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DISTINCTION OF MACROPHAGE SUBPOPULATIONS: MEASUREMENT OF FUNCTIONAL CELL PARAMETERS BY FLOW CYTOMETRY

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INTRODUCTION

Macrophages represent a functionally heterogeneous group of cells which belong to the mononuclear phagocyte system (1). Heterogeneity may exist between macrophages from different organs as well as among macrophages within one organ (2,3). Heterogeneity has been defined by differences of Ia-antigen expression (4,3); monoclonal antibodies (5,6) against cell surface determinants; receptors for the C3 complement component or the Fc part of IgG molecules; cell size (7,3); enzyme activities e.g. phosphatase, nucleotidase (8) peroxidase (9) and transglutaminase (10); wheat germ lectin binding (11); tumor cytoxicity (12); or phagocytosis (13) and adherence. Classification according to several parameters is necessary to identify small subpopulations of macrophages (1). Flow cytometry is a particularly useful method for this purpose, especially because functional parameters of living cells can be measured simultaneously at the single cell level in a fast and accurate way. Such parameters include cytoplasmic (14,15) or lysosomal (16) enzyme activities, transmembrane potential (17,18), intracellular pH (19) and phagocytosis. The use of vital stains also permits cell sorting. Sorted cells can be recultivated and further analyzed. Macrophages are often characterized as cells with high esterase activity (20,21,22), although there are some reports on low esterase activity in macrophages (23,24,25). It was the purpose of this study to characterize the low activity macrophages in more detail.

MATERIAL AND METHODS

Preparation and Incubation of Peritoneal Macrophages: Resident peritoneal macrophages or paraffin oil induced macrophages were re-

moved from the peritoneal cavity under sterile conditions. Paraffin oil induced macrophages were collected between 3 and 7 days after injection of 20 ml sterile paraffin oil into the peritoneal cavity of 600 to 900 g female Pirbright guinea pigs. The cells were eluted with RPMI 1640 medium buffered with 20mM HEPES to pH 7.35, washed once by centrifugation for 5 minutes at 200 x g and maintained in the same medium. Two ml containing 1.5 to 1.8×10^6 cells/ml were incubated in 35x10 mm uncoated plastic petri dishes (Greiner, Solingen, Germany) at 37°C in humified air. Non-adherent cells were removed at various times by gentle shaking and aspiration of the supernatant. Adherent cells were either mechanically scraped off or enzymatically detached using 1% Dispase (Boehringer, Mannheim, Germany) for 8 minutes at 37°C. Cell viability was assessed by exclusion of trypan blue (4.5 mg/ml), or exclusion of DNA stain mithramycin (100 µg/ml, 15 mM $MgCl_2$ Serva, Heidelberg, Germany). May-Grünwald-Giemsa stained cytocentrifuge slides were prepared to determine the portion of lymphocytes and granulocytes.

Cell Staining: The esterase activity and the intracellular pH were both determined with the recently developed dye 1,4-diacetoxy-2,3-dicyanobenzene (ADB), final concentration 25 µg/ml (19). The intracellular pH was determined from the ratio of fluorescent light of intracellularly hydrolyzed ADB collected between 420 to 440 and 500 to 580 nm. In some experiments fluorescein-diacetate (FDA), final concentration 4 µg/ml (15), was used for the esterase activity measurement. Esterase inhibition studies were performed by incubating the cells for 50 minutes at 4°C with 70 mM NaF (26). The phagocytic activity of macrophages was quantified from the red fluorescence of ingested monosized 1.2 µm latex particles.

Flow Cytometry: Flow cytometric measurements were performed at 25°C with a Fluvo-Metricell flow cytometer developed earlier in our laboratory (27). The electronic cell volume and two fluorescence signals of each cell were measured simultaneously, amplified by 2.5 decade logarithmic amplifiers, and collected in list-mode on magnetic tape. Data acquisition and display were accomplished by FORTRAN IV computer programs (28). A hydrodynamically focused orifice of 95 µm diameter and 100 µm length with a current of 0.229 mA was used for the electronic cell volume determination.

RESULTS

The volume versus esterase activity (ADB as substrate) display of six day paraffin stimulated peritoneal exudate cells immediately after cell preparation (Figure 1A) showed four cell populations: Population I to III consisted of small, middle and large macrophages (10%, 42%, 39%) with high esterase activity, while Population IV comprised 9% of all cells and contained macrophages with esterase activity approximately 3% that of high activity cells. Their volume was between the

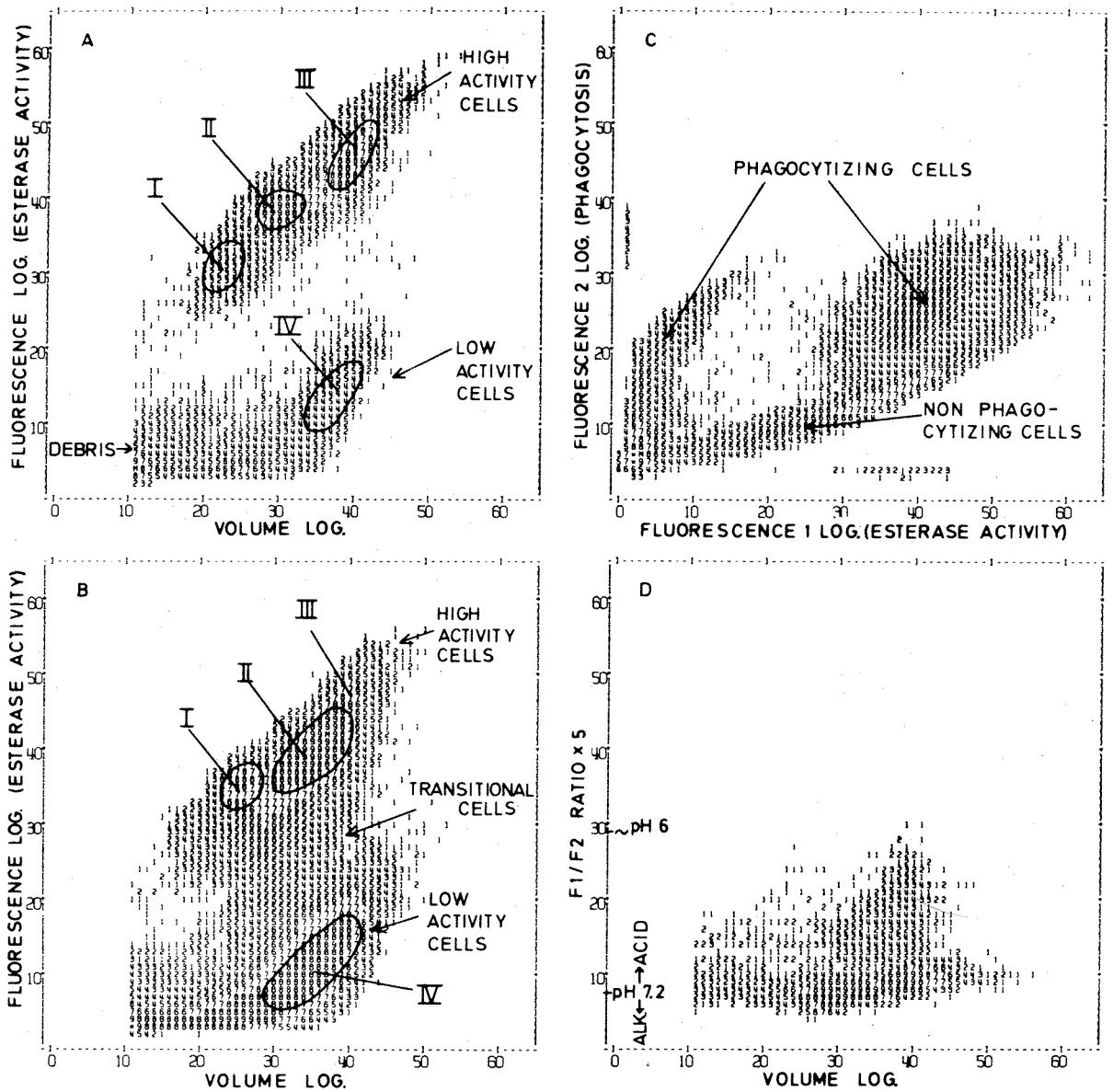

Fig. 1. A- Esterase activity versus cell volume of g. pig peritoneal macrophages 6 days after paraffin oil injection. Each scale comprises 2.5 log decades divided into ten equal steps and numbers between 1 and 9 assigned according to the number of cells in the channel. Volume class 23 corresponds to 160 μm^3. Approximately 6 classes correspond to doubling of the cell volume or fluorescence signals. Each histogram is standardized to the channel 'M'. The histograms contain between 40 to 80,000 cells. Four populations of macrophages (I-IV) are distinguishable. B- Esterase activity of macrophages after 24 h culture in uncoated plastic petri dishes. Transitional cells are apparent between the high and low activity cells. C- Esterase activity versus phagocytosis of rhodamine stained latex particles after 24 h of culture. D- Intracellular pH versus cell volume of the cells of Fig. 1A.

values of population II and III. Similar esterase activity patterns were obtained with FDA as substrate. The esterase activity was not significantly reduced by 50 minutes incubation of the cells with 3 mg/ml NaF. The macrophage preparation contained 17% lymphocytes, 4% monocytes and 1% granulocytes. Comparative measurements of the leukocytes from the peripheral blood of the guinea pig showed that lymphocytes and granulocytes were superimposed upon macrophage population I and II.

