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Respiratory burst activity in brain macrophages: a flow cytometric study on cultured rat microglia

R. B. BANATI, G. ROTHE*, G. VALET* AND G. W. KREUTZBERG
*Department of Neuromorphology, Max-Planck-Institute for Psychiatry and Cellular Biochemistry Group and *Max-Planck-Institute for Biochemistry, Martinsried, FRG*

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A new flow cytometric method for the investigation of the respiratory burst of macrophages/microglia isolated from neonatal rat brain has been established. Respiratory burst activity was measured quantitatively in single viable cells by the intracellular oxidation of non-fluorescent dihydrorhodamine 123 (DHR) to fluorescent rhodamine 123. Cultured microglia exhibited high spontaneous respiratory burst activity already before stimulation. After maximal stimulation with phorbol myristate acetate, DHR oxidation rose by 40–95%. The respiratory burst activity in resident or inflammatory, i.e. thioglycolate elicited, peritoneal macrophages was significantly lower than in cultured brain macrophages suggesting a high potential of microglia for oxidative tissue destruction.

Keywords: brain macrophages, microglia, peritoneal macrophages, respiratory burst, free radicals, flow cytometry, dihydrorhodamine 123

INTRODUCTION

Microglia activated in pathological conditions of the central nervous system assume a macrophage-like morphology, migrate to the site of tissue damage, proliferate and phagocytose (McGeer *et al.*, 1988b; Streit, Graeber & Kreutzberg, 1988). The activation may lead to the production of reactive oxygen intermediates (Giulian & Baker, 1986; Colton & Gilbert, 1987; Sonderer *et al.*, 1987; Woodroofe, Hayes & Cuzner, 1989). Following stimulation, for example by phagocytosis, the NADPH oxidase which is characteristic for macrophages, monocytes and polymorphonuclear leucocytes reduces O_2 to the superoxide anion (O_2^-). The O_2^- dismutates together with H^+ to H_2O_2 and O_2. H_2O_2 is reduced to hypochlorous acid by myeloperoxidase. This cascade of metabolic steps typically found in phagocytic cells is well known as 'respiratory burst' (Bellavite, 1988).

Respiratory burst activity of rat brain-derived microglia and macrophages has been demonstrated by luminol- or lucigenin-enhanced chemiluminescence and the measurement of the superoxide dismutase-(SOD)-inhibitable reduction of cytochrome C (Colton & Gilbert, 1987; Giulian & Baker, 1986; Sonderer *et al.*, 1987; Woodroofe *et al.*, 1989). The luminol-

Correspondence to: Professor G. W. Kreutzberg, Max-Planck-Institute for Psychiatry, Am Klopferspitz 18a, D-8033 Martinsried, FRG.

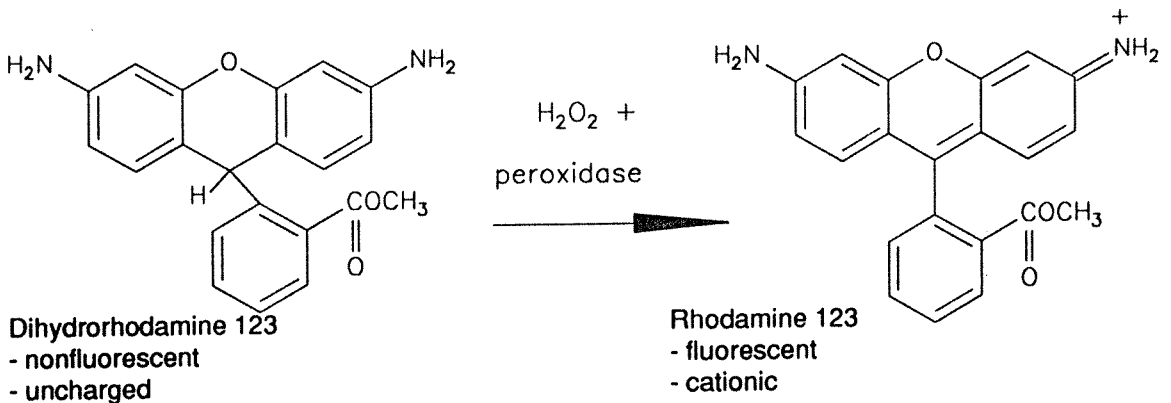

Figure 1. The membrane permeable non-fluorescent dihydrorhodamine 123 (DHR) is oxidized to the positively charged and membrane impermeable fluorescent rhodamine 123 in the simultaneous presence of H_2O_2 and peroxidase.

