Acta Biologica Hungarica 54 (3–4), pp. 347–355 (2003)

QUANTITATIVE EVALUATION OF MACROPHAGE PHAGOCYTOSING CAPACITY BY A FLUOROMETRIC ASSAY

VALĖ MILIUKIENĖ,[1]* GENĖ BIZIULEVIČIENĖ,[2,3] AUDRONĖ PILINKIENĖ[3]

[1] Department of Developmental Biology, Institute of Biochemistry, 12 Mokslininkø Street, LT-2600, Vilnius, Lithuania; [2] Department of Plant Physiology and Microbiology, Faculty of Natural Sciences, University of Vilnius, 21/27 M. K. Čiurlionio Street, LT-2009, Vilnius, Lithuania; [3] Laboratory of Pharmacology, Institute of Immunology, University of Vilnius, 29 Molëtø plentas, LT-2021, Vilnius, Lithuania

(Received: October 1, 2002; accepted: March 10, 2003)

This paper reviews sensitive and simple quantitative evaluation of macrophage phagocytosing capacity by applying fluorescein-labeled *Sacharomyces cerevisiae* cells. Yeast cells were conjugated with fluoresceinisothiocyanate (FITC) and used as fluorescent particles. A time course analysis within this method showed that phagocytosis of yeast cells was temperature dependent and that the number of that ones ingested by macrophages increased rapidly during the initial 60 min of incubation at 37 °C. Free fluorescent cells can be effectively removed by aspiration from the well. Furthermore, yeast cells required preopsonization with serum to achieve optimal uptake of the cells. The uptake of nonopsonized yeast cells by macrophages was significantly lower than that of opsonized cells ($P < 0.05$). We propose that about 50% of mouse macrophages can carry functionally active FcR responsible for phagocytosis.

Keywords: Phagocytosis – yeast – macrophage – fluorometric assay

INTRODUCTION

Phagocytes play a crucial role in host defense through their ability to recognize, ingest and destroy invading microorganisms. Process of phagocytosis is initiated by the interaction of specific receptors on the surface of the phagocyte with ligands on the particle [3].

Macrophages are "professional phagocytes" that play a critical role in innate and acquired immunity due to their unique ability to internalize and degrade pathogens in couple with the release of inflammatory mediators [3]. Macrophage phagocytosis can be mediated by the receptors for the Fc from IgG and for the complement (such as CR3) [10].

Interaction between phagocytic cells and target cells is often studied by light microscopy [14, 15] or fluorescence microscopy [4] both with the disadvantage of being cumbersome. Moreover, the direct counting of particles inside phagocytic cells under the microscope is fastidious and depends on the quality of the human observ-

* Corresponding author; e-mail: aiste@ifo.lt

er. Phagocytosis evaluation technique with the use of radioactively labeled particles [12, 15] require specialized laboratories and personnel. Although this technique is easier to perform, it provides no information about important parameters ascribing phagocytosis, such as the percentage of phagocytosing cells or the distribution of intracellular particles per individual cell. Furthermore, it is very difficult to analyze the time course of particle uptake properly during the phagocytic process. But the main disadvantages of this method for laboratory use are health hazards as well as cost associated with radioisotope use and disposal. Confocal scanning fluorescence microscopy [9] and flow cytometry [2, 5, 6, 8] have been used as well. Both the methods require a high degree of technical sophistication and expensive equipment as well as costly monoclonal antibodies [8]. Thus all the previously mentioned methods possess disadvantages for the routine laboratory use.

Here we describe a new rapid and objective fluorometric method. This method enables to assess quantitatively phagocytosis of *Saccharomyces cerevisiae* by mouse macrophages in a suspension, using fluorescein-labeled yeast cells.The present method will not only overcome the above-mentioned problems, but will also provide more reproducible and more accurate data on the events taking place in phagocytic processes. In addition, we suppose that mouse macrophages and *S. cerevisiae* cells will remain suitable models for studying cell-particle interactions *in vitro*. It is important to add that the presented method can be readily applied for analysis of other factors involved in phagocytosis, such as role of opsonins and temperature as well as rate of phagocytosis. This method can be suitable, for example, for screening the opsonization capacity of sera of vaccinated people. It can be used to study the interactions between other microorganisms and phagocytes as well.