The following experiments were performed with resident and paraffin oil induced macrophages to determine whether macrophages with low esterase activity were living or dying cells.

a. Adherence: The portion of cells with low esterase activity during the first 25 hours of culture increased both in the non-adherent and adherent cell fraction from 5 to 27% of all cells (Figure 2A,B) although the viability (trypan blue exclusion) only slightly declined from 95 to about 80% (Figure 2C). Mechanically detached, adherent cells behaved similarly. A corresponding decrease in the proportion of intermediate and small cells occurred at the same time (Figure 2A-C). The transition of small and intermediate cells from a higher to lower esterase activity is also seen in Figure 1B.

b. Viability: Adherent and non-adherent cells with low esterase activity excluded trypan blue. Low esterase activity macrophages also excluded the fluorescent DNA stain mithramycin which brightly stained the nuclei of dead cells.

c. Phagocytosis: FDA esterase activity when plotted against phagocytosis showed that approximately 50% of both the low and high esterase activity cells were phagocytic (Figure 1C).

d. Intracellular pH: Figure 1A shows that a portion of intermediate and large cells had an acid intracellular pH (Figure 1D). By thresholding the esterase data of Figure 1A, it became apparent that the high esterase activity cells had a pH around 7.2, whereas the majority of the low esterase activity cells had an acidic pH between 6 and 6.9.

DISCUSSION

A significant number (5 to 15%) of macrophages with low esterase activity exist in guinea pig resident and paraffin induced peritoneal macrophage populations. Similar results were obtained in the rat (unpublished data). The low esterase activity cells are living cells as assessed by adherence, phagocytosis, and trypan blue and mithramycin dye exclusion. They have a low intracellular pH, occur in the non-adherent and adherent cell fractions and increase in number during

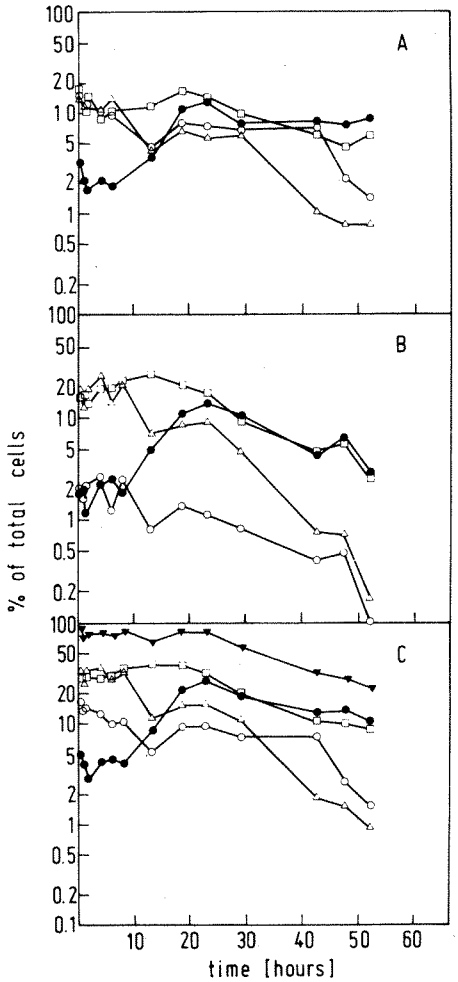

Fig. 2. Viability and esterase activity of non-adherent (A) and adherent (B) cells as a % of total cells in culture. Data are for paraffin oil induced g. pig peritoneal macrophages. Total cells (▼); high esterase activity macrophages: small population I (○), intermediate sized population II (△), and large sized population III (□); low esterase activity population IV macrophages (●).

the first 24 hours of culture by recruitment mostly from intermediately sized cells. The esterases which cleave FDA and ADB are located mainly in the cytoplasm of the cell (15). The localization of these esterases is similar to the non specific esterases which cleave alpha-naphthyl substrates.

It is of interest that flow cytometry distinguished several populations of high and low activity cells. Almost no cells with intermediate activity were observed immediately after removing resi-

dent or paraffin oil induced macrophages from the peritoneal cavity (Figure 1A). The low activity cells were often slightly enriched in the adherent cell fraction and their number increased by 24 hours cultivation.

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DISCUSSION

OLIVER: I'm concerned about a technical point. The fluorescein diacetate substrate which you used to measure esterase produces fluorescein whose emission is strictly pH dependent. Is it possible that you have the same esterase activity in both populations but different pH's which could account for the apparent difference in activity?

RAFFAEL: You might be right in this case, but not with the substrate ADP.

OLIVER: But that only measures pH, not esterase.

RAFFAEL: We measured simultaneously the esterase activity and the intracellular pH.

VAN FURTH: I'm surprised that in culture your esterase activity goes down. With guinea pig cells using a conventional assay method after 24 hours incubation, most of the cells are heavily stained for the non-specific esterase. Do you have any explanation for this observation?

RAFFAEL: Perhaps it is dependent on the substrate you use to measure the esterase activity. The non-specific esterase activity of macrophages is localized only on the membrane, but we are measuring intracellular enzyme activity.