Acta vet. scand. 1987, *28*, 333–342.

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Flow Cytometric Measurement of Bovine Milk Neutrophil Phagocytosis

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Saad, A. M.: Flow cytometric measurement of bovine milk neutrophil phagocytosis. Acta vet. scand. 1987, 28, 333–342. – A flow cytometric method for the evaluation of the phagocytic capacity of bovine milk polymorphonuclear neutrophils (PMN) is described. Milk PMN were isolated from stripping milk collected from udder quarters fitted with abraded intramammary devices (AIMD). A significant increase in the milk somatic cell count was observed in the stripping milk after the insertion of AIMD (308×10^3 and 1447×10^3 cells/ml milk before respectively after the insertion of the AIMD, $p < 0.001$).

PMN were also isolated from blood by a discontinous gradient of Percoll. Blood and milk PMN were incubated for 15 min with FITC-labeled bacteria in a ratio of 1 PMN:20 bacteria and a final serum concentration of 10 %. The number of extracellular bacteria and the percentage of phagocytic cells were measured by a flow cytometer.

Percentage of phagocytized bacteria by milk PMN was significantly lower than that by blood PMN ($p < 0.05$). A smaller number of active phagocytes was present among cells isolated from milk than among cells isolated from blood. The phagocytic capacity of milk PMN reflects that of blood PMN in the same animal. A large variation in the phagocytic capacity of blood and milk PMN among animals was observed.

intramammary device; milk cells; blood PMN.

Introduction

Elimination of microorganisms from the mammary gland depends mainly on the combined function of humoral components and phagocytosis. Phagocytosis is the function of polymorphonuclear neutrophils (PMN) and the monocyte-macrophage system. In milk from a noninfected mammary gland 80–90 % of the cells are PMN and macrophages (*Lee et al.* 1980).

Large numbers of milk PMN are required to conduct studies on "in vitro" phagocytosis. Diapedesis of blood PMN into udder compartments and milk can be temporarily stimulated by intramammary infusions such as saline containing Escherichia coli endotoxin or oyster glycogen (*Russell & Reiter* 1975, *Paape et al.* 1976).

The insertion of an intramammary polyethylene device (IMD) into the gland cistern has been found to increase the milk somatic cell count (MSCC) in strippings (*Paape et al.* 1981). Abrasion of the device (AIMD) was found to intensify the response (*Paape & Norman* 1984). IMD may provide a consistent and efficient way of obtaining milk PMN for experimental studies on their functions.

Bovine milk PMN have less efficient phago-

cytic and bactericidal capabilities than blood PMN. The reduced efficiency has been attributed to the ingestion of fat globules (*Paape & Guidry* 1977) or casein (*Russell & Reiter* 1975), to the poor glycogen reserves in milk leukocytes (*Newbould* 1970) and to the low opsonising ability of milk as compared with that of blood serum (*Wisniowski et al.* 1965).

Various procedures have been used to assess phagocytosis "in vitro". Many of these techniques are based on the time-consuming process of microscopical counting of phagocytized bacteria. A flow cytometric (FCM) method for the evaluation of bovine blood PMN phagocytosis has been described by *Saad & Hageltorn* (1985). The method allowed a rapid and accurate simultaneous determination of different parameters of phagocytosis. The purpose of the present study was to describe the modifications of the FCM method in use for the evaluation of milk PMN phagocytosis. The possibility of using IMD as an aid for the collection of milk PMN was investigated. Further standardization of the FCM method and comparison of the phagocytic competence of blood and milk PMN were also included in the present report.

Materials and methods

Animals

Eight non-pregnant cows (Swedish Red and White or Swedish Friesian cross breed) 2 1/2–5 1/2 years of age, in different stages of lactation and without clinical symptoms of mastitis were used. In each cow a randomly selected udder quarter was fitted with an abraded intramammary device (AIMD). The AIMD was made of molded polyethylene, 2 mm in diameter, 115 mm long and formed into a loop of 25 mm in diameter in the udder cistern (*Paape & Norman* 1984). All AIMD-fitted quarters were free of bacteria according to bacteriological examination of foremilk samples before and after the insertion of the AIMD.

Milk and blood samples

Stripping milk samples from the AIMD-fitted quarters and blood samples from the jugular vein were taken immediately after the morning milking. Milk samples were used for determination of MSCC by the FCM and for isolation of PMN for the phagocytosis assay. The blood samples were collected in vacutainer tubes containing sodium heparin.

Utensils and solutions

If not stated otherwise, all tubes and glassware used were previously siliconized (Sigmacote, Sigma Chemicals, St. Louis, USA). Solutions used in the experiment were filtered (av pore diam. 0.2 μm, Microflow 25, Flow Laboratories) immediately after preparation.

Isolation of PMN

PMN were isolated from the blood by a one step discontinous Percoll gradient technique (*Saad & Hageltorn* 1985).

Stripping milk (20–25 ml) from the AIMD-fitted quarters was diluted in ice cold phosphate-buffered saline (PBS) to a final volume of 100 ml, thoroughly mixed and then filtered through a nylon net of 50 μm porosity. After centrifugation (15 min, 4°C, 1500xg) the cream layer and skimmed milk were discarded and the cell pellet was resuspended in 7 ml ice-cold PBS. Some red blood cells (RBC) were often found in the cell pellet. Lysis of RBS was induced by treating all cell suspensions with 2 volumes of cold distilled water for 45 s. Isotonicity was restored by adding 1 volume of cold, phosphate-buffered (0.0132 M, pH 6.8) 2.7 % sodium chloride. Cells were washed twice (centri-

fugation, 4°C, 200xg) and then resuspended in cold Hank's balanced salt solution (HBSS) containing 0.05 % human serum albumin (pH = 7.4). The cell suspension was kept iced and was used within 30 min. The cells were at least 90 % viable according to the trypan blue exclusion test.

Phagocytosis test

PMN isolated from blood or milk were incubated with FITC-labeled S. aureus in a ratio of 1 PMN:20 bacteria and a final serum concentration of 10 % for 15 min at 37°C (*Saad & Hageltorn* 1985). Phagocytosis was terminated by the addition of 6 ml 0.9 % saline solution containing 0.02 % EDTA.

In preliminary studies it was noticed that milk PMN tend to aggregate after incubation with bacteria, particularly the active phagocytes (microscopial observation). Therefore, after the addition of EDTA and just before the FCM analysis, all samples were sucked off and pushed gently by a plastic syringe through a narrow injection needle (0.6 mm diam.).

Flow cytometry

Cytofluorograf 50H (Ortho Diagnostic Instruments, Westwood, Mass., USA) equipped with a 3W argon ion laser (Spectra-Physics INC., California, USA) with an excitation wavelength of 488 nm was used. The pulse area values were used throughout the entire FCM measurements.

The red and green fluorescences of acridine orange-stained cells were measured at 600–650 nm and 515–575 nm, respectively. The total and differential counts of cells in milk and cells isolated from blood or milk were performed by FCM after staining with acridine orange (*Saad & Hageltorn* 1985, *Hageltorn & Saad* 1986).

In phagocytosis assays, the FITC-fluorescence was measured at 515–575 nm combined with forward light scatter. The percentage of phagocytic cells and the number of extracellular bacteria after the incubation were determined by the FCM (*Saad & Hageltorn* 1985).

Statistical analysis

All results are expressed as mean ± standard error. The statistical significance of differences between means was determined by paired t-test analysis.

Results

Significant increase in the MSCC, represented mainly by an increase in the number of PMN was observed in the stripping milk after the insertion of the AIMD. The mean MSCC of the 8 cows increased significantly ($p < 0.001$) from $308 \pm 66.5 \times 10^3$ (3 determinations 1–2 weeks before the insertion of AIMD) to $1447 \pm 133 \times 10^3$ cells/ml of stripping milk (7 determinations 3–7 weeks after the insertion of AIMD).

Mean percentage recovery of total somatic cells isolated from stripping milk of AIMD-fitted quarters after the removal of the cream and skimmed milk and after subsequent washings was 36.6 ± 2.2 % (10 isolations from each cow). Mean percentage recovery of PMN was higher than those of lymphocytes and monocyte-macrophages (43.9 ± 3.1, 21.7 ± 2.3 and 26.6 ± 2.3 %, respectively).

Mean differential cell count of stripping milk from AIMD-fitted quarters was 73.3 ± 1.5 % PMN, 11.8 ± 1.5 % lymphocytes and 14.9 ± 1.2 % monocyte-macrophages. After the isolation of cells from the stripping milk, the mean differential count of isolated cells in suspension was 83.2 ± 0.4 % PMN, 6.3 ± 0.4 % lymphocytes and 10.5 ±1.2 % monocyte-macrophages.

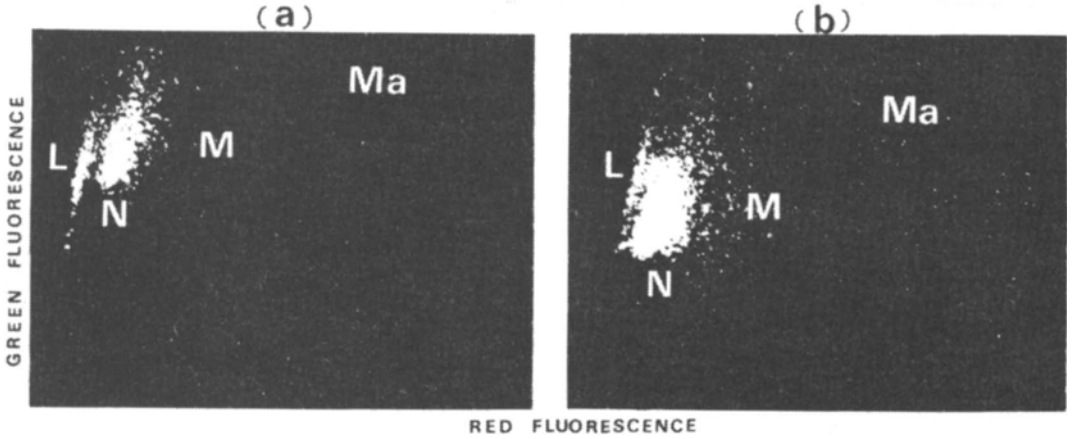

Figure 1. Cytofluorograms of somatic cells stained with acridine orange in bovine milk. Cell populations in stripping milk from AIMD-fitted udder quarter (a) and after the isolation of the cells from the stripping milk (b).
L = lymphocyte population; N = neutrophil population; M = monocyte population and Ma = macrophage population.

Fig. 1 shows cytofluorograms representing cell populations in stripping milk from an AIMD-fitted quarter and cell populations after the isolation of cells from that milk (Fig. 1a and b, respectively). The PMN population increased from 80.2 % (Fig. 1a) to 88.2 % (Fig. 1b).

After the incubation of blood or milk PMN with the bacteria, the number of extracellular bacteria and the proportion of phagocytic cells were determined by the FCM. The percentage of phagocytized bacteria and the number of bacteria per phagocyte were calculated from the FCM measurements. The coefficient of variation for percentage of bacteria phagocytized by blood PMN was 5.5 % of the mean as determined by duplicate analysis (n = 30).

Table 1 shows a comparison between the phagocytic competence of PMN isolated from blood and milk in the same animal. Each value represents the mean of 3 days measurements for a given animal. Tests were conducted in duplicates. The percentage of bacteria phagocytized by milk PMN was significantly lower than that by blood PMN ($p < 0.05$). Fewer active phagocytes were present among milk cells than among blood cells. Variation among animals in the phagocytic capacity of blood and milk PNM was observed.

The effects of various incubation times and serum concentrations on the parameters of the phagocytosis are presented in Figs. 2 and 3, respectively. The percentage of bacteria phagocytized by blood and milk PMN increased rapidly during the first 15 min of incubation approaching values of 55 % and 50 %, respectively (Figs. 2A and B, respectively). The number of bacteria per phagocyte after 15 min incubation was 10 and 11.7, respectively. When the incubation time was extended to intervals up to one hour, the above mentioned parameters continued to increase, though at a lower rate. After 1 h of incubation, 78 % and 69 % of

Table 1. Phagocytic capacity of PMN isolated from blood and stripping milk collected from AIMD-fitted quarters. Each value represents the mean of 3 days measurements run in duplicate.

Cow no.	Source of PMN	% of PMN[a]	% of phagocytic cells	% of phagocytized bacteria	Number of bacteria/ phagocyte
1	Blood	79.2	75.3	59.8	11.4
	Milk	87.1	76.7	53.7	12.2
2	Blood	82.6	70.9	40.5	9.3
	Milk	86	80.6	38.1	8.2
3	Blood	68.4	61.2	55.1	12.5
	Milk	79.2	61.7	50.6	13.6
4	Blood	73.9	76.9	43.2	8.3
	Milk	78.8	57.1	36.3	9.9
5	Blood	81.8	82.2	52.4	10.5
	Milk	82.3	61.1	51.9	14.1
6	Blood	61.9	79.7	54.3	8.5
	Milk	84.6	69.2	46.4	11.4
7	Blood	72	79.2	47.8	8.3
	Milk	88.1	68.3	48.2	12.7
8	Blood	42.5	72.3	60.2	7.1
	Milk	61	52.3	47.2	11.2
Mean ± SE	Blood	70.3 ± 4.7	74.7 ± 2.3	51.7 ± 2.6	9.5 ± 0.6
	Milk	80.9 ± 3.1	65.9 ± 3.4	46.6 ± 2.2*	11.7 ± 0.7*

[a]: % of PMN in the cell suspension after isolation.
*: Significantly different from blood PMN ($p < 0.05$).

the bacteria were phagocytized by blood and milk PMN, respectively; corresponding values for the number of bacteria per phagocyte were 14.9 and 16.4. However, maximal percentage of phagocytic cells was reached at 15 min incubation.

Serum concentrations lower than 10 % in the incubation mixture caused decreases in most of the phagocytosis parameters when blood and milk PMN were used (Fig. 3A and B, respectively). Little increase in the percentage of phagocytized bacteria and in the number of bacteria per phagocyte was observed when serum concentration ranging from 15 % to 40 % was prepared in the medium. Percentage of phagocytic cells, however, did not change after increasing the serum concentration to more than 10 %. It should be pointed out that blood and milk cells, used for the studies of effects of various incubation times and serum concentrations were not isolated from the same animal.

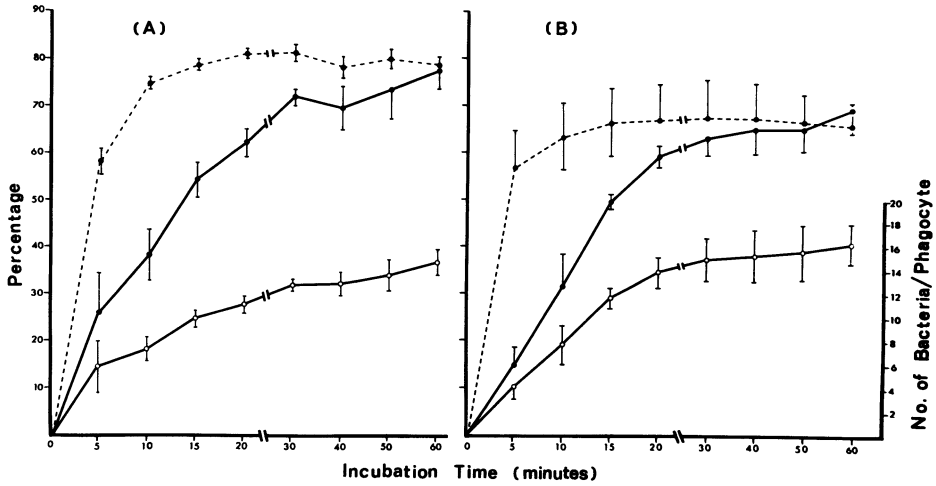

Figure 2. Effects of different incubation time on % of phagocytic cells (●--●), % of phagocytized bacteria (●—●) and the number of bacteria per phagocyte (○—○) when blood (A) and milk (B) PMN were used. Each value represents the mean ± SE of 9 cows (A) and 3 cows (B). Mean % of PMN in the incubation mixture ± SE = 72.9 ± 4 (A) and 77.9 ±7.6 (B).

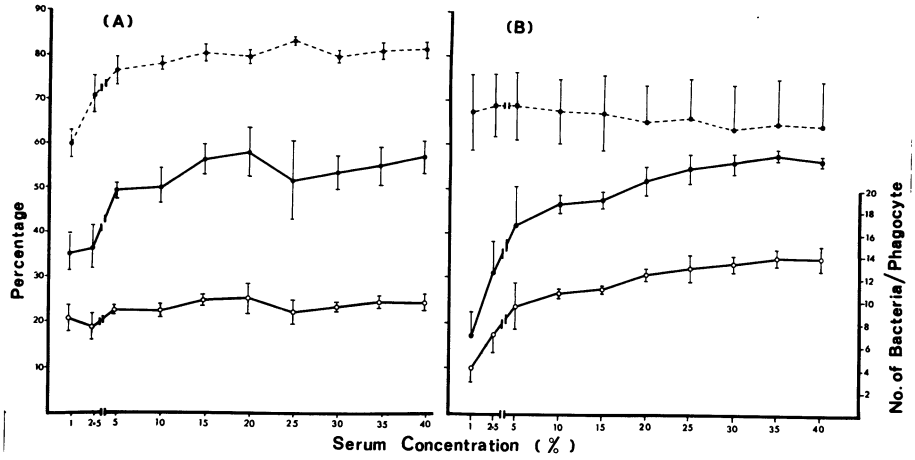

Figure 3. Effects of different serum concentration in the incubation mixture on the % of phagocytic cells (●--●), % of phagocytized bacteria (●—●) and the number of bacteria per phagocyte (○—○) when blood (A) and milk (B) PMN were used. Each value represents the mean ± SE of 5 cows (A) and 3 cows (B). Mean % of PMN in the incubation mixture ± SE = 69.9 ± 3.9 (A) and 76.6 ± 7.9 (B).

Discussion

In a previous study, *Saad & Hageltorn* (1985) described an FCM method for the evaluation of bovine blood PMN phagocytosis. In order to employ the FCM method for the evaluation of milk PMN phagocytosis, certain precautions have to be taken into consideration. Firstly, milk PMN tend to aggregate after incubation with the bacteria much more than the blood PMN. As single cell suspension was a prerequisite for the FCM analysis, all samples were gently passed through a narrow needle in order to minimize cell aggregation. *Bryant et al.* (1966) noticed that cells were less motile and tend to clump after "in vitro" phagocytosis. Human neutrophils aggregate while phagocytizing bacteria (*Henricks et al.* 1984). Milk fat globules attached to the cell membrane of the neutrophils may act as ligands between the cells and thus induce milk PMN aggregation. Secondly, during the isolation of the cells from the milk by centrifugation, some cell debris and other particles sediment in the cell pellet. These will be registered by the FCM as long as they cause light scattering when passing the laser beam. The patterns of light scattering by small particles and cells indicate their size or internal structure (*Brunsting & Mullaney* 1974). Therefore, the debris will be registered quite apart from the intact cells on the scattered light intensity axis. The FCM should be adjusted to eliminate the cell debris from the cytogram by the use of the noise discrimination threshold.

The present investigation has shown that percentage of bacteria phagocytized by milk PMN was significantly lower than that by blood PMN in the same animal (46.6 ± 2.2 % and 51.7 ± 2.6 %, respectively, $p < 0.05$, Table 1). More cells were actively phagocytic among blood cells than among milk cells. Consequently, more extracellular bacteria would be available to the active milk phagocytes which might explain the higher number of bacteria per phagocyte value of milk PMN (11.7 ± 0.7 and 9.5 ± 0.6 bacteria/phagocyte for milk and blood cells, respectively).

Kent & Newbould (1969) also reported fewer active phagocytes among bovine milk PMN than among blood PMN, while the active milk phagocytes were engulfing as many bacteria as the blood PMN. It is well established that PMN isolated from bovine milk have a reduced "in vitro" phagocytic capacity compared with PMN isolated from blood (*Wisniowski et al.* 1965, *Newbould* 1970, *Kent & Newbould* 1969, *Russell & Reiter* 1975, *Paape & Guidry* 1977). Reduced phagocytic activity has also been noticed in PMN in human colostrum (*Pickering et al.* 1980).

From the data presented in Table 1 it seems reasonable to suggest that the phagocytic capacity of milk PMN reflects that of blood PMN in the same cow. This is in agreement with the findings of *Newbould* (1973). Variation among cows in the ability of blood and milk PMN to phagocytize bacteria was observed (Table 1). Such variation has also been observed by other investigators using other techniques (*Paape et al.* 1978, *Williams et al.* 1984).

This study has shown that the percentage of phagocytic cells among both milk and blood PMN reached a plateau value after 15 min of incubation. Percentage of phagocytized bacteria and the number of bacteria per phagocyte, however, continued to increase when the incubation time was extended for up to 1 h. A considerable number of blood and milk cells adhered to the incubation tubes after 50 to 60 min of incubation.

Serum concentrations of more than 10 % in the incubation mixture had no large influence on the phagocytosis parameters. It

should be pointed out, however, that the bacteria tended to aggregate when a high serum concentration and a long incubation time were used. The bacterial aggregation was indicated by the appearance of a second peak in the histogram of extracellular bacteria.

Large numbers of viable milk PMN are required to conduct "in vitro"studies on the function of these cells. Different sorts of intramammary infusions are used to induce diapedesis of blood PMN into the milk. In this study stripping milk from AIMD-fitted quarters has been used as a continous source of milk PMN in order to avoid repeated infusions in the udder.

The mean MSCC in stripping milk of 8 cows was 308×10^3 and 1447×10^3 cells/ml milk before and after the insertion of AIMD, respectively. Only 36.6 % of the total milk cells and 43.9 % of milk PMN were recovered after the removal of cream and subsequent centrifugations. *Paape et al.* (1976) reported a yield of 58 % and 33 % of milk PMN from milk taken 11 h and 35 h respectively, after intramammary infusion of saline containing 0.1 % oyster glycogen. The low recovery of milk cells after centrifugation was most probably due to the loss of cells laden with milk fat globules in the cream layer. PMN and macrophages are known to phagocytize milk fat globules (*Paape & Guidry* 1977, *Lee et al.* 1980). In the present study the recovery of milk PMN was unexpectedly higher than in other cell types. A possible explanation would be that the PMN appearing in the stripping milk of AIMD-fitted quarters were cells freshly coming into the milk and they may have contained less ingested fat globules.

Different methods for stimulation of influx of PMN into the milk may exert some influence on PMN function. Considering the length of exposure of PMN to the milk components "in vivo". PMN just coming into the milk may have the same or little lower phagocytic capacity than those in the blood, because they have not been in a prolonged contact with the factors known to inhibit phagocytosis in milk (i.e. casein and fat globules). Prolonged incubation of blood or milk PMN in milk impairs their phagocytic competence (*Russell & Reiter* 1975). In addition, milk PMN isolated 11 h after saline-glycogen infusion are more efficient in phagocytizing yeast cells than PMN collected 35 h after the infusion (*Paape et al.* 1976).

In flow cytometric analysis of bovine milk (*Hageltorn & Saad* 1986), the neutrophil cluster on the oscilloscope screen was most distinct when stripping or residual milk was analysed. Storage of foremilk, stripping and residual milk for 5–6 h before the FCM analysis resulted in an indistinct neutrophil cluster definition and sometimes overlapped the one representing the lymphocytes. That was due to a decrease in the red fluorescence emission, presumably as a result of lysosomal losses in the neutrophils because of continuous phagocytosis of fat and casein.

IMD causes no apparent damage to the udder parenchyma but a significant increase in the concentration of RBC in bucket milk occurs (*Paape et al.* 1985). In the present study, however, some stripping milk samples from the AIMD-fitted quarters contained some RBC. As presence of RBC together with the isolated cells might influence the FCM measurement of phagocytosis (*Saad & Hageltorn* 1985), a hypotonic shock was performed to lyse the RBC.

There are no differences between the percentage of phagocytized bacteria by milk PMN isolated from AIMD-fitted quarters and from control quarters (*Young et al.* 1984). AIMD is useful as an aid for collection of freshly infiltrating PMN in the milk

during a long experimental study on the function of these cells.

The FCM method for studying the milk and blood PMN phagocytosis supplies a reliable and rapid means for the evaluation of phagocytosis. Since up to 5000 cells may be counted and differentiated per second the results can be obtained immediately and multiple parameters of phagocytosis can be measured in a single determination.

Acknowledgement

Prof. Göran Åström is cordially thanked for helpful discussion and advices. Mrs. Gudrun Mellberg-Wieslander is thanked for typewriting the manuscript. This study was supported by grants from the Swedish Council for Forestry and Agricultural Research.

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Sammanfattning

Flödescytometrisk bestämning av fagocytos hos neutrofila leukocyter isolerade från mjölk.

I arbetet beskrivs en flödescytometrisk metod för bestämning av fagocytoskapaciteten hos neutrofila leukocyter (PMN) i komjölk. PMN isolerades från eftermjölksprov från juverfjärdedelar i vars juvercisterner spiraler gjorda av plasttråd med skrovlig yta (AIMD) hade placerats. Cellkoncentrationen i mjölken ökade från 308 × 10^3 til 1447 × 10^3 celler/ml efter införandet av plastspiralerna i juverfjärdedelarna. Ökningen var signifikant ($p < 0{,}001$).

För isolering av PMN från blod användes en diskontinuerlig Percollgradient.

PMN från blod och mjölk inkuberades i 10 % serum med FITC-märkta bakterier i proportionen 1 PMN:20 bakterier. Inkubationstid, 15 min. Antalet extracellulära bakterier och frekvensen fagocyterande celler mättes med en flödescytometer.

PMN isolerade från mjölk fagocyterade signifikant lägre andel av bakterierna än PMN isolerade från blod ($p < 0{,}05$). En lägre andel av fagocyterna i mjölken var aktiva jämfört med i blodet.

Fagocytoskapaciteten hos PMN isolerade från mjölken speglar den hos blodet från samma djur. Variationen mellen djur är stor.

(Received March 16, 1987).

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