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Flow cytometry measurement of *Saccharomyces cerevisiae* phagocytosis by neutrophils in mouse blood

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We used the flow cytometric method for measuring the phagocytosis of *Saccharomyces cerevisiae* (*S. cerevisiae*) cells by mouse neutrophils. This method allows us to identify different cell populations by their light scattering properties. The heterogeneity of the mouse blood cell population was determined by forward scatter (FSC) and side scatter (SSC) cytometric profile which showed two distinct cell populations. The experimental conditions were selected in such a way that it was possible to analyse the phagocytosing function of neutrophils in pheripheral blood without time-consuming cell separation. Only 100 µl of heparinized whole blood samples was required for this determination. The phagocytosis was not dependent on the opsonins at 37 °C for 30 min. This method allowed to differentiate between the cells adherent to neutrophils and the ingested yeast cells by quenching fluorescein-labeled extracellular yeast cells with ethidium bromide. Trypan blue did not alter in fluorescence quenching the effect on ingested and adherent yeast cells. In addition, the results of this study show that cytochalasin D by 45–46% inhibited the phagocytosis of ingested *S. cerevisiae* cells by mouse neutrophils. These data offer a sensitive technique which should be very useful in the further studies and estimation of the interactions between normal phagocytes and other microorganisms in pheripheral blood.

Key words: flow cytometry, neutrophils, phagocytosis, *Saccharomyces cerevisiae*

INTRODUCTION

Phagocytosis represents a central element of the host defense system against bacterial and fungal invasion [1]. As is known, “professional phagocytes” such as neutrophils are primarily responsible for maintaining normal host defenses against bacterial and fungal intruders by a process known as phagocytosis [2]. Evaluation of blood cell phagocytosis is of interest to many clinical and research laboratories. The clinical interest of studies of the phagocytic function of neutrophils is presently well established [3]. Defective neutrophil phagocytic function can occur in children with immunodeficiency states, particularly chronic granulomatous disease (CGD); specific alterations in neutrophil function including abnormal phagocytosis can predict the development of AIDS in HIV-infected patients.

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Interaction between phagocytic cells and target cells is often studied by light microscopy, fluorescence microscopy or use of radioactively labeled target cells. But classic techniques present several disadvantages: they are indirect, expensive, complex and often poorly reproducible. However, time-consuming and often highly subjective methods do not always distinguish between adherent and ingested particles and are often not reproducible.

In most instances, neutrophil functions are individually measured, and consequently purified neutrophils are necessary for most *in vitro* studies [4]. This inevitably requires a relatively large amount of blood, which poses particular difficulties for studies when small amounts of blood samples are available. Another common problem with the usual methods resides in the preparation of the cells and deals with modification of receptors and activation of the cells [5].

Many of the methods used for neutrophil function assay pose difficulties in differentiation of in-

gested yeast cells from those adherent. Flow cytometric study of phagocytosis has addressed several cell types, such as neutrophils or monocytes following internalization of bacteria, fluorescent microspheres or immunomicrospheres, nanoparticles [6]. These instruments combine cytofluorometry with the identification of different cell subpopulations by differences in the light scattering properties. As is known, flow cytometry has improved the prior methods and enabled us to analyze cellular kinetics or interactions between host and microorganisms with a much smaller number of cells requiring smaller volumes of blood. In our assay we used FITC labeled yeast cells for measuring their uptake by neutrophils.

The aim of our study was to evaluate the phagocytosis of *Saccharomyces cerevisiae* cells by mouse neutrophils, using two-colour flow cytometric analysis, and to distinguish adherent and ingested yeast cells without prior cell separation. In addition, we suppose that mouse neutrophils and *S. cerevisiae* cells will remain suitable models for studying cell–particle interactions *in vitro*. It will be valuable for rapid measurement of functional phagocytosis parameters of neutrophils in heterogeneous living cellular samples for immunology, pharmacology, toxicology and clinical research.

MATERIALS AND METHODS

Mice. All procedures were carried out in accordance with the European Union guidelines and were allowed by the Ethical Committee of the Institute of Immunology, University of Vilnius, Lithuania. BALB/c mice of either sex age 4 to 8 weeks, weighing 24 ± 2 g, were obtained from the same organization. The animals were kept in standard conditions with food and water intake *ad libitum*.

Sera. Normal mouse sera were obtained from the BALB/c mice. The mice were irretrievably anesthetized by inhaled chloroform and exsanguinated by cardiac puncture. The whole blood was allowed to clot for 30 min at room temperature following the exposure for 2 h at 4 °C. Serum was removed by centrifugation at 1000 g for 20 min at 4 °C, subdivided into 100 µl aliquots and stored at –70 °C until use. An aliquot was thawed immediately prior to being used in each experiment.