or lucigenin-enhanced chemiluminescence signal, however, is of unknown origin and chemiluminescence was detected at equal levels in astrocytes and in microglia (Badwey *et al.*, 1986; Sonderer *et al.*, 1987). The SOD-inhibitable cytochrome C reduction, in contrast, is a highly specific, but not very sensitive, method for the measurement of the extracellular release of superoxide anion. While this method has established the capability of cells present in microglial preparations to release superoxide anion, this method has significant limitations in the interpretation of the cellular origin of the measured reactive oxygen metabolites due to the lack of control for the heterogeneity of cellular preparations and the large number of dead cells normally found in plated microglial culture.

The measurement of respiratory burst activity in single neutrophils of heterogeneous white blood cell preparations was demonstrated in a recently published flow cytometric assay, which is based on the intracellular oxidation of the membrane permeable and nonfluorescent dihydrorhodamine 123 (DHR) to the cationic and intracellularly trapped, green fluorescent rhodamine 123 (Rothe, Oser & Valet, 1988) (Figure 1). DHR is oxidized to rhodamine 123 in the simultaneous presence of H_2O_2 and peroxidase but not by O_2^- or H_2O_2 alone, as shown in cell free assays (Rothe & Valet, 1990). Dead cells are discriminated in the flow cytometric assay by simultaneous counterstaining with propidium iodide. The goal of our study was the adaption and use of this method for the characterization of the respiratory burst activity of cultured microglia in comparison to peritoneal macrophages, which are the most commonly used cell preparation in macrophage research.

MATERIALS AND METHODS

Cultures of newborn rat brain were prepared as described previously (Giulian & Baker, 1986; Frei *et al.*, 1987). Following mechanical dissociation of the tissue in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 g/l $NaHCO_3$ and 20% heat-inactivated fetal calf serum, primary cultures were kept in 75 cm^2 culture flasks at 3% PCO_2 and 37°C for 2–4 weeks. Cells growing on top of a confluent cell layer were removed by vigorous shaking, pelleted and resuspended (3×10^6 cells/ml) in Hepes buffered saline (5 mM Hepes, 0.15 M NaCl, pH 7.35, Serva Feinbiochemica, Heidelberg, FRG) supplemented with 2 mM EDTA (HBS-EDTA). HBS-EDTA was used to avoid loss of cells through adherence.

Peritoneal macrophages were obtained from 12 week old male white Wistar rats by peritoneal lavage with 10 ml HBS-EDTA, either as resident cells or as elicited cells 4 days after intraperitoneal injection of 3 ml thioglycolate (Serva). The cells were sedimented at 200 g and

20°C for 5 min and resuspended in HBS-EDTA to 4×10^6 cells/ml. All cells were stored at 4°C for a maximum of 2 h until flow cytometric analysis.

DHR was synthesized from rhodamine 123 (Sigma Chemie, Deisenhofen, FRG) according to Kinsey *et al.* (1987) and dissolved to a 40 mM stock solution in *N,N*-dimethylformamide (DMF; Merck, Darmstadt, FRG). DHR is now also available from Molecular Probes (Eugene, Oregon, USA).

The macrophage suspensions (10 µl) were further diluted with 1 ml HBS-EDTA and stained for 5 min at 37°C with 1 µl of the 40 mM DHR solution in DMF. The DHR loaded cells were split into two aliquots of 500 µl and further incubated for 20 min at 37°C with or without 5 µl of a 15 µM solution of phorbol 12-myristate 13-acetate (PMA) (1/100 prediluted with HBS from 1.5 mM PMA in DMF, Sigma). The DNA of dead cells was counterstained with 5 µl of 3 mM propidium iodide (Serva) solution in HBS 3 min before the flow cytometric measurement.