MATERIALS AND METHODS

Mice

From 4 to 8 weeks old BALB/c mice of either sex, weighing 25–30 g, were obtained from the Institute of Immunology, University of Vilnius, Lithuania. The animals were kept on standard diets; the water was given *ad libitum*.

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 lyophilized.

Preparation of fluorescent yeast cells

10 mg of lyophilized yeast cells were suspended at 4 °C in 2.5 ml 0.1 M carbonate-bicarbonate buffer, pH 9.0, containing 0.9% sodium chloride. 0.5 mg of fluorescein-isothiocyanate (FITC, Sigma, St. Louis, MO, USA) were added to this solution. After mixing for 30 min at 4 °C, the cells were washed vigorously several times with cold PBS 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. Fluorescein-labeled yeast cells were stored in small aliquots of Krebs–Ringer phosphate buffer (KRP; pH 7.4) at 4 °C. The labeling was stable for 1 year at –20 °C.

Peritoneal mouse macrophages

Macrophages were stimulated by intraperitoneal injection of 0.5 ml Freund's adjuvant per mouse. After two days the mice were killed by rapid dislocation of the neck. The skin was lifted upward from anterior abdominal wall with forceps and snipped making 50 mm long incision. 5 ml of Hanks medium, containing 2 µl of heparin, were injected into the mouse peritoneal cavity. Peritoneal excudate cells were exsanguinated from peritoneum lavage. Cells suspension was incubated in Hanks medium at 5% CO_2, 37 °C for 3 h. After 3 h exposure mouse macrophages were firmly adhered to glass surface. Then the medium was removed and the adherent cells were washed vigorously 3 times with Hanks medium to remove nonadherent cells. Removal of irrelevant non-glass-adherent cells can be achieved by replacing the Hanks medium after gentle shaking of the Petri dish. Cell viability was consistently >98% as determined by trypan blue exclusion. About 96% of these adherent cells exhibited the morphological and staining characteristics of macrophages.

Sera

Normal mice 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 2h at 4 °C. Serum was removed by centrifugation at 1000 g for 20 min at 4 °C, subdivided into 0.1 ml aliquots and stored at –70 °C. To measure opsonin-dependent phagocytosis, the sera were heat inactivated at 56 °C for 30 min and stored at –70 °C. An aliquot was thawed immediately prior to being used in each experiment.

Opsonization of yeast cells

Pooled mice sera were used as a convenient source of opsonins for mouse macrophage phagocytosis. Fluorescein-labeled yeast cells were opsonized by incubation

for 1 h at 37 °C with 5% heat-inactivated mouse sera or with 5% half-diluted pooled mouse sera. After opsonization, the serum was removed by centrifugation to eliminate the excess of opsonins.

Measurement of phagocytosis

Ingestion of fluorescein-labeled yeast cells by phagocytic cells was measured as follows. Fluorescein-labeled yeast cells suspended in KRP were added to adherent phagocytic cells at a concentration of 5×10^5 cells/well. The final yeast to macrophage ratios of 10 : 1, 50 : 1 and 500 : 1 was verified in experiments in a final volume of 1 ml. After incubation at 0 °C and 37 °C for various time periods, phagocytic cells were washed 3 times with PBS with aspiration to remove non-cell-ingested particles. When adapting the fluorescent quenching method, 100 μl of 0.04% trypan blue (MERCK, Darmstadt, Germany) in PBS, pH 4.5 were added to the suspension and kept for 2 min. Cells were then solubilized by addition of 1 ml of 25 mM Tris-HCl buffer, pH 8.5, containing 0.2% sodium dodecyl sulfate (SDS, Sigma). The solution was then transferred to a cuvette for measurement of the fluorescent intensity by a fluorescence spectrophotometer (MPF-4, Hitachi, Tokyo, Japan) with an excitation wavelength of 493 ηm and an emission wavelength of 520 ηm.