Cultivation of *S. cerevisiae*. Yeast cells were grown in complete YEPD medium (1% yeast extract, 2% peptone, 2% glucose) at 30 °C on a reciprocal shaker at 150 rpm for 24 h to the OD_{590} = 0.4. Solidified media contained 2% agar (Difco Laboratories Detroit, MI, USA). The cells were harvested by centrifugation, washed twice with distilled water and heat-killed (heated on a boiling water-bath for 30 min), washed, resuspended in physiological saline and stored at –20 °C in small aliquots.

Preparation of fluorescent yeast cells. Heat-killed yeast cells were labeled with fluoresceinisothiocyanate (FITC) (Sigma, St. Louis, MO, USA) in a 0.5 M carbonate buffer pH 9.5 containing 10^8 yeast cells and 100 µg of FITC/ml. The reaction mixture was incubated at 37 °C for 30 min and washed four times in phosphate-buffered saline (PBS), pH 7.4 and once with distilled water. Each washing was followed by centrifugation. The supernatant obtained after the last centrifugation was found to be free from fluorescent dye. The FITC-labeled yeast cells were suspended to a final concentration of 5×10^8 cells/ml in PBS and were stored in small aliquots at 4 °C. The labeling was stable for 1 year at –20 °C.

Phagocytic assay. The phagocytosis assay was determined in 12 × 75 polypropylene tubes with a final volume of 1 ml/tube. 100 µl of heparinized blood samples was added to each tube. The stock of FITC-labeled yeast cells at a concentration of 5×10^8 cells/ml in a volume of 20 µl were added to all tubes. A tube with blood sample only served as a negative control. To adapt the opsonization conditions, 20 µl of freshly thawed pooled mouse serum was added to each tube. PBS was added to all tubes to a final volume of 1 ml. Parafilm was placed over the tubes, which were incubated in a shaking water bath at 37 °C, with continuous agitation for 5, 10, 20, 30 and 60 min, respectively. Samples were removed to each time period and phagocytosis was stopped in ice. All collected samples were centrifuged in a microcentrifuge for 1.5 min at 300 × g and washed twice with ice-cold PBS to remove excess fluorescein-labeled yeast cells. Erythrocytes were removed with 2 ml by hypotonic lysis (ammonium chloride 150 mM / l, potassium bicarbonate 12 mM/l and EDTA 0.1mM/l) for 20 min at room temperature. The cells were resuspended in 2 ml of ice-cold PBS and then split into two tubes 1 ml each and kept at 4 °C. To one tube, 10 µl of ethidium bromide (EB) (Sigma) was added to a final concentration of 50 µg/ml or 10 µl trypan blue (TB) (MERCK, Darmastadt, Germany) in PBS, pH 4.5 at a concentration of 0.04% to quench adherent yeast cells. This procedure differentiated between yeast cells that were ingested (green fluorescence cells) and adherent (red fluorescence cells). To analyze the quenching capacity on adherent yeast cells, 5 µmol / l cytochalasin D (Sigma Chemical Co.) was used to inhibit phagocytosis. The samples were then allowed to equilibrate at room temperature for at least 2 min before analysis. A stock solution of EB was prepared with water at 5 mg/ml, and TB was prepared in PBS, pH 4.5 at a concentration 0.04% and stored at room temperature.

Flow cytometry. FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, Calif., USA) emitting an argon laser beam at 488 nm was used to detect both FITC and EB fluorescence. In flow cytometry, each cell is assigned two values, forward scatter and side scatter, depending on light scattering

properties as a cell passes the laser beam. Based on these two values each cell is represented by a point in a rectangular co-ordinate system where granulocytes, lymphocytes and monocytes are represented by well-separated clusters. A total of 10000 cells were analyzed per tube. Green fluorescence was measured at 530 ± 15 nm and red fluorescence was measured at 585 ± 42 nm. The events were acquired in a linear mode for FSC and SSC and in a logarithmic mode for FL-1 and FL-3.

RESULTS AND DISCUSSION

In the last 30 years great advance has been made towards understanding the interactions between microorganisms and hosts. Increasing interest in recent years has been shown in the flow cytometric evaluation of phagocytic events [7]. In this light, interactions between neutrophils and yeast cells are still very poorly understood.

There are hundreds of different species of yeast identified in nature which exist in/on all living matter: water, soil, plants, air, etc., but the most important species from human standpoint are yeast-pathogens of immunocompromised people. The ongoing problems associated with the antibiotic resistance of pathogenic microorganisms, as well as immunodeficiency, allergic, autoimmune and cancer diseases impel to search after new prophylactic and therapeutic approaches.

Preliminary experiments were done to develop and optimize flow cytometry assay to quantify phagocytosis of *S. cerevisiae* cells by mouse neutrophils without the requirement for manipulation of the cells. In the cytogram the cells are distributed according to the forward and side scattered light. To set gates, FSC (size) and SSC (granularity) were recorded. Then blood cells were identified and gated as shown in Fig. 1. The granulocytes were characterized as medium-sized cells with high granularity (area R1), 95% of them being neutrophils. Mononuclear cells were identified as large cells with lower granularity (area R2). Our findings are in agreement with data reported by Emmendor and colleagues [8]. They showed an example, in the analysis of a correct gate setting of cells adjusted by flow cytometry in whole human blood where two cell populations could be discriminated. Recently we have published the results of fluorometric assay for the quantitative evaluation of macrophage phagocytosing capacity, using *S. cerevisiae* cells as a target [9]. In our experiments, we assessed the phagocytosis kinetics of isolated macrophages by fluorometric assay. In the present study we have simplified our evaluation by using only small amounts of whole blood for the analysis of the interaction between *S. cerevisiae* cells and mouse neutrophils. However, evaluation of neutrophil function when purified and washed neutrophils are used may have many disadvantages, especially when analysis is carried out in pediatric patients [10].

In order to study the binding and uptake of yeast cells by phagocytes, we used opsonins freshly thawed from pooled mouse serum. The amount of phagocytosis of yeast cells by neutrophils was determined after 5, 10, 20, 30 and 60 min of incubation at 37 °C. The results (Fig. 2) showed that phagocytosis increased with time up to 30 min and re-

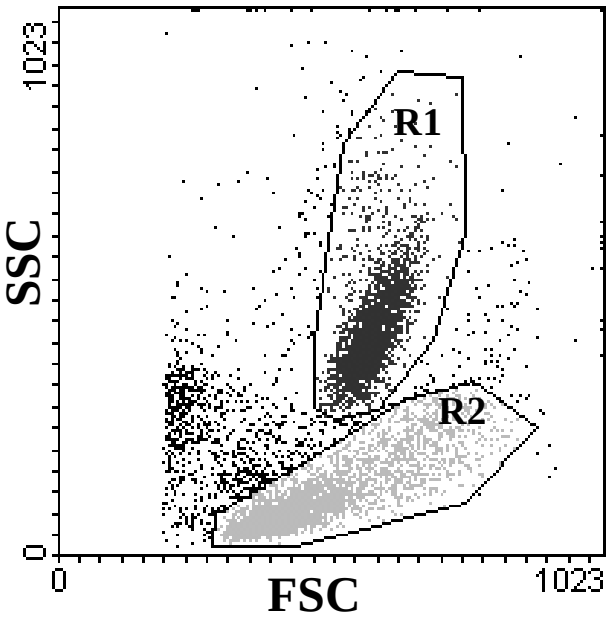

Fig. 1. FSC/SSC dot plot of a whole blood sample. The distribution of cells of a whole blood sample characterized by size (FSC) and granularity (SSC) is shown. Polymorphonuclear cells – area R1, mononuclear cells – area R2

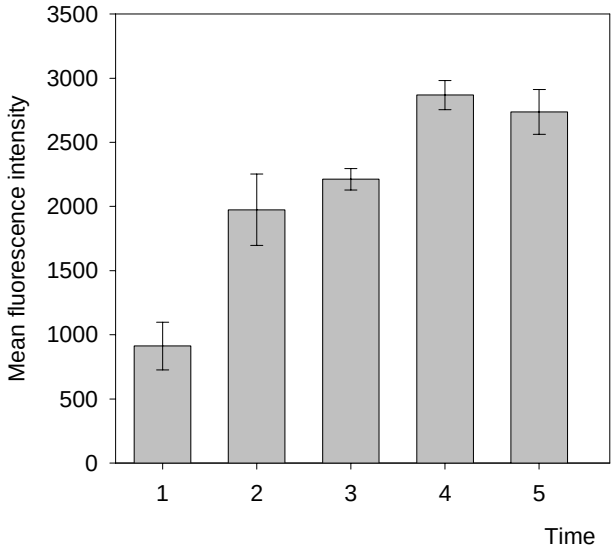

Fig. 2. Time course analysis of phagocytosis of *S. cerevisiae* cells by mouse neutrophils in whole blood (1 – 5 min; 2 – 10 min; 3 – 20 min; 4 – 30 min; 5 – 60 min). Each point represents a mean ± SEM of three experiments performed in triplicate

mained at the same level after prolonged incubation up to 60 min. Our previous results showed that the maximal rate was observed at 37 °C for 60 min [9]. We found that 30 min of incubation of *S. cerevisiae* cells with neutrophils is enough for complete phagocytosis to occur. The same experiments were repeated with or without opsonins. A possible explanation for this faster phagocytosis process in whole blood may be the contribution of other blood components. It is possible that in whole blood phagocytic cells produce cytokines during incubation and they influence the phagocytic function [10]. The results of the phagocytosis experiments performed with heat-killed *S. cerevisiae* cells and neutrophils showed that the phagocytic process is independent of opsonins. This could mean that opsonins don't play any role when neutrophils are present in whole blood. It was confirmed by our experimental data, when the results of phagocytosis at 37 °C after 60 min of incubation with or without opsonins were compared (data not shown). Hence, opsonization of yeast cells was unnecessary, since the incubation time within whole blood during the test ensured sufficient opsonization of yeast cells for phagocytosis in the physiological environment. Our findings are in agreement with data of Perticarari et al. [10] who have described phagocytosis of bacteria *Staphylococcus aureus* and *Candida albicans* by polymorphonuclear leukocytes and monocytes.

In other series of experiments we described the fluorescence quenching method which differentiates between attached and ingested yeast cells in individual neutrophils, testing the ability of dyes to cause quenching of the fluorescence of attached yeast cells without affecting the fluorescence of ingested yeast cells due to the dye exclusion of viable cells. It should be noted that trypan blue as a quenching agent is not applicable in this assay because of its inability to distinguish between adherent and ingested cells. It is important to select a quenching dye that does not penetrate the membrane of the phagocyte. As Heinzelmann et al. [4] have noted, EB is suitable for this purpose. The disadvantage of EB in penetrating dead cells, however, could be changed into an advantage by excluding those cells from the flow cytometric analysis because of their high red fluorescence. A typical flow cytometric analysis of phagocytosis by neutrophils of fluorescein-labeled yeast cells is shown in Fig. 3. The extracellular yeast cells adhered to neutrophils were clearly distinguished from intracellular yeast cells that had been ingested by neutrophils using quenching with EB. Simultaneous measurements of both green and red fluorescence allowed quantitation of yeast cells adhered to neutrophils or ingested by neutrophils. Red EB fluorescence was equivalent to the number of neutrophils with attached yeast cells. Green FITC fluorescence was representative of the number of neutrophils with ingested yeast cells. The expression of both red and green fluorescence was associated with both attached and ingested yeast cells.

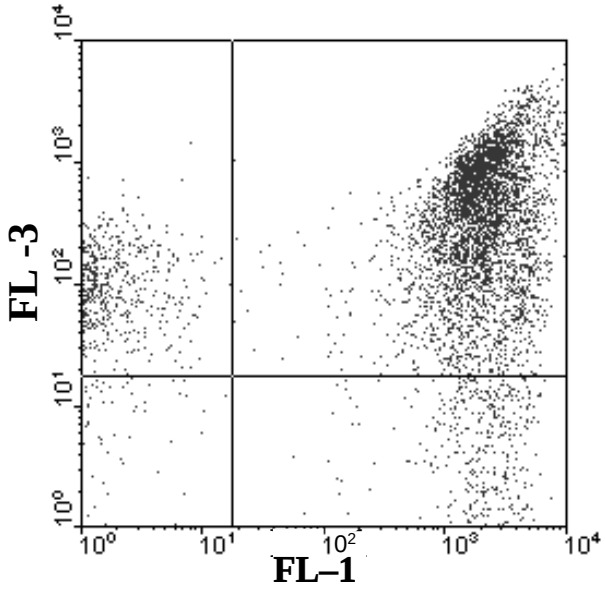

Fig. 3. Effect of EB on the phagocytosis of *S. cerevisiae* cells by mouse neutrophils for 30 min at 37 °C. Typical cytogram demonstrating phagocytosis (FL-1) and fluorescence (FL-3)

Finally, we tested the effect of cytochalasin D on phagocytosis, as measured in this assay. Others have reported that phagocytosis is sensitive to the microfilament disrupting effects of cytochalasins [11]. They showed that while cytochalasins inhibit the ingestion process, they do not inhibit adherence. After treatment of neutrophils with cytochalasin D prior to exposure to *S. cerevisiae* for 30 min, the result was a green fluorescence mean channel (MC) of 2796, reflecting the lack of phagocytosis (compared with 6087 without cytochalasin D) and subsequent quenching with EB decreased the MC to 2684 (*versus* 5992 without cytochalasin D). The relative difference in MC after addition of EB was 112 for neutrophils pretreated with cytochalasin D *versus* 95 for neutrophils without cytochalasin D pretreatment. These results reflect the MC values of adherent *S. cerevisiae* cells (Fig. 4). Our results show that cytochalasin D inhibited phagocytosis by 45–46% with and without quenching with EB. Granfeldt's group demonstrated similar findings using cytochalasin B in the phagocytosis of yeast cells by human neutrophils [2]. The presence of cytochalasin B in a phagocytic system is often used to inhibit the ingestion phase. In that case our results confirm this proposition. When trypan blue at pH 4.5 was used in the flow cytometric assay, there were no statistically significant differences in the fluorescence intensity of neutrophils that were quenched or not with trypan blue. The quenching effect was in good agreement with the results obtained on macrophages and yeast cells in our previous work [9].

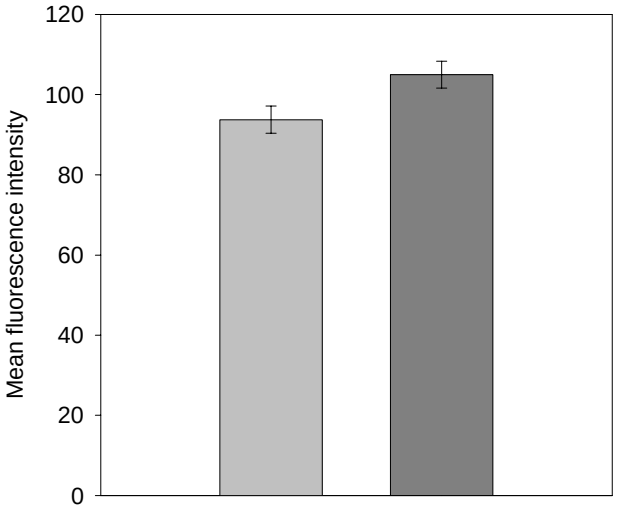

Fig. 4. Effect of cytochalasine D on the phagocytosis of *S. cerevisiae* cells by mouse neutrophils for 30 min at 37 °C. Columns represent green fluorescence intensity mean channel values of adherent *S. cerevisiae* ▫ – with and ▪ – without quenching with EB

In conclusion, we have shown that using FITC-labeled yeast cell samples, the fluorescence quenching technique, small amounts of whole blood and two-colour flow cytometric technique could be an effective diagnostic tool in evaluating a functional impairment of neutrophils. Also, our results suggest that they can be useful for studying the interactions between normal phagocytic cells and other microorganisms.

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SACCHAROMYCES CEREVISIAE LÀSTELIØ FAGOCITOZËS TËKMËS CITOMETRINIS TYRIMAS PELIØ NEUTROFILAIS KRAUJYJE

Santrauka

Tëkmës citometrinis metodas buvo pritaikytas tirti *Saccharomyces cerevisiae* làsteliø fagocitozæ peliø neutrofilais; jo dëka làsteliø populiacijos atskirtos pagal ðviesos sklaidà. Làsteliø populiacijø heterogeniðkumas nustatytas pagal citometrinius tiesinës (FSC) ir ðoninës (SSC) ðviesos sklaidos parametrus. Pasirinktos eksperimentinës sàlygos leido tirti *S. cerevisiae* làsteliø fagocitozæ peliø neutrofilais periferiniame kraujyje netaikant daug laiko trunkanèiø làsteliø iðskyrimo procedûrø, kurios neigiamai veikia neutrofilø funkcijà. Tam tikslui panaudota 100 µl ðvieþiai iðskirto, heparinizuoto peliø kraujo. Nustatyta, kad fagocitozë, vykstanti 30 min. 37°C temperatûroje, nepriklauso nuo opsoninø. Panaudojæ etidþio bromidà (EB) ávertinome prie neutrofilo prilipusias ir prarytas mieliø làsteles. EB gesino fluorescinu þymëtø prilipusiø mieliø làsteliø fuorescencijà, kai tuo tarpu tripano mëlis (TM) ðiam procesui átakos neturëjo. Be to, nustatyta, kad fagocitozës inhibitorius citochalazinas D 45–46% inhibavo prarytø *S. cerevisiae* làsteliø fagocitozæ peliø neutrofilais. Manome, kad gauti duomenys ir pritaikyto metodo privalumai bus naudingi tiriant bei ávertinant kitas sàveikas tarp fagocitø ir kitø rûðiø mikroorganizmø periferiniame kraujyje.