The electrical cell volume and two fluorescences of more than 2000 cells per sample were measured simultaneously with a FLUVO-II flow cytometer (HEKA-Elektronik, Lambrecht/Pfalz, FRG). The electrical cell volume was measured with HBS as the sheath fluid after hydrodynamic focusing of the cells through the center of a cylindrical orifice of 80 µm diameter and 80 µm length at an electrical current of 0·15 mA and a suction of 10^4 nm^{-2}. Rhodamine 123 green fluorescence (500–530 nm) and propidium iodide red fluorescence (590–700 nm) were measured with the light (470–500 nm) from a HBO-100 high pressure mercury arc lamp (Osram, Augsburg, FRG). The flow cytometer was calibrated with standardized yellow-green fluorescent microspheres of 4.3 µm diameter (Polysciences, St Goar, FRG). The three signals of each cell were amplified logarithmically and digitized at maximum pulse height by a 4096-step analog to digital converters. The list mode data were evaluated by the DIAGNOS1 program system for display, calculation and databasing of flow cytometric data (Valet, Warnecke & Kahle, 1986).

RESULTS

The published method for the measurement of respiratory burst activity of human neutrophils (Rothe, *et al.*, 1988) was not applicable to cultured microglia without significant modifications. Microglial cells showed significant autofluorescence over a wide range of excitation between 400 and 500 nm. The level of autofluorescence was substantially reduced with a narrow excitation bandwidth filter from 470 to 500 nm. When microglia were brought into suspension without EDTA the concentration of viable cells strongly decreased. This was most likely due to adherence to the walls of the Eppendorf vial. To prevent cell adherence a 2 mM EDTA supplemented HBS-EDTA solution was prepared for the cellular suspension. The respiratory burst activity was unaffected by EDTA and siliconization of the vials resulted in no further improvement.

The cell suspensions were incubated at 37°C with DHR at a concentration ranging from 4–40 µM for 5 min and then stimulated with PMA (150 nM) for 10–50 min in order to establish the optimal DHR concentration for DHR oxidation. At 40 µM DHR a maximal fluorescence for stimulated cells was reached. Beyond this level the cellular DHR oxidation increased only slightly showing that the DHR concentration was no longer a limiting factor. Stimulation with PMA at a concentration of 150 nm for 20 min caused a maximal increase of fluorescence. Toxic effects did not occur as no rise in propidium iodide positive dead cells, which usually amounted to 5–10% of the total cell population, was seen for up to 60 min.

The following results were obtained with the established protocol: the microglia population (Figure 2) was homogeneous with respect to cell volume and respiratory burst activity but had

