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FLOW CYTOMETRIC CHARACTERIZATION OF BOVINE BLOOD NEUTROPHIL PHAGOCYTOSIS OF FLUORESCENT BACTERIA AND ZYMOSAN PARTICLES

By

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SAAD, ALAA M. and M. HAGELTORN: *Flow cytometric characterization of bovine blood neutrophil phagocytosis of fluorescent bacteria and zymosan particles.* Acta vet. scand. 1985, *26*, 289—307. — A Flow Cytometric method for the evaluation of the phagocytic capacity of bovine blood neutrophils is described. The neutrophils were isolated from bovine blood by a one step discontinuous gradient of Percoll. By this technique of isolation, 90 ± 2.8 % (mean ± s) of the granulocytes in the whole blood were recovered.

Isolated neutrophils were incubated with FITC labeled S. aureus or zymosan particles in a ratio of 1:20 and 1:10, respectively, and a final serum concentration of 10 %. Phagocytosis was terminated after 15 min and the number of extracellular bacteria or zymosan particles and the percentage of phagocytic granulocytes were registered by Flow Cytometry (FCM). FCM and microscopic studies revealed that eosinophils play a minor role in the phagocytosis of bacteria. The neutrophils were the main population of the granulocytes which were actively phagocytic. Variation among cows in the ability of their blood neutrophils to phagocytize bacteria was evident.

Percoll; FCM; PMN phagocytosis; eosinophil; separation.

Phagocytosis of microorganisms by the granulocyte and mononuclear-phagocytic system is one of the most important mechanisms of body-defense against infection.

Information about this process has been obtained by both 'in vitro' and 'in vivo' techniques. The latter technique is, however, limited by the fact, that measurements can be only performed on experimental animals.

Studies carried out 'in vitro' have the advantage that homogenous well defined and characterized phagocytes, particles or serum factors can be used. On these bases, techniques of several

kinds have been developed to measure and evaluate phagocytosis 'in vitro'. For instance the number of particles ingested per cell can be determined microscopically (*Jain & Lasmanis* 1978), but also by the use of radiolabeled organisms after removal of the extracellular organisms (*Paape et al.* 1975, *Roth & Kaeberle* 1981). The ingestion of viable microorganisms can be measured microbiologically as a function of their extracellular disappearance (*Solberg* 1972).

Recently flow cytometric (FCM) technology has expanded to a variety of fields (see review article by *Braylan* 1983), including studies concerning phagocytosis. Different kinds of particles have been used in flow cytometric studies of phagocytosis as fluorescing latex particles (*Parod & Brain* 1980, *Dunn & Tyrer* 1981, *Steinkamp et al.* 1982), Baker's yeast (*Dérer et al.* 1982), zymosan particles (*Bjerknes & Bassøe* 1983), and fluoresceinated bacteria (*Braunsteip et al.* 1976, *Bassøe et al.* 1980, 1983a, 1983b). The technique seems to be very promising due to its efficiency. It has been successfully applied in studies of certain immunologic defects in man (*Bassøe* 1984) and will most probably be important in veterinary research concerning variations in immunological efficiency among individuals and among breeds as well as immunological defects. In the future, selective breeding for improved resistance against diseases may be accomplished on the base of these studies.

The purpose of the present study was to apply the flow cytometric technique for studying phagocytosis by normal bovine blood granulocytes.

MATERIALS AND METHODS

Blood

About 10 ml of peripheral venous blood were collected from clinically healthy cows in their first lactation period (Swedish Red and White and Swedish Friesian cross breed) in vacutainer tubes containing sodium heparin (Becton Dickinson Co). Blood samples were also collected for differential and total leukocyte counts using standard haematological techniques.

Utensils

If not stated, all tubes and glassware used in the experiments were previously siliconized (Sigmacote, Sigma Chemicals, St Louis, USA).

Serum

Pooled fresh bovine serum (further on only "serum") from 11 clinically healthy cows from the same herd was stored in 1 ml aliquots at —20°C and used within 4—5 weeks. Immediately before use, 1 ml of freshly thawed serum was diluted by 3 ml Hank's balanced salt solution (HBSS).

Bacteria

Staphylococcus aureus (S. aureus) were heat-killed at 60°C for 30 min and labeled with fluorescein isothiocyanate (FITC) (Sigma Chemicals, St Louis, USA) according to the method of *Gelfand et al.* (1976). The bacteria suspension was then divided into small volumes and stored at —80°C. Immediately before use, a small volume of FITC-labeled bacteria was diluted with HBSS, sonicated for 30 s at 2.5 A, counted by the FCM according to *Bassøe et al.* (1983b) and adjusted to 5×10^8 bacteria/ml in HBSS.

Zymosan particles

Zymosan particles (Sigma Chemicals, St Louis, USA) were labeled with FITC according to the method of *Gelfand et al.* (1976) without adding gelatin. The FITC labeled zymosan particles were then pelleted, resuspended in HBSS, divided into small volumes and stored at —20°C. Immediately before use, a small volume was thawed, diluted with HBSS, sonicated for 30 s at 2.5 A, counted by the FCM (*Bjerknes & Bassøe* 1983) and adjusted to 2.5×10^8 zymosan particles/ml in HBSS.

Granulocyte separation technique

Discontinous gradients were formed by placing two Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) solutions of different densities, 10 ml density: 1.103 g/ml and 20 ml density: 1.087 g/ml, on top of each other in 50 ml plastic tubes (Conical tube, 2070 F, Falcon). The densities of the solutions were prepared according to the formula given by the manufacturer (in: Percoll® Methodology and applications: Density Marker Beads for calibration of gradients of Percoll®). Nine ml of blood were diluted in ratio 1:1 with a phosphate buffered isotonic saline, pH 7.4 and carefully layered on the top of the gradient (17 ml per tube).

Afterwards the tubes were centrifuged for 15 min at 1800 × g at 20°C in a swing out rotor. After centrifugation the diluted plasma and buffy interface, the latter containing predominantly the mononuclear cells, plus one centimeter of the lower density Percoll solution were discarded. The remaining volume of the lower density Percoll solution plus the interface between the two Percoll fractions contained almost all granulocytes evenly distributed but with a higher proportion of neutrophils in the lower part of it. This fraction was sucked off with a Pasteur pipette for further treatment.

The lowest fraction, containing mainly erythrocytes, was discarded. Erythrocytes contaminating the granulocyte fraction were lysed by mixing 1 volume of granulocyte fraction with 2 volumes of distilled water (+4°C) for 45 s followed by the addition of 1 volume of 0.0132 mol/l phosphate buffered (pH 6.8) 2.7 % NaCl solution.

The granulocytes were pelleted, washed two times with HBSS supplemented with 2 % Bovine Serum Albumin (BSA) (Sigma Chemicals, St Louis, USA) by centrifugation at 150 × g for 10 min and resuspended in HBSS with 2 % BSA. Total and differential leukocyte counts were established by Flow Cytometry (FCM) and the granulocyte suspensions were adjusted to 5×10^6 neutrophils per ml in HBSS containing 2 % BSA.

Viability tests were carried out by the trypan blue exclusion test (microscopical analysis).

Smears were made after separation of granulocytes and stained with May-Grünwalds Giemsa stain.

Phagocytic systems

Granulocyte-bacteria mixture. Half a ml of the granulocyte suspension, 0.1 ml of the FITC-labeled bacteria suspension and 0.4 ml of the diluted serum were mixed in a small disposable plastic tube. This mixture provided 20 bacteria per 1 neutrophil and 10 % serum. After incubation for 15 min at 37°C with an end-over-end rotation to promote contact between bacteria and granulocytes, 6 ml 0.9 % NaCl solution containing 0.02 % EDTA was added in order to interrupt phagocytosis.

Smears from granulocyte-bacteria mixtures after incubation were made, quickly air-dried and fixed in methanol. Microscopic counting of the numbers of cells phagocytizing bacteria and the total numbers of bacteria associated with 100 neutrophils

and 100 eosinophils were performed after the smears had been stained with May-Grünwalds Giemsa stain. Some smears were also examined by combined light and fluorescence microscopy before Giemsa staining.

Granulocyte-zymosan mixture. The same procedure as for the granulocyte-bacteria mixture was applied except that 0.1 ml of FITC-labeled zymosan particle suspension instead of bacteria was used. The mixture provided 10 zymosan particles per neutrophil and 10 % serum.

Flow cytometry

A cytofluorograf 50L (Ortho Diagnostic Instruments, Westwood, Mass., USA), equipped with a 100 mW argon ion laser with an excitation wavelength of 488 nm was used. The red and green fluorescence of acridine orange (AO) stained leukocytes was measured at 600—650 nm and 515—575 nm, respectively and the laser output was adjusted to 20 mW.

In phagocytosis assays the output power was adjusted to 30 mW. The FITC fluorescence was measured at 515—575 nm combined with forward light scatter, when the FITC-labeled bacteria were used, or with 90° light scatter when the FITC labeled zymosan particles were used. The pulse area values were used throughout the entire FCM analysis.

FCM measurements

Differential and total cell counts. A volume of 0.05 ml of the granulocyte suspensions were mixed with 3 ml of acridine orange solution (AO) according to the formula by *Adams & Kamentsky* (1974) and incubated for 3 min at room temperature before FCM analysis. The pulse area of cellular red and green fluorescence was displayed in the X and Y directions, respectively, on the oscilloscope screen. Differential cell counts were digitally registered within appropriate windows and the total cell counts were established by counting the total cell number in 0.4 ml of the mixtures. The same procedure was used for the total and differential cell counts of the whole blood. Cells were sorted on slides by the FCM from all leukocyte populations and identified directly by fluorescence microscopy.

Parameters of phagocytosis. After phagocytosis, extracellular bacteria or zymosan particles, phagocytizing cells

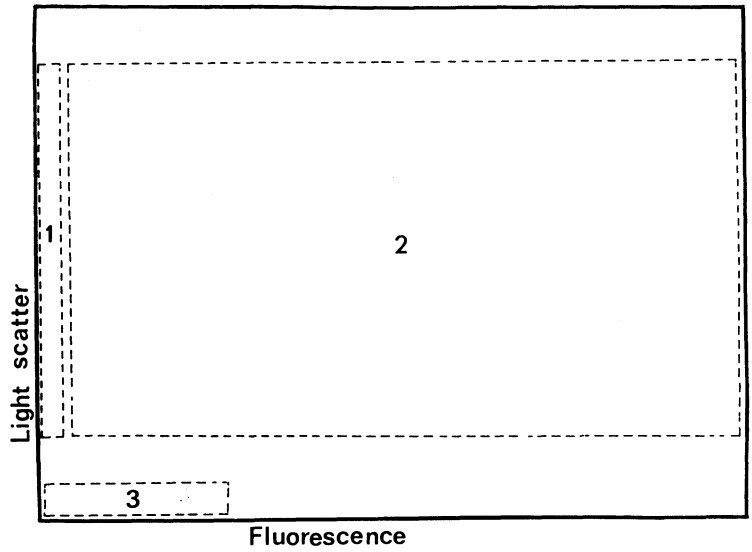

Figure 1. A schematic drawing of the cytogram showing the regions of setting sorting windows for different populations after the termination of phagocytosis, region (1) non-phagocytes, region (2) phagocytes and region (3) free bacteria or zymosan particles.

(fluorescent cells) and non-phagocytizing cells (region 3, 2 and 1, respectively in Fig. 1; see also Figs. 6 and 7) were demarcated by two sorting windows and quantified.

In the first step of measurements, the percentage of the extracellular bacteria or zymosan particles (region 3) and the percentage of the total cell population (region 1 and 2) were determined simultaneously. With the knowledge of the original absolute total cell number per volume unit, the absolute number of extracellular bacteria or particles was established. In the second step, the extracellular bacteria or zymosan particles (region 3) were excluded from the cytogram by means of the total count threshold adjustment, and the percentage of phagocytizing cells (region 2) and non-phagocytizing cells (region 1) was recorded simultaneously.

Quantification of phagocytosis was performed by calculating the following parameters:

1. Percentage phagocytosis: the ratio of the number of fluorescent cells to the total number of cells.
2. The number of bacteria or zymosan particles per phagocyte:

the difference between initial and final counts of extracellular bacteria or zymosan particles divided by the number of phagocytizing cells.

3. Percentage of lost bacteria or zymosan particles (ingested and adherent): equal to the ratio of the initial minus the final to the initial counts of the extracellular bacteria or zymosan particles.

RESULTS

Separation of blood granulocytes

By FCM measurements of AO stained leukocytes from whole bovine blood, five populations could easily be recognized in the cytofluorograms (Fig. 2). After separation, the FCM differential cell counts of the separated granulocytes from the blood of 9 cows were as follows: 93 ± 2.1 % granulocytes (mainly neutrophils and eosinophils but also a few basophils), 3.1 ± 0.6 % lymphocytes, 1.7 ± 0.4 % monocytes (mean ± s) and less than 2 % were registered as doublets or larger cell aggregates (Fig. 3).

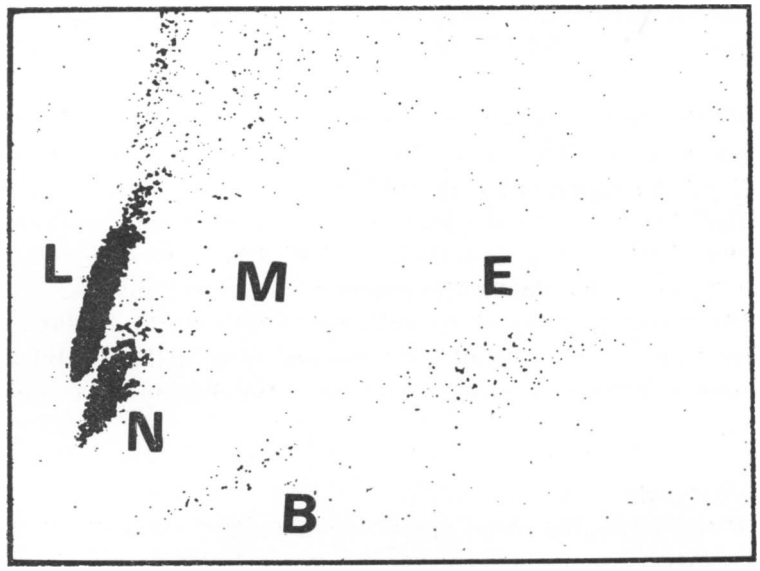

Figure 2. Cytofluorogram of normal bovine blood stained with hypotonic Acridine orange solution, each leukocyte is represented by a dot. L = lymphocyte population; B = basophil population; M = monocyte population; N = neutrophil population. Abscissa: Red fluorescence; ordinate: green fluorescence. (This animal has a low proportion of monocytes).

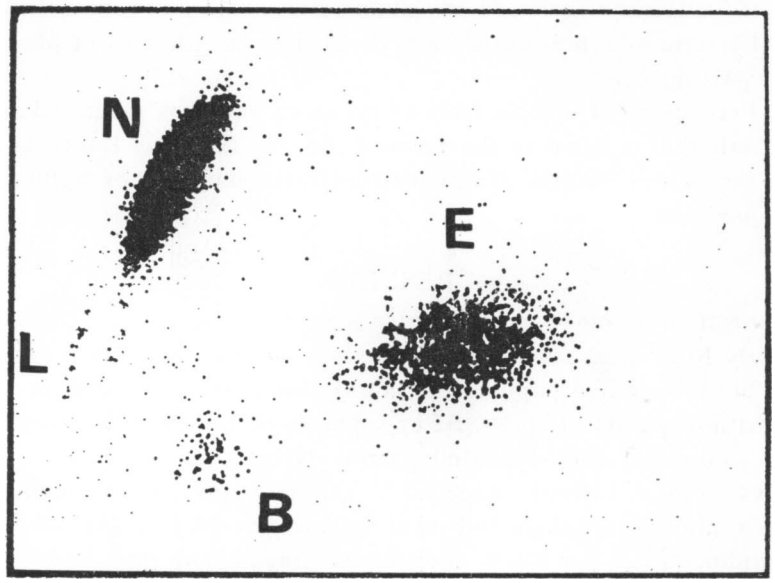

Figure 3. Cytofluorogram of separated leukocytes from the blood of the same cow used in Fig. 2. L = lymphocyte population; N = neutrophil population; E = eosinophil population; B = basophil population. Abscissa: Red fluorescence; ordinate: green fluorescence.

By this technique of separation, 90 ± 2.8 % (mean ±s) of the granulocytes in the whole blood were recovered. Ninety-six to 98 % of the separated leukocytes were viable according to the trypan blue test. The percentage of neutrophils and eosinophils after separation was depending on the initial percentage of each population in the blood. The recovery of neutrophils was 85.5 ± 2.1 % and that of the eosinophils was 95.8 ± 3.1 % (mean ± s). There were only few erythrocytes remaining in the granulocyte suspensions after separation and lysis (microscopical examination).

Phagocytosis

Using separated blood granulocytes from 6 clinically healthy cows to quantify phagocytosis by using FITC-labeled bacteria and the FCM, a good correlation was found between the percentage phagocytosis and the percentage of AO stained neutrophils ($r = 0.975$, $P < 0.001$, $n = 6$) (Fig. 4).

The percentage phagocytosis followed and did not exceed the

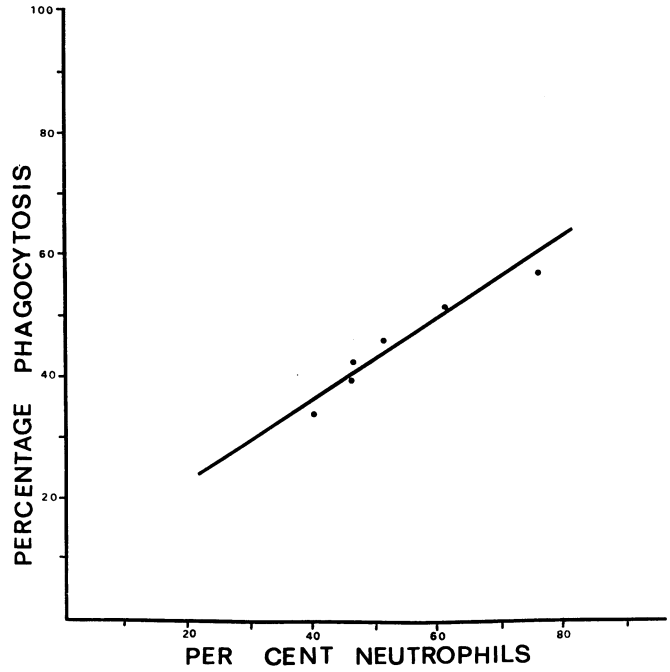

Figure 4. Correlation between percentage of neutrophils and percentage phagocytosis. Each dot represents leukocytes from different animals (mean of 2 measurements per animal). The percentage of neutrophils was determined by AO-staining.

percentage of neutrophils even when the percentage of neutrophils was further decreased in one of the animals which had a low proportion of neutrophils from the beginning (Fig. 5). The extra decrease was performed by taking only the upper half of the granulocyte fraction from the gradient after separation. These findings indicate that the neutrophils were the main population phagocytizing bacteria in the separated granulocytes. Granulocytes from 2 animals chosen from the total of 6 animals were used to quantify phagocytosis of FITC-labeled bacteria (Fig. 6). Based on the percentage of neutrophils after separation, one animal was selected for its relatively high percentage of neutrophils and the other for its low percentage of neutrophils.

The two animals exhibited variations in their neutrophil function by differences in the percentage of lost bacteria from the mixture and the number of bacteria per phagocyte (Table 1).

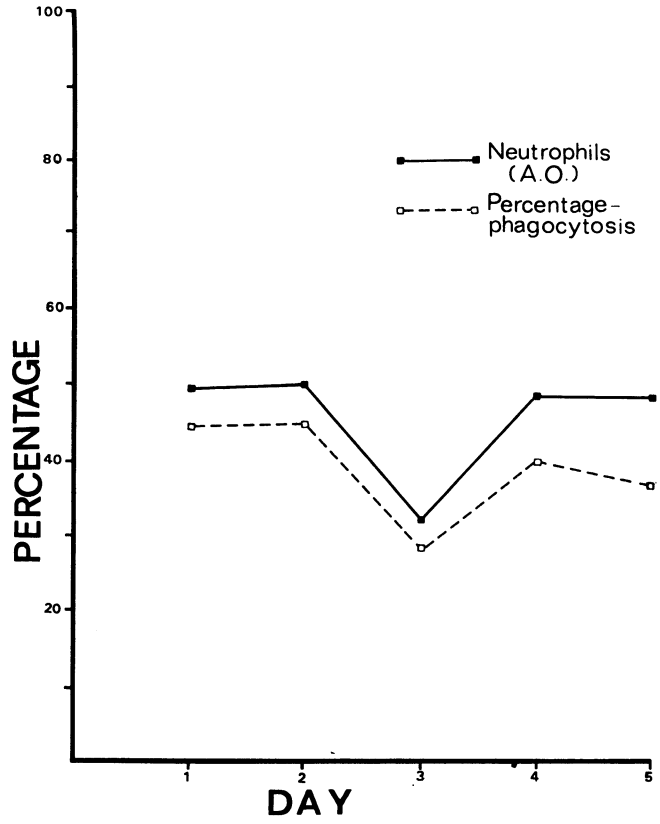

Figure 5. The relationship between the percentage of neutrophils determined by AO-staining and the percentage phagocytosis in one animal follow for 5 successive days. (This animal has a low proportion of neutrophils in the whole blood).

Table 1. Parameters of phagocytosis measured by FCM. Separated bovine leukocytes and FITC-labeled bacteria were used. Each value represents the mean ± s of 5 measurements for each animal.

Animal No.	% neutrophil (FCM)	% phagocytosis (FCM)	% of lost bacteria*	No. of bacteria per phagocyte
1	45.5 ± 8	38.6 ± 6.9	43.6 ± 3.2	10.26 ± 1.12
2	71.7 ± 2.4	54.8 ± 6.1	32.4 ± 2.4	8.56 ± 0.8

ingested and adherent.

In order to study the effect of the test particle on the parameters of phagocytosis, samples from the above mentioned 2 selected animals were used for the phagocytosis of FITC-labeled zymosan particles (Fig. 7). The variations between the animals were approximately the same as when the labeled bacteria were used as a test particle (Table 2).

Table 2. Parameters of phagocytosis measured by FCM. Separated bovine leukocytes and FITC-labeled zymosan particles were used. Each value represents the mean ± s of 3 measurements for each animal.

Animal No.	% neuthophil (FCM)	% phagocytosis (FCM)	% of lost zymosan particles*	No. of zymosan particle per phagocyte
1	42.6 ± 4.93	40.7 ± 5.13	60.6 ± 6.1	6.2 ± 0.5
2	76.6 ± 2.88	50.1 ± 8.64	28.5 ± 6.6	4.3 ± 1.04

* ingested and adherent.

Figure 6. Cytogram showing bovine leukocytes and FITC-labeled bacteria after the termination of phagocytosis. Abscissa: Green fluorescence; ordinate: light scatter. Region (1) no-phagocytes; region (2) phagocytes; region (3) free bacteria (refer to Fig. 1).

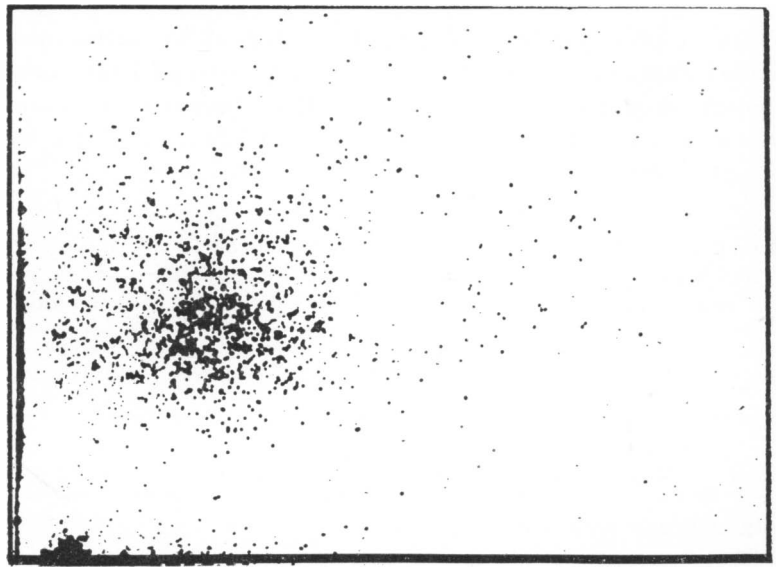

Figure 7. Cytogram showing bovine leukocytes and FITC-labeled zymosan particles after the termination of phagocytosis. Abscissa: Green fluorescence; ordinate: light scatter. Region (1) non-phagocytes; region (2) phagocytes; region (3) free zymosan particles (refer to Fig. 1).