The number of phagocytosed cells was determined in accordance to relationship between the fluorescent intensity and number of particles determined by applying the standard curve. The extent of phagocytosis was expressed as a number of ingested particles per cell. All experiments were performed at least in triplicate.

Statistical analysis

A mean ±SD was calculated for all representative experiments. Statistical significance between mean values was determined using Student's test. Values having $P<0.05$ were considered as significant. All calculations were performed using *Microsoft Excel, version 7.0.*

RESULTS AND DISCUSSION

We have developed a method which allows analyze the phagocytosis of yeast cells by mouse macrophages. The simple method presented here involves application of fluorescein-labeled yeast cells and permits quantitative measuring of phagocytosis with an adequate sensitivity. An advantage of the present method is employment of the fluorescein-labeled yeast cells and simple preparation of the latter. The fluorescein ation does not require expensive materials or apparatus for fluorescence detection.

To quantitate internal vs. external yeast cells after phagocytosis we used trypan blue as a quencher of the non-internalized FITC-labeled yeast cells. Such an approach was adapted from a method being used for studying monocytes and macrophages phagocytosis of fluorescein – labeled *Cryptococcus neoformans* by a fluorescence multi-well reader [15]. The fluorescence intensity of the FITC-labeled yeast cells was measured by a fluorescence spectrophotometer before and after addition of trypan blue. This enabled us to distinguish yeast cells that had been taken up by adherent phagocytes from those that were simply attached. It is interesting to note that there were no statistically significant differences in fluorescence intensity of macrophages that were quenched with trypan blue or not (results not shown). This was verified by fluorescence microscopy. Such an observation is in agreement with the data, obtained by Walenkamp et al. [15] who analyzed macrophage phatocytosing capacity of *C. neoformans*.

In order to determine the fluorescent intensity of the labeled yeast cells by fluorescent spectrophotometer, we constructed a standard curve using various concentrations of yeast cells (10^3 to 10^6 per ml). The fluorescence in the test samples was determined by interpolation of the results in accordance with standard curve and thus the particle number was determined quantitatively. The fluorescence intensity of these particles was directly proportional to their concentration (Fig. 1).

The further purpose of our study was to estimate the conditions of the phagocytosis: rate, influence of temperature and opsonization. To study the effect of variation of any of these factors the others should be kept constant. We determined that the ratio of yeast cells to macrophages of 50 : 1 produced a maximal rate of phagocytosis in our experiments (data not shown). A time course analysis within this method showed that phagocytosis of yeast cells was temperature dependent and that number of particles ingested by cells increased rapidly during the initial 30 min incubation at 37 °C. Our results show that the maximal rate was observed at 37 °C for 60 min,

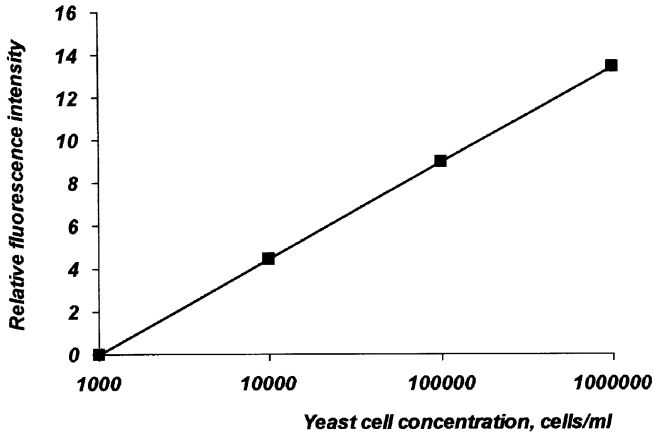

Fig. 1. Relationship between intensity of fluorescence and yeast cell concentration

when yeast cells were opsonized with half diluted mice sera at the final concentration of 5% (Fig. 2). Macrophages ingested opsonized yeast cells in a temperature dependent fashion. At 37 °C the rate of ingestion of yeast cells by these phagocytic cells was essentially linear with time up to 60 min and then reached a plateau after 120 min. In contrast, uptake of yeast cells at 0 °C was minimal and the amount of ingested particles after 60 min was 5–10% of that at 37 °C.