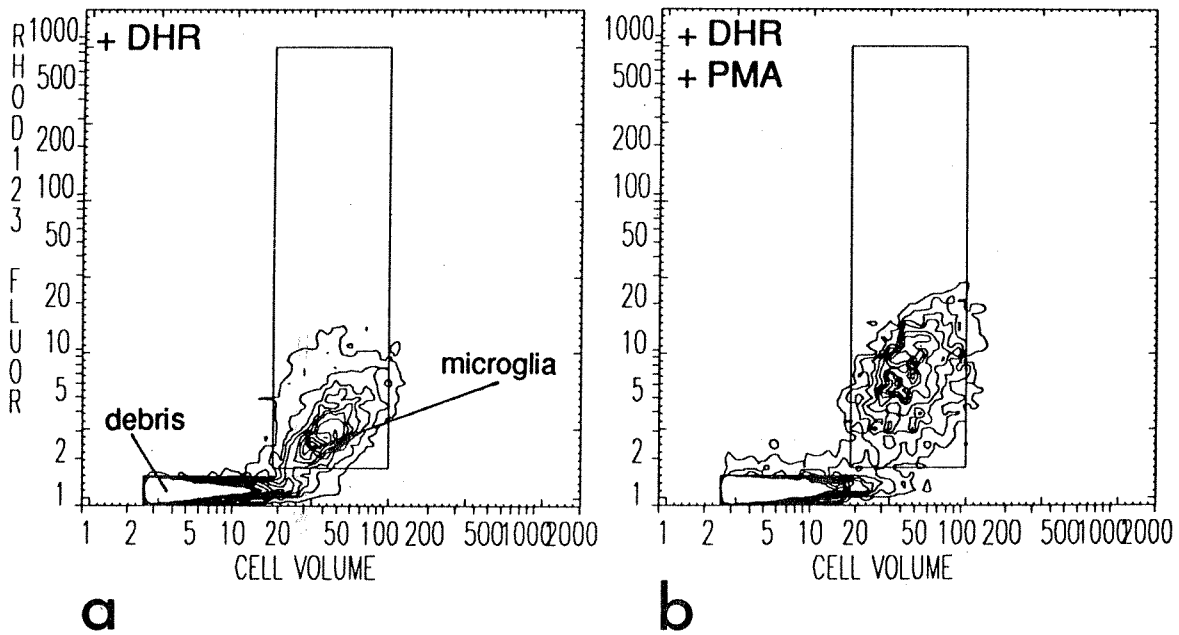

Figure 2. Cultured brain macrophages (microglia). Flow cytometric measurement of the cell volume (x-axis, arbitrary cell volume units) and intracellular oxidation of DHR to rhodamine 123 (y-axis, arbitrary cellular green fluorescence units) of microglia incubated with 40 μM DHR at 37°C for 5 min. **a**, Microglia spontaneously exhibited high respiratory burst activity even without stimulation. **b**,After stimulation with 150 nm PMA for 20 min a twofold increase of respiratory burst activity is indicated by the increase of cellular R123 fluorescence. The graphs are standardized to the maximum logarithmic channel content which lies in the centre of the contour plot and is defined as 100%. The contour lines are then drawn downwards in linear steps of 10%. The complete histogram contains the data of more than 2000 cells.

a 2.8 fold higher mean cell volume than the peritoneal macrophages (Figure 4). Microglia spontaneously exhibited a high respiratory burst activity as indicated by the oxidation of DHR (Figure 2**a**). This spontaneous activity was markedly higher than in both resident and elicited peritoneal macrophages (Figures 2**a**, 3). PMA stimulation of microglia (Figure 2**b**) resulted in a 1.4–2 fold increased respiratory burst activity which was homogeneous over the entire cell population and independent of cell cycle. Upon this stimulation with PMA, microglia also had a significantly higher respiratory burst activity than stimulated resident or elicited peritoneal macrophages (Figures 2**a**, 3 and 4). The respiratory burst activity of elicited peritoneal macrophages after PMA stimulation was higher than in PMA stimulated resident peritoneal macrophages (Figure 4**a**,**b**).

The investigation of peritoneal cell populations showed the advantage of the flow cytometric single cell analysis. When harvesting the peritoneal cells slight haemorrhagic contamination sometimes occurred. In the flow cytometric measurement contaminating granulocytes that are characterized by a high respiratory burst rate after PMA stimulation could readily be distinguished and separately analysed from the population of macrophages (Figure 4). The method thus gives reliable mean values of the respiratory burst rates of macrophages that otherwise would have been heavily influenced even by only a few contaminating cells with different respiratory burst characteristics.

DISCUSSION

Microglia form a network of potential immunoeffector cells in the brain (Perry & Gordon, 1988; Streit *et al.*, 1988). There is ample documentation that activation of this cell type is involved in

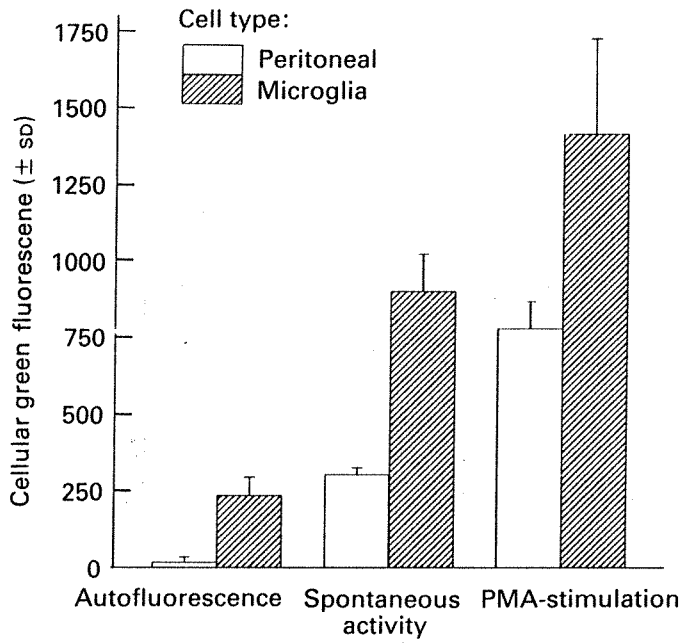

Figure 3. Hydrogen peroxide production by macrophages. The spontaneous and PMA-stimulated respiratory burst activity of rat peritoneal macrophages and cultured rