants of fresh cell suspensions and expressed as percentage of HC. All measurements were performed in triplicate.

To determine the cytolysis, the average absorbance values of triplicate measurements were calculated, and from each of these the background absorbance values were subtracted. The resulting values were substituted into the following equation:

$$\text{Cytolysis (\%)} = \frac{\text{experimental value} - \text{low control}}{\text{high control} - \text{low control}} \times 100$$

In a preliminary experiment, the linearity of the relationship was determined for the following variables: lysed cell counts (cells/ml) and LDH activity (absorbance) (Figure 1). The correlation coefficient was determined as $r = 0.998$.

Flow cytometric (FCM) assessment of neutrophil apoptosis and secondary necrosis. Apoptotic and secondary necrotic apoptotic neutrophils were enumerated by FCM (FACScan, Becton Dickinson Immunocytometry Systems, San Marcos, Ca, USA). Two different staining procedures were used: Simultaneous staining with Annexin V labelled with fluorescein isothiocyanate (FITC) and propidium iodide (PI) as described by Vermes et al. (1995) and SYTO 13 staining as described by Dosogne et al. (2003).

The commercial Annexin-V-FLUOS Staining Kit (Boehringer Mannheim, GmbH, Mannheim, Germany) was used according to the manufacturer's instructions. After the staining, the suspension was analysed by FCM by counting at least 10 000 cells. Dot plots were analysed by a procedure described elsewhere (Sladek et al., 2001) and evaluated qualitatively and quantitatively using the CellQuest software analysis (Beckton Dickinson; Mountain View, CA; USA).

The commercial SYTO 13 green fluorescent nucleic acid stain (Molecular Probes, Eugene, OR, USA) was used as described by Dosogne et al. (2003) in a slight modification: 490 µl of cell suspension in RPMI 1640 was stained with 10 µl of diluted (1:40) SYTO 13 solution. After incubation at laboratory temperature in the dark for 15 min, the specimens were placed on ice and stored up to the FCM measurement. The dot plots were analysed by a procedure shown on Figure 2. The analysis of neutrophil apoptosis and secondary necrosis was performed by back gating.

Cell suspensions resuspended after removing the supernatants assessment were used for the determination of apoptosis and secondary necrosis.

Statistics. Before statistical analysis, the outlying values were excluded. The logarithmic transformation of cell counts was performed (with logarithms base 10). For the detection of statistical differences between the control arithmetic mean and the mean

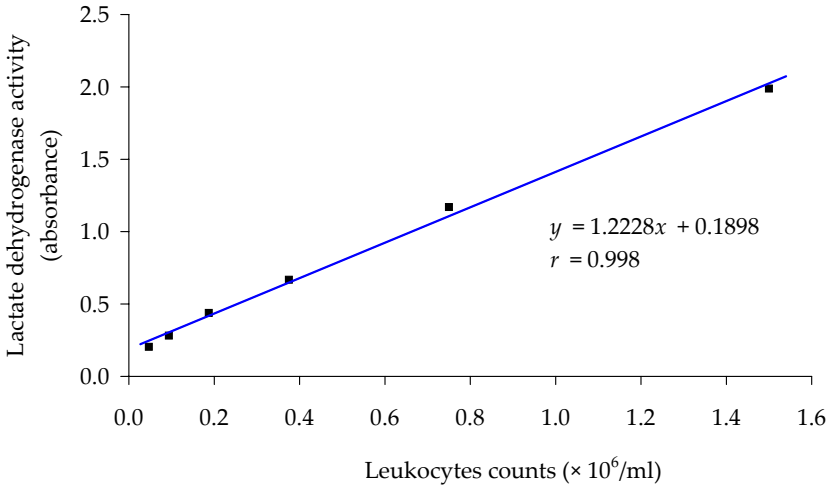

Figure 1. Correlation of leukocytes counts and activity of lactate dehydrogenase released by lysed leukocytes. Fresh mammary gland leukocytes were adjusted to 1.5 × 10^6/ml using haemocytometer. Two-fold dilutions were performed. Leukocytes were lysed by Triton X-100 (1%). The lactate dehydrogenase activities were determined by colorimetric assay end express in absorbance values. Correlation coeficient $r = 0.998$

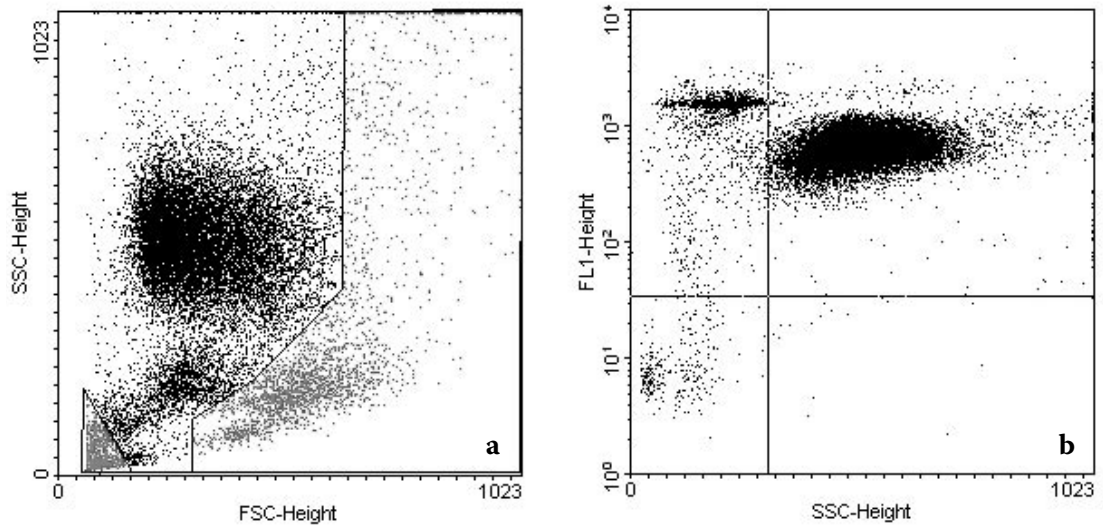

Figure 2. Leukocytes in flow cytometry. Figure (**a**) represent dot plot of leukocytes gated for scatter parameters (FSC/SSC). Subpopulation of neutrophils (black) obtained by backgating technique is represented on dot plot (b) with SSC/FL1 axes. The bit map (**b**) is set for neutrophils only. Upper left apoptotic neutrophills (12.5%) and low left necrotic neutrophils (2.1%) are discriminated

values of experimental variables, the Student's *t*-test or paired *t*-test were used with the support of Microsoft Excel XP (Microsoft Corp., Redmond, Washington D.C., USA) software. The correlation analysis was performed with the support of the same software.

RESULTS

Direct microscopy – trypan blue test

Exposure of mammary gland leukocytes to a temperature of 60°C for 30 min (HT), as well as the exposure to ultraviolet radiation for 30 min (UT) *in vitro* resulted in a fall in the cell number and in an increased percentage of trypan blue positive cells in comparison with the controls (Table 1). The reduction in the leukocyte count after heat stress was statistically highly significant after influx induction with PBS, but it was not statistically significant if the leukocyte influx was induced with MDP or LPS, probably owing to the greater value variability in these experiments. Heat exposure increased the number of trypan blue positive leukocytes significantly more than ultraviolet irradiation of the cells ($HT_{PBS; MDP; LPS}$, $P < 0.01$; $UV_{PBS; MDP}$, $P < 0.05$; UV_{LPS}, $P > 0.05$).

Also, spontaneous leukocyte aging *in vitro* resulted in a reduction in the leukocyte number and in an increase in the representation of trypan blue positive leukocytes in comparison with the controls, dependent on the time of incubation (Table 2). The fall in the leukocyte number was statistically highly significant after influx induction with PBS or MDP, but only statistically significant in experiments with the induction of leukocyte influx with LPS ($4h_{PBS}$; $24h_{PBS}$; $4h_{MDP}$; $24h_{MDP}$, $P < 0.01$; $4h_{LPS}$; $24h_{LPS}$, $P < 0.05$). The increase in the percentage share of trypan blue positive leukocytes in comparison with the control was only statistically significant ($4h_{PBS}$; $24h_{PBS}$; $24h_{MDP}$; $24h_{LPS}$, $P < 0.05$), or insignificant ($4h_{MDP}$; $4h_{LPS}$, $P > 0.05$).

Cytolysis – lactate dehydrogenase activity

The effect of heat stress and ultraviolet irradiation was also studied on the basis of cytolysis determined indirectly as LDH activity released from the lysed cells into the cultivation medium and expressed as the percentage share of lysed cells (Table 3). The statistically highly significant increase in the percentage share of cytolysis after HT was demonstrated ($P < 0.01$). The effect of UT was not statistically significant ($P > 0.05$). The leukocyte influx inductors used did not specifically affect the significance of the differences between the two experimental treatments.

Spontaneous leukocyte aging *in vitro* resulted in a statistically highly significant increase in the percentage of cells undergoing cytolysis ($P < 0.01$) at both time points of the monitoring (4 h and 24 h) (Table 4).

Table 1. Effects of heat treatment and ultraviolet irradiation on leukocytes number and percentage of death leukocytes in course of *in vitro* cultivation

Control			Experimental variables				Statistics			
n	a. m.	SD		*n*	a. m.	SD	$t_{tab\ (0.05)}$	$t_{tab\ (0.01)}$	*t*	S
Number of cells										
Leukocytes influx induced by phosphate buffered saline										
4	6.444	0.076	HT	4	6.323	0.039	3.182	5.841	5.982	++
			UT	4	6.296	0.093	3.182	5.841	3.658	+
Leukocytes influx induced by muramyl dipeptide										
4	5.888	0.079	HT	4	5.757	0.083	3.182	5.841	2.311	–
			UT	4	5.668	0.046	3.182	5.841	8.452	++
Leukocytes influx induced by lipopolysaccharide										
4	6.004	0.083	HT	4	5.873	0.119	3.182	5.841	2.936	–
			UT	4	5.846	0.065	3.182	5.841	15.981	++
Percentage of trypan blue positive (death) cells										
Leukocytes influx induced by phosphate buffered saline										
4	2.545	0.933	HT	4	91.813	2.144	3.182	5.841	60.872	++
			UT	4	7.555	3.798	3.182	5.841	3.293	+
Leukocytes influx induced by muramyl dipeptide										
4	2.935	3.252	HT	4	94.393	3.851	3.182	5.841	91.141	++
			UT	4	5.343	2.522	3.182	5.841	5.258	+
Leukocytes influx induced by lipopolysaccharide										
4	7.515	2.346	HT	4	100.00	0.000	3.182	5.841	78.833	++
			UT	4	13.988	5.252	3.182	5.841	2.405	–

n = number; a. m. = arithmetic mean; SD = standard deviation; S = significance;
HT = heat treatment; UT = ultraviolet irradiation

The significance of the difference in leukocyte cytolysis between different leukocyte influx inductors was tested in the process of spontaneous cell aging *in vitro* (Table 5). Statistically significant differences between influx inductors were recorded only after 4 h of cell incubation (PBS × MDP; PBS × LPS, $P < 0.01$; MDP × LPS, $P < 0.05$). The lowest level of cytolysis was recorded after influx induction with PBS, the highest cytolysis after influx induction with LPS. For fresh leukocytes (time point 0) or for leukocytes after 24 h of incubation (time point 24 h), no statistically significant difference between influx inductors was recorded with respect to the leukocyte cytolysis ($P > 0.05$).

Flow cytometry

Manifestations of apoptosis and necrosis of neutrophilic leukocytes as shown by flow cytometry (FCM) were studied on leukocytes obtained by influx induction with PBS and LPS (Tables 6 and 7), namely by the action of HT, UT and spontaneous aging after 4 h and 24 h incubation.

After leukocyte influx induction with PBS and after staining with FITC Annexin V + PI and SYTO 13, a highly statistically significant increase in the percentage share of apoptotic neutrophils was demonstrated after exposure to UT and after 24 h cell incubation ($P < 0.01$) as distinct from the control. After SYTO 13 staining, a statistically highly significant increase in the representation of apoptotic neutrophils both after exposure to UT, and to HT, and also after the 4 h and 24 h incubation in contrast to the control ($P < 0.01$) was demonstrated (Table 6).

A statistically significant increase in the share of necrotic neutrophils in comparison with the control could only be demonstrated after staining with FITC Annexin V + PI plus SYTO 13 (HT, $P < 0.01$; UT, $P < 0.05$), but not after staining with SYTO 13.

Table 2. Effects of spontaneous aging on leukocytes number and percentage of death leukocytes in course of *in vitro* cultivation

Control			Experimental variables				Statistics			
n	a. m.	SD		*n*	a. m.	SD	$t_{tab\ (0.05)}$	$t_{tab\ (0.01)}$	*t*	S
Number of cells										
Leukocytes influx induced by phosphate buffered saline										
4	6.444	0.076	4 h	4	6.315	0.071	3.182	5.841	7.649	++
			24 h	4	6.241	0.037	3.182	5.841	7.751	++
Leukocytes influx induced by muramyl dipeptide										
4	5.888	0.079	4 h	4	5.647	0.083	3.182	5.841	6.433	++
			24 h	4	5.503	0.098	3.182	5.841	8.237	++
Leukocytes influx induced by lipopolysaccharide										
4	6.004	0.083	4 h	4	5.963	0.069	3.182	5.841	3.729	+
			24 h	4	5.849	0.082	3.182	5.841	3.763	+
Percentage of trypan blue positive (death) cells										
Leukocytes influx induced by phosphate buffered saline										
4	2.545	0.933	4 h	4	11.158	4.334	3.182	5.841	4.618	+
			24 h	4	27.178	13.277	3.182	5.841	3.881	+
Leukocytes influx induced by muramyl dipeptide										
4	2.935	3.253	4 h	4	3.015	2.175	3.182	5.841	0.061	–
			24 h	4	32.095	11.639	3.182	5.841	5.755	+
Leukocytes influx induced by lipopolysaccharide										
4	7.515	2.346	4 h	4	10.878	3.195	3.182	5.841	1.424	–
			24 h	4	43.960	14.002	3.182	5.841	4.476	+

n = number; a. m. = arithmetic mean; SD = standard deviation; S = significance
4 h = spontaneous aging for 4 hours; 24 h = spontaneous aging for 24 hours

Table 3. Effects of heat treatment and ultraviolet irradiation on leukocytes cytolysis in course of *in vitro* cultivation

Control			Experimental variables				Statistics			
n	a. m.	SD		*n*	a. m.	SD	$t_{\text{tab (0.05)}}$	$t_{\text{tab (0.01)}}$	*t*	S
Leukocytes influx induced by phosphate buffered saline										
3	11.07	0.86	HT	9	56.84	9.11	2.228	3.169	14.871	++
			UT	8	9.36	1.47	2.262	3.250	1.849	–
Leukocytes influx induced by muramyl dipeptide										
4	10.82	2.30	HT	11	53.17	10.20	2.160	3.010	12.890	++
			UT	5	8.56	1.76	2.365	3.450	1.678	–
Leukocytes influx induced by lipopolysaccharide										
5	9.56	1.86	HT	2	40.00	2.69	2.571	4.032	17.706	++
			UT	6	7.43	1.28	2.262	3.250	2.248	–

n = number; a. m. = arithmetic mean; SD = standard deviation; S = significance
HT = heat treatment; UT = ultraviolet irradiation

Table 4. Effects of spontaneous cell aging on leukocytes cytolysis in course of *in vitro* cultivation

Control			Experimental variables				Statistics			
n	a. m.	SD		*n*	a. m.	SD	$t_{tab\ (0.05)}$	$t_{tab\ (0.01)}$	*t*	S
Leukocytes influx induced by phosphate buffered saline										
3	11.07	0.86	4 h	8	21.40	3.77	2.262	3.250	4.559	++
			24 h	9	74.87	13.02	2.228	3.169	14.608	++
Leukocytes influx induced by muramyl dipeptide										
4	10.82	2.30	4 h	7	28.74	4.03	2.262	3.250	8.063	++
			24 h	12	79.31	14.58	2.140	2.980	15.692	++
Leukocytes influx induced by lipopolysaccharide										
5	9.56	1.86	4 h	9	34.43	4.07	2.179	3.055	12.751	++
			24 h	12	73.00	13.18	2.130	2.950	16.288	++

n = number; a. m. = arithmetic mean; SD = standard deviation; S = significance
4 h = spontaneous aging for 4 hours; 24 h = spontaneous aging for 24 hours

After leukocyte influx induction with LPS (Table 7) and after staining with FITC Annexin V + PI and SYTO 13, a highly statistically significant increase in the percentage proportion of apoptotic neutrophils caused by HT, UT and by 4 h and 24 h cell incubation by contrast to the control was demonstrated ($P < 0.01$).

After leukocyte influx induction with LPS and after staining with FITC Annexin V + PI, we succeeded in proving a statistically significant increase in the share of necrotic neutrophils in comparison with the control after the action of HT or UT ($P < 0.01$), and after 4 h and 24 h incubation ($P < 0.05$). After SYTO 13 staining, a statistically significant increase in necrotic neutrophils in contrast to the control was observed only owing to the action of HT ($P < 0.01$) and to the effect of the 24 h incubation ($P < 0.05$).

Correlation analysis

The percentage of apoptotic and necrotic neutrophilic leukocytes correlated with the percentage of lysed cells as studied on leukocytes obtained by influx induction with PBS and LPS, namely during spontaneous aging after 0, 4 and 24 hours of incubation (Table 8).

A statistically highly significant correlation between the percentage share of apoptotic cells and the share of lysed leukocytes after PBS or LPS influx induction and after staining with FITC Annexin V + PI and SYTO 13 was demonstrated. Statistically significant positive correlation between the share of necrotic neutrophils and the share of lysed leukocytes after influx induction with LPS and staining with FITC Annexin V + PI and SYTO 13 was also shown.

Seemingly paradoxical results of the correlation analysis were obtained after influx induction with PBS. After staining with SYTO 13, no statistically significant correlation could be demonstrated. After staining with FITC Annexin V + PI, a statistically significant correlation between the share of necrotic neutrophils and the share of lysed leukocytes was even demonstrated.

The results obtained involve the following conclusions: The leukocytes of the mammary gland of virgin heifers obtained by induced influx undergo cytolysis during *in vitro* incubation due to spontaneous aging accompanied by cytolysis. The share of lysed leukocytes increases with the time of incubation *in vitro* and it reaches 21% up to 34% after 4 h and 73% up to 79% after 24 h, depending on the influx inductor used. Among the influx inductors, PBS resulted in the lowest incidence of cytolysis, LPS in the highest incidence. Differences in the effect of influx inductors on leukocyte cytolysis became manifest in the first 4 h of incubation; at 24 h, the differences between inductors became insignificant. HT, unlike UT, resulted in a significant increase in the proportion of lysed leukocytes. UT induced neutrophil apoptosis in a dominant way with no effect of the influx inductors and of the staining method used (FITC Annexin V + PI/SYTO 13). HT also induced neutrophil apoptosis but to a lesser extent than the UT. Spontaneous

Table 5. Effects of certain inducers of leukocytes influx on leukocytes cytolysis during spontaneous aging in course of *in vitro* cultivation

Data Files	Data File 1			Data File 2			Statistics			
	n	a. m.	SD	*n*	a. m.	SD	$t_{tab\ (0.05)}$	$t_{tab\ (0.01)}$	*t*	S
Time 0										
PBS × MDP	3	11.07	0.86	4	10.82	2.30	2.571	4.032	0.170	–
PBS × LPS	3	11.07	0.86	5	9.56	1.86	2.447	3.707	1.289	–
MDP × LPS	4	10.82	2.30	5	9.56	1.86	2.365	3.499	0.914	–
Time 4 hours										
PBS × MDP	8	21.40	3.77	7	28.74	4.03	2.160	3.012	3.648	++
PBS × LPS	8	21.40	3.77	9	34.43	4.07	2.131	2.947	6.816	++
MDP × LPS	7	28.74	4.03	9	34.43	4.07	2.145	2.298	2.785	+
Time 24 hours										
PBS × MDP	9	74.88	13.02	12	79.31	14.58	2.093	2.861	0.720	–
PBS × LPS	9	74.88	13.02	12	73.00	13.18	2.093	2.861	0.325	–
MDP × LPS	12	79.31	14.58	12	73.00	13.18	2.074	2.819	1.112	–

PBS = phosphate buffered saline; MDP = muramyl dipeptide; LPS = lipopolysaccharide
n = number; a. m. = arithmetic mean; SD = standard deviation; S = significance

leukocyte aging during *in vitro* incubation resulted in an increase in the share of apoptotic neutrophils increasing with the duration of the incubation. The rise in the proportion of necrotic neutrophils was significant only after influx induction with LPS, not after induction with PBS. Highly significant positive correlation between the percentage representation of apoptotic neutrophils and the proportion of lysed leukocytes was demonstrated. Statistically significant positive correlation was shown to exist between the share of necrotic neutrophils and the proportion of lysed leukocytes only after influx induction with LPS. After influx induction with PBS, a statistically significant negative correlation between the percentage share of necrotic neutrophils and the percentage of lysed leukocytes was recorded. This means that situations may come up in which the more leukocytes succumb to cytolysis, the smaller the part of necrotic neutrophils that can be detected with FCM.

DISCUSSION

The current paradigm states that inflammatory cells disappear from inflammatory tissues through apoptosis and phagocytosis. However, cells may also be cleared through primary cytolysis (Uller et al., 2004). We have stated (Rysanek and Sladek, 2006), that leukocytes were often dying due to cytolysis in the area of inflammation during which coalescent spots (confluents) were formed from the released cell contents. In such spots the process of digestion of the phagocytosed bacteria and neutrophils, released from macrophages, continued. This shows that the leukocyte cytolysis has an important role in the inflammatory process. Therefore we have focused this study on it.

As far as we know, the senescence process of the bovine mammary gland neutrophils *in vitro* has not yet been studied with respect to the neutrophil cytolysis.

Special interest was paid to the cytolysis of bovine neutrophils only in studies devoted to leukotoxin of *Mannheimia* (*Pasteurella*) *haemolytica*, which specifically kills the leukocytes of ruminants (for details see Leite et al., 2002).

The results of this study have shown that, during the *in vitro* incubation of leukocytes, the number of cells falls due to cytolysis, namely both when the cell number is determined directly, and when it is determined indirectly as LDH activity in the medium. No doubt, the cause of this is the aging of neutrophils after the supplies of energy and nutrients are used up, as stated by Savill et al. (1989) and Payne et al. (1994). Undoubtedly, even histotoxic substances released in the process of cytolysis can have an effect. In agreement with this fact and with this study is the demonstrated increase in trypan-

Table 6. Effects of heat treatment, ultraviolet irradiation and spontaneous cell aging on flow cytometry detection of neutrophil apoptosis and necrosis in course of *in vitro* cultivation after leukocytes influx induction by phosphate buffered saline

Control			Experimental variables				Statistics			
n	a. m.	SD		*n*	a. m.	SD	$t_{tab\ (0.05)}$	$t_{tab\ (0.01)}$	*t*	S
Apoptosis of neutrophils										
Annexin-V and Propidium iodide										
4	23.03	1.94	HT	4	29.83	10.18	2.447	3.707	1.313	–
			UT	4	65.08	0.63	2.447	3.707	41.275	++
			4 h	8	21.24	3.80	2.228	3.169	0.871	–
			24 h	8	67.81	7.80	2.228	3.169	15.326	++
SYTO 13										
4	11.10	1.50	HT	4	31.50	5.61	2.447	3.707	7.030	++
			UT	4	74.08	6.25	2.447	3.707	19.584	++
			4 h	8	25.31	3.87	2.228	3.169	6.948	++
			24 h	8	66.10	6.01	2.228	3.169	24.397	++
Necrosis of Neutrophils										
Annexin-V and Propidium iodide										
4	14.00	4.62	HT	4	50.10	5.73	2.447	3.707	9.808	++
			UT	4	6.33	4.11	2.447	3.707	2.482	+
			4 h	8	11.64	1.87	2.228	3.169	0.983	–
			24 h	8	6.86	1.73	2.228	3.169	2.985	–
SYTO 13										
4	4.32	2.29	HT	4	3.98	3.35	2.447	3.707	0.256	–
			UT	4	4.38	1.67	2.447	3.707	0.042	–
			4 h	8	11.49	6.18	2.228	3.169	2.201	–
			24 h	8	9.85	7.51	2.228	3.169	1.409	–

n = number; a. m. = arithmetic mean; SD = standard deviation; S = significance

HT = heat treatment; UT = ultraviolet irradiation; 4 h = spontaneous aging for 4 hours; 24 h = spontaneous aging for 24 hours

blue positive leukocytes, increasing with the duration of the incubation. The high percentage share of lysed leukocytes after 24 h of *in vitro* incubation comes as a surprise. It means that a selection of cells takes place whereby the actually present leukocytes are probably more vital that those succumbing to cytolysis. The actually present neutrophils then undergo apoptotic death and secondary necrosis, reflected in the FCM picture.

The high percentage share of lysed leukocytes may also be effected by the substitution of the bovine fetal serum used for the supplementation of the cultivation medium with bovine serum albumin of low concentration, which was done on the basis of the manufacturer's recommendation in order to prevent interference of the high LDH background level with the measurement. Actually it has been known that the absence of the blood serum in the cultivation medium significantly increases the proportion of apoptotic neutrophils during cultivation for 24 hours (Guejes et al., 2003).

This study has confirmed that spontaneous leukocyte aging during *in vitro* cultivation results in an increasing proportion of apoptotic neutrophils depending on the time of incubation, as it has been known that spontaneous apoptosis resulting from natural aging of these cells had been demonstrated first in *in vitro* cultivations of human blood neutrophils (Savill et al., 1989) and also bovine blood neutrophils and mammary gland neutrophils (Van Oostveldt et al., 1999, 2001; Chin et al., 2000; Sladek et al. 2002; Rysanek et al., 2005). It has also been found that apoptosis expression was directly proportional to the length of neutrophil cultivation

Table 7. Effects of heat treatment, ultraviolet irradiation and spontaneous cell aging on flow cytometry detection of neutrophil apoptosis and necrosis in course of *in vitro* cultivation after leukocytes influx induction by lipopolysaccharide

Control			Experimental variables				Statistics			
n	a. m.	SD		*n*	a. m.	SD	$t_{tab\ (0.05)}$	$t_{tab\ (0.01)}$	*t*	S
Apoptosis of neutrophils										
Annexin-V and Propidium iodide										
4	6.10	2.07	HT	4	23.88	4.35	2.447	3.707	7.380	++
			UT	4	42.48	7.47	2.447	3.707	9.391	++
			4 h	8	14.20	4.45	2.228	3.169	3.397	++
			24 h	8	65.40	5.56	2.228	3.169	20.227	++
SYTO 13										
4	13.58	3.69	HT	4	47.00	7.74	2.447	3.707	7.798	++
			UT	4	55.25	7.72	2.447	3.707	9.738	++
			4 h	8	27.88	6.57	2.228	3.169	3.985	++
			24 h	8	54.35	11.88	2.228	3.169	6.566	++
Necrosis of neutrophils										
Annexin-V and Propidium iodide										
4	5.88	2.83	HT	4	53.80	7.51	2.447	3.707	11.940	++
			UT	4	30.38	8.97	2.447	3.707	5.209	++
			4 h	8	10.98	3.13	2.228	3.169	2.740	+
			24 h	8	11.10	3.86	2.228	3.169	2.384	+
SYTO 13										
4	3.98	1.96	HT	4	43.10	6.02	2.447	3.707	12.349	++
			UT	4	6.43	1.82	2.447	3.707	1.830	–
			4 h	8	4.95	1.51	2.228	3.169	0.960	–
			24 h	8	10.94	5.00	2.228	3.169	2.630	+

n = number; a. m. = arithmetic mean; SD = standard deviation; S = significance
HT = heat treatment; UT = ultraviolet irradiation; 4 h = spontaneous aging for 4 hours; 24 h = spontaneous aging for 24 hours

(Savill et al., 1989; Payne et al., 1994; Van Oostveldt et al., 1999; Sladek et al. 2002).

We have demonstrated that HT results in a significant increase in lysed leukocytes, and, in the microscopic picture, in an increase of trypan-blue positive leukocytes reaching up to 100% of all cells. The UT has not caused significant leukocyte lysis; nevertheless it leads to a significant increase in trypan-blue positive cells. We have not been able to find data relevant to this finding in the literature.

Ultraviolet irradiation induces neutrophil apoptosis in a dominant way. The heat stress also induces apoptosis of neutrophils, but to a lesser extent than ultraviolet irradiation. These data are consistent with the data by Watson et al. (1996), who have demonstrated that heat shock is an inductor of neutrophil apoptosis, and with data of Callahan et al. (1999) who have demonstrated a significant early increase in the rate of apoptosis in the cells subjected to the hyperthermic treatment and with data by Sweeny et al. (1997) and Zheng et al. (2004), state that UT induces apoptosis of neutrophils.

Our findings can also be utilised for a selection of a positive control in *in vitro* studies. The UT seems to be best suited for this purpose.

The different effect of the leukocyte influx inductors in the process of cell aging *in vitro* was recorded only after 4 hours of incubation in this study. The lowest degree of cytolysis was recorded after influx induction with PBS, the highest cytolysis after influx induction with LPS. These findings cannot be confronted with the data in the literature, as such data are not available. Nevertheless, the higher level of neutrophil cytolysis can be explai-

Table 8. Correlation among results of flow cytometry detections of neutrophil apoptosis, secondary necrosis and results of leukocytes cytolysis detected by lactate dehydrogenase activity after leukocytes influx induction by phosphate buffered saline and lipopolysaccharide

Influx inductors	Staining	Apoptosis Necrosis	Correlation coefficient	Regression slope	*P*	S
PBS	Annexin-V + Propidium iodide	apoptosis	0.767	0.9014	0.00359	++
		necrosis	–0.653	–4.2485	0.021432	+
	SYTO 13	apoptosis	0.932	0.9321	0.000084	++
		necrosis	0.312	1.1701	0.379520	–
LPS	Annexin-V + Propidium iodide	apoptosis	0.966	1.2211	0.0000003	++
		necrosis	0.579	5.1601	0.048325	+
	SYTO 13	apoptosis	0.922	1.7202	0.000020	++
		necrosis	0.765	6.0092	0.003739	++

P = probability; S = significance

ned by the fact that LPS, unlike PBS, results in a more intensive neutrophil activation in the influx induction. Neutrophils release reactive oxygen species and proteolytic enzymes during the activation process (Guthrie et al. 1984; Hughes et al. 1997). These substances have histotoxic effects and thus they potentiate the cytolysis.

It is not surprising that highly significant correlation between the percentage representation of apoptotic neutrophils and the percentage share of lysed leukocytes was demonstrated in this study. Significant positive correlation was demonstrated between the share of necrotic neutrophils and the share of lysed leukocytes. More surprisingly, statistically significant negative correlation between the percentage of necrotic neutrophils and the percentage of lysed leukocytes after influx induction with PBS was found. This can mean that situations may occur under which the more leukocytes are subject to cytolysis, the smaller the proportion of necrotic neutrophils that can be detected by flow cytometry.

Moreover, we have verified an *in vitro* model for parallel quantitative determination of apoptosis and secondary neutrophil necrosis as well as of primary neutrophil necrosis that can be used in the study of interactions of these immunocompetent cells and pathogenic bacteria.

Acknowledgements

The authors thank Ing. L. Leva, Mrs M. Knakalova for technical assistance.

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Received: 2005–12–11
Accepted after corrections: 2006–04–20

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