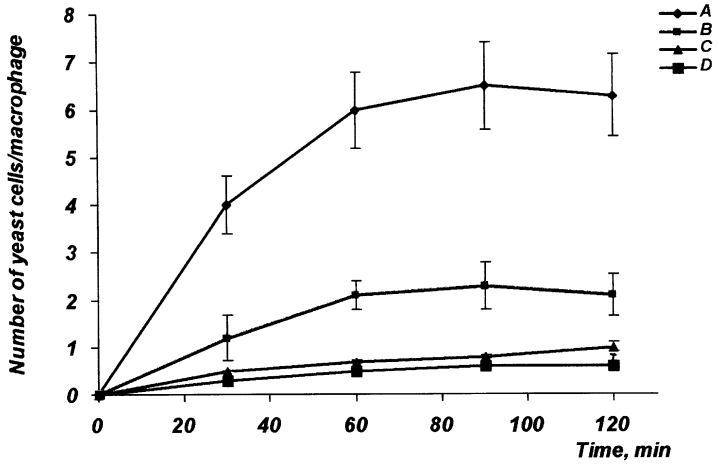

Fig. 2. Time course analysis of phagocytosis of yeast cells by mouse macrophages. Phagocytosis of opsonized (A) or non-opsonized (B) particles at 37 °C and opsonized (C) or non-opsonized particles (D) at 0 °C

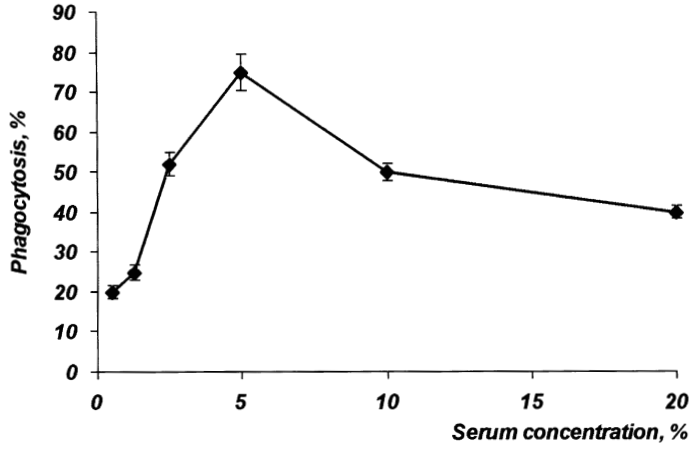

Fig. 3. Effect of serum concentration of the percentage of phagocytosing macrophages measured by fluorometry. Experiments were performed with yeast: macrophage ratio 50 : 1

Furthermore, phagocytic cells required opsonization with serum to achieve optimal uptake of yeast cells. Our results show (Fig. 2B) that the uptake of nonopsonized particles by macrophages was significantly lower at 37 °C than that of opsonized particles (Fig. 2A) ($P < 0.05$). In contrast, the uptake of yeast cells at 0 °C both opsonized with serum (Fig. 2C) and non-opsonized ones (Fig. 2D), was pretty much the same ($P > 0.5$). The quantitative analysis of maximum phagocytosis has recently been performed by other investigators [13] applying flow cytometry. For over than a 60 min period the isolated human polymorphonuclears (PMN) were exposed to *Staphylococcus aureus* which were rapidly phagocytosed and *Klebsiella pneumoniae* which were slowly phagocytosed. These differences in phagocytic susceptibility may explain the clinical course of the bacterial infections. Other authors reported that amount of bacteria attached to the phagocytic surface membrane was important as well [7]. Each phagocyte was associated maximally with 80 and 45 *S. aureus* cells when measured by the flow cytometry (FCM) and microbiological method, respectively. Accordingly, the difference between the number of bacteria per phagocyte, measured by FCM and microbiological method should give the estimation of the amount of bacteria attached to the phagocyte surface membrane.

Our other experiments were carried out in order to determine the optimal sera dilution to be used for opsonization. Final serum concentrations, ranging from 1% to 20% were made up in a medium. Figure 3 indicates that maximal phagocytosis occurs when a serum concentration is equal to 5%. Results of present studies of the opsonic effect on the uptake of yeast cells by macrophages were used in all experiments. Our results indicate that in the presence of low sera concentrations, the main factor, limiting phagocytosis is lack of attachment of yeast cells to the phagocyte surface membrane. On the other hand, we consider that the higher IgG concentration may block Fc receptors and prevent the opsonized yeast cells from gaining access to them. Our results fulfill the data of other investigators [1], who found that serum concentration influences the differences between the number of bacteria per phagocyte measured by FCM and the microbiological methods.

Our data suggest that opsonization is required for effective phagocytosis. FcR and CR3 receptors represent two classes of phagocytic receptors that are involved in the phagocytic process [10]. The results, presented here show that in case of heated mouse sera the percentage of phagocytosing macrophages was approximately 50% lower than that of the control value (Fig. 4). Heating of the sera leads to the inactivation of complement and thus inhibits complement receptor mediated phagocytosis. As known, mouse macrophages possess surface membrane receptors for Fc region of IgG [10, 11]. When heat-labile serum opsonins were inactivated IgG served as the main opsonin for yeast cells. Hence, our results suggest that about 50% of mouse macrophages carry functionally active FcR responsible for phagocytosis. In such a situation, the percentage of phagocytosing macrophages, determined by fluorometric assay offers an estimation of the percentage of macrophages only half phagocytosing by FcR and assumes half macrophages phagocytosing by other receptors, most probably by complement receptors. However, we did not directly measure complement

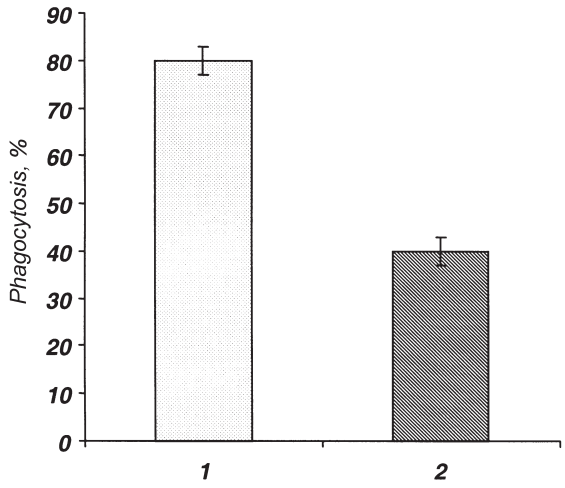

Fig. 4. Effect of control (1) and inactivated at 56 °C, 30 min (2), mouse sera on the percentage of macrophages phagocytosing yeast cells as measured by fluorometric assay after 60 min

deposition, and further experiments are needed to investigate the mechanism of complement mediated opsonization. This will be the object of our further studies.

Our fluorometric method offers multiple advantages: it is rapid, sensitive, precise and uses inexpensive readily available equipment and reagents are used. This procedure is neither technically difficult, nor does it require sophisticated equipment; the results are accurate and reproducible. In addition, the method could be used to study the interaction between other microorganisms and adherent phagocytes.

ACKNOWLEDGEMENT

We thank Dr. Gediminas Arvydas Biziulevičius for critically reading the manuscript and Miss Rima Martinkėnaitė for technical assistance.