In general, histograms of extracellular bacteria or zymosan particles before and after incubation exhibit nearly a single peak.

The coefficient of variation of FCM determinations of the bacteria concentration was 6.8 % ($n = 10$). The coefficients of variation of repeated FCM determinations of the number of bacteria per phagocyte and the percentage of lost bacteria from the mixture for one animal on the same day were found to be 1.8 % and 5.9 %, respectively ($n = 3$).

Correlation of microscopy and FCM measurements

A good correlation was found between the percentage of neutrophils after granulocyte separation measured by FCM and by microscopic studies of smears stained with Giemsa ($r = 0.966$, $P < 0.001$, $n = 11$) (Fig. 8).

A good correlation was also found between the percentage phagocytosis measured by FCM and microscopic studies of smears stained with Giemsa ($r = 0.989$, $P < 0.001$, $n = 11$) (Fig. 9).

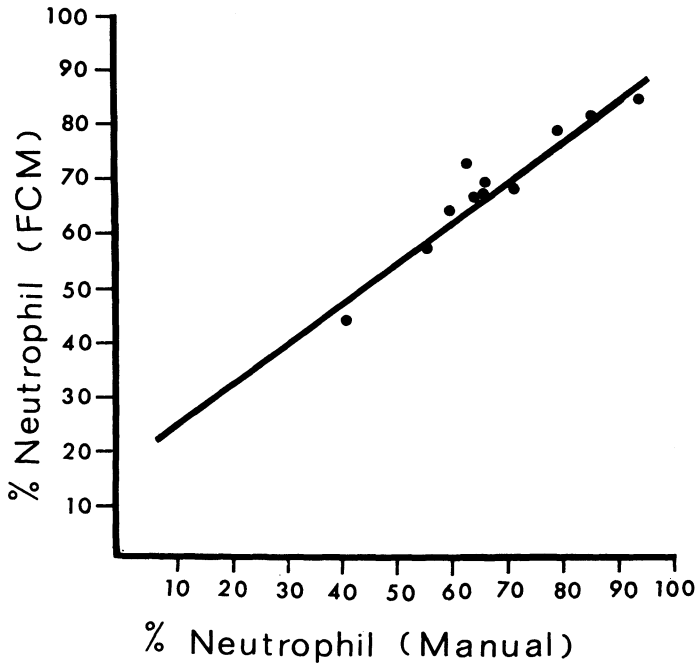

Figure 8. Correlation between percentage of neutrophils after granulocyte separation measured by FCM and microscopic examination of stained smears (Manual) ($r = 0.966$, $P < 0.001$, $n = 11$).

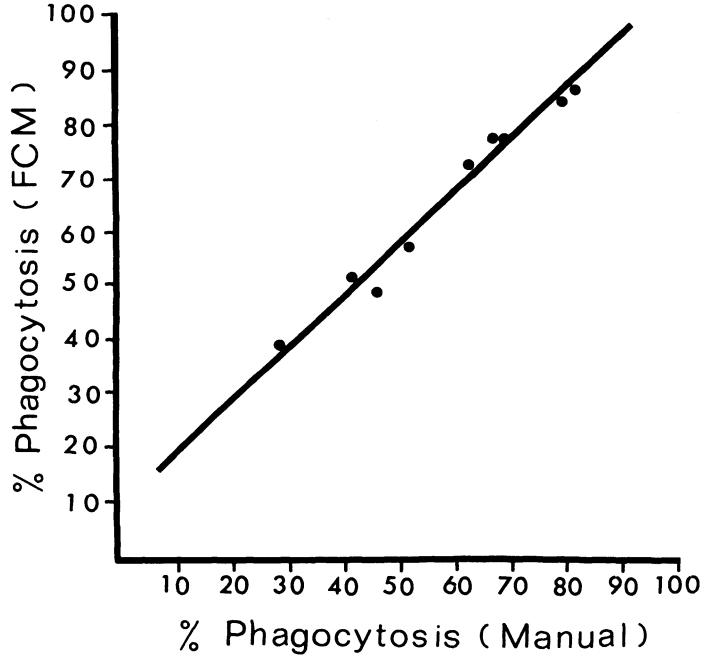

Figure 9. Correlation between percentage phagocytosis measured by FCM with that measured by microscipic studies of stained smears (Manual). ($r = 0.989$, $P < 0.001$, $n = 9$).

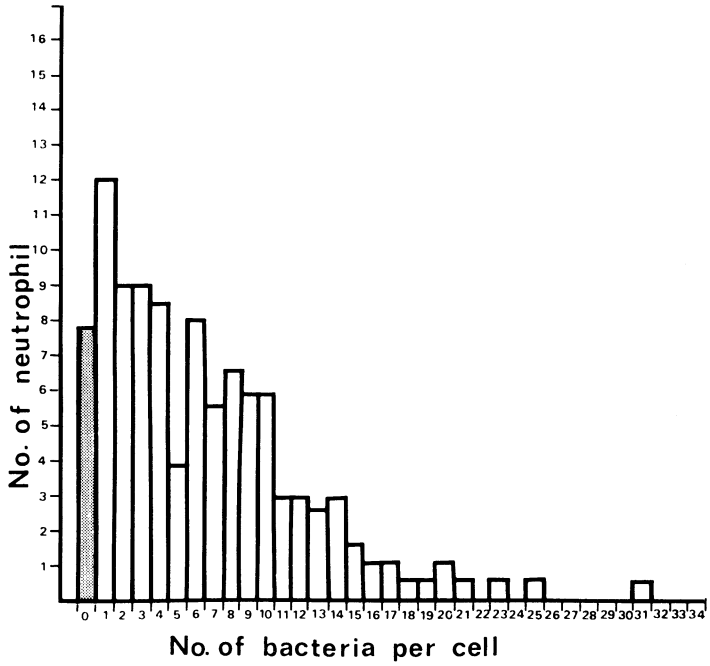

Figure 10. Frequency distribution of the number of bacteria associated with neutrophils estimated from microscopic studies of stained smears (mean of 3 animals).

The number of bacteria per phagocyte measured by FCM show a good correlation with the average number of bacteria associated with one phagocyte estimated from microscope studies of dried smears without staining ($r = 0.953$, $P < 0.01$, $n = 6$).

Fig. 10 and Fig. 11 show the frequency distribution of the number of bacteria associated with neutrophils and eosinophils respectively (microscopic studies). The mean percentage of phagocytic eosinophils was 6.7 ± 5.4 % while that of neutrophils was 91.0 ± 4.8 (mean $\pm$ s, $n = 9$). The mean number of bacteria per phagocytic eosinophil was found to be 1.4 ± 0.4 bacteria/phagocytic eosinophil (mean $\pm$ s, $n = 9$).

However, by microscopic studies of smears stained with Giemsa, it became clear that some neutrophils did not associate with bacteria and their percentage was 9 ± 4.8 % of the total neutrophils (mean $\pm$ s, $n = 9$). By FCM measurement, if the minor effect of phagocytic eosinophils on the percentage phagocytosis was neglected, the percentage of neutrophils which failed

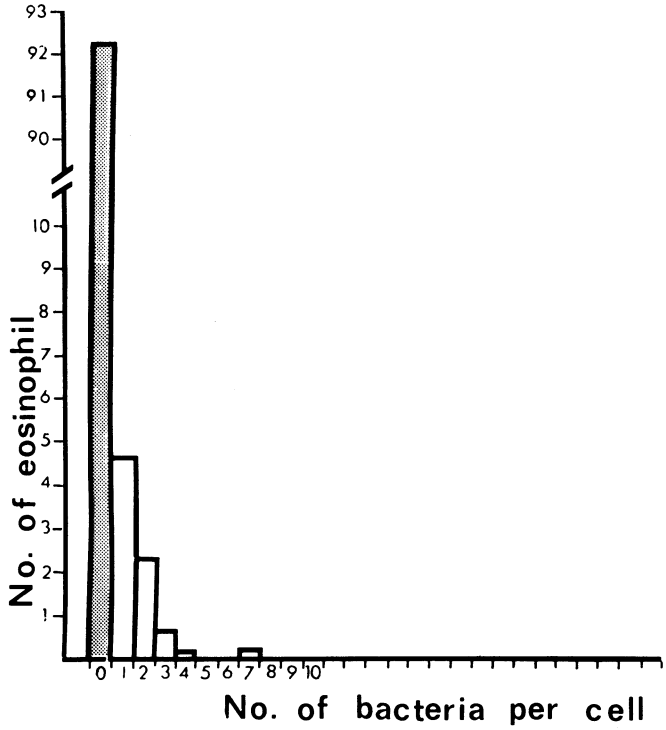

Figure 11. Frequency distribution of the number of bacteria associated with eosinophils estimated from microscopic studies of stained smears (mean of 9 animals).

to associate with bacteria was 6.7 ± 3.7 % of the total neutrophils (mean ± s, n = 9).

Erythrocytes were rarely found among smears prepared after granulocyte separation.

DISCUSSION

FCM investigations on the phagocytosis by granulocytes depends on the isolation of an adequate number of viable pure phagocytes.

In the bovine species this requirement is complicated by the fact that the erythrocytes do not sediment readily on standing. Another problem is that the eosinophils in the cow have a density range over lapping that of the neutrophils and differ in this respect from corresponding cells in other species like man and horse. *Riding & Willadsen* (1981) found that the extremes of the

density range for bovine eosinophils were 1.096—1.111 g/ml and for neutrophils 1.106—1.120 g/ml. In our investigations the neutrophils are the interesting cell population. According to the available literature there are no methods for separating pure neutrophils from the blood of the cow with a sufficient rate of recovery even though certain attempts have taken place (*Carlson & Kaneko* 1973, *Carson et al.* 1975, *Riding & Willadsen* 1981, *Roth & Kaeberle* 1981b). Hence, to obtain the highest possible recovery of the neutrophils, a one step discontinous gradient separation technique was developed, which also provided the opportunity for manipulating the proportion of neutrophils and eosinophils.

According to our results, the contaminating eosinophils play a minor role in the phagocytosis assays, at least when using bacteria as test particles. That was also confirmed by microscopic studies of stained smears (Figs. 10 and 11).

The eosinophils do not play an important role in the phagocytosis and destruction of bacterial organisms (*Butterworth* 1977). *Roth & Kaeberle* (1981) reported that bovine eosinophils did not ingest S. aureus as efficiently as neutrophils. *Grewal & Babiuk* (1979) reported lower affinity Fc receptors on bovine eosinophils, than that of the neutrophils and monocytes which may explain the poor phagocytic capacity of eosinophils (*Kay* 1976).

The ratio of bacteria to the neutrophils influence the results obtained in different assays of phagocytosis. The numbers of ^{125}IUdR labeled S. aureus ingested by one bovine polymorphonuclear leukocyte (PMN) reported by *Roth & Kaeberle* (1981a) were 5.3, 11.2, 24.4, 37.3 and 41.5 when they used bacteria to PMN ratio of 15, 30, 60, 120 and 240 to one PMN, respectively. *Paape et al.* (1975) incubated PMNs isolated from bovine blood with ^{32}P labeled S. aureus, in a ratio of 1:16 and 10 % bovine serum for 30 min, 71 % of the bacteria were phagocytized, which means that the average number of bacteria ingested by one neutrophil was 11.4.

Jain & Lasmanis (1978) used coliform bacteria to blood leukocyte ratios ranging between 10:1 to 20:1 in a tube test for phagocytosis. About 80 % of the neutrophils were actively phagocytic and each neutrophil ingested 9.2 bacteria.

FCM measurements correlated well with estimates using microscopy both with regard to the percentage of phagocytizing

leukocytes and the number of bacteria or zymosan particles per phagocyte, which is in agreement with *Bassøe et al.* (1980).

Red blood cells are not detected in the differential cell counts by FCM. They may influence the results by increasing the proportion of non-phagocytizing cells. Consequently, the lysis treatment must be performed with care.

Histograms of extracellular bacteria or zymosan particles after 15 min of incubation with granulocytes and serum exhibit a single peak, indicating a limited agglutination of bacteria or zymosan particles.

Repeated sampilng and analysis of phagocytosis by FCM have revealed differences between individuals in their phagocytic capacities of S. aureus. Such differences have also been noticed by other investigators using other techniques (*Paape et al.* 1978, *Williams et al.* 1978, 1984). Similar differences were also found when zymosan particles were used instead of bacteria. However, further experiments are needed to clarify the factors involved.

A new method described by *Bjerknes & Bassøe* (1983) makes the differentiation between adherent and ingested zymosan particles by the FCM possible. The automatic FCM measurements of bacteria and zymosan particle degradation by phagocytes was also recently performed by *Bassøe* (1984). These two methods, however, require a computer assistance.

The technique used in this paper permits a rapid and precise determination of several important parameters of phagocytic activity of normal peripheral bovine blood granulocytes.

ACKNOWLEDGEMENT

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SAMMANFATTNING

Flödescytometrisk karaktärisering av bovina neutrofila leukocyters fagocytos av fluorescerande bakterier och zymosan partiklar.

En flödescytometrisk metod användes för att studera fagocytoskapaciteten hos neutrofila leukocyter som isolerades från bovint blod. För isolering av cellerna från blodet användes en enstegs diskontinuerlig Percoll gradient. Med metoden kunde 90 ± 2,8 % (medeltal ± SD) av granulocyterna utvinnas ur blodet.

De neutrofila leukocyterna inkuberades med FITC (fluorecin isothiocyanat) märkta S. aureus eller zymosanpartiklar i proportionerna 1:20 respektive 1:10 i 10 % serum. Fagocytosen avslutades efter 15 minuter och antalet extracellulära bakterier eller zymosanpartiklar och fagocyterande granulocyter mättes medelst flödescytometri (FCM). FCM och mikroskopiska studier visade att de eosinofila leukocyterna fagocyterar bakterier i ringa utsträckning. Den granulocytpopulation som var aktivt fagocyterande utgjordes till allra största delen av neutrofila luekocyter.

De neutrofila leukocyternas förmåga at fagocytera bakterier varierade mellan kor.

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