REFERENCES

1. Bassoe, C. F., Solberg, C. O. (1984) Phagocytosis of *Staphylococcus aureus* by human leukocytes: Quantitation by a flow cytometric and microbiological method. *Acta Pathol. Microbiol. Immunol. Scand. 92,* 43–50.
2. Bjerknes, R., Bassoe, C. F., Sjursen, H., Laerum, O. D., Solberg, C. O. (1989) Flow cytometry for the study of phagocyte functions. *Rev. Infect. Dis. 11,* 16–33.
3. Gold, E. S., Underhill, D. M., Morrisette, N. S., Guo, J., McNiven, M. A., Aderem, A. (1999) Dynamin 2 is required for phagocytosis in macrophages. *J. Exp. Med. 190,* 1849–1856.
4. Gordon, S. B., Irving, G. R. B., Lawson, R. A., Lee, M. E., Read, R. C. (2000) Intracellular trafficing and killing of *Streptococcus pneumoniae* by human alveolar macrophages are influenced by opsonins. *Infect. Immunol. 68,* 2286–2291.

5. Heinzelmann, M., Gardner, S. A., Jones, M. M., Roll, A. J., Polk, H. C. Jr. (1999) Quantification of phagocytosis in human neutrophils by flow cytometry. *Microbiol. Immunol. 43,* 505–512.
6. Lehmann, A. K., Sornes, S., Halstensen, A. (2000) Phagocytosis: measurement by flow cytometry. *J. Immunol. Methods 243,* 229–242.
7. Leijk, P. C., van der Barselaar, M. T., van Zwet, T. L., Doubbeldman-Rempt, J., Furth, R. (1979) Kinetics of phagocytosis of *Staphylococcus aureus* and *Eschericia coli* by human granulocytes. *Immunology 37,* 453–465.
8. Martin, E., Bhakdi, S. (1992) Flow cytometric assay for quantifying opsonophagocytosis and killing of *Staphylococcus aureus* by peripheral blood leucocytes. *J. Clin. Microbiol. 30,* 2246–2255.
9. Matsui, H., Eguchi, M., Kikuchi, Y. (2000) Use of confocal microscopy to detect *Salmonella typhimurium* within host cells associated with Spv-mediated intracellular proliferation. *Microb. Pathogenesis 29,* 53–59.
10. May, R. C., Caron, E., Hall, A., Machesky, L. M. (2000) Involvement of the Arp 2/3 complex in phagocytosis mediated by FcgR or CR3. *Nature Cell Biol. 2,* 246–248.
11. Mejorada, G. S. (1998) Signal transduction by immunoglobulin Fc receptors. *J. Leukocyte Biol. 63,* 521–533.
12. Raley, M. J., Lennartz, M. R., Loegering, D. J. (1999) A phagocytic challenge with IgG-coated erythrocytes depresses macrophage respiratory burst and phagocytic function by different mechanisms. *J. Leukocyte Biol. 66,* 803–808.
13. Sveum, R. J., Chused, T. M., Frank, M. M., Brown, E. J. (1986) A quantitative fluorescent method for measurement of bacterial adherence and phagocytosis. *J. Immunol. Methods 90,* 257–264.
14. Van den Dobbelsteen, G. P. J. M., Van Rooijen, N., Sminia, T., Van Rees, E. P. (1991) The immune response in the rat to *Streptococcus pneumoniae* types 3 and 4 capsular polysaccharide. Detection by double immunocytochemical staining of antibody-containing cells in situ and ELISA. *J. Immunol. Methods 145,* 93–99.
15. Walenkamp, A. M. E., Scharringa, J., Schramel, F. M. N. H., Coenjaerts, F. E. J. (2000) Quantitative analysis of phagocytosis of *Cryptococcus neoformans* by adherent phagocytic cells by fluorescence multi-well plate reader. *J. Microbiol. Methods 40,* 39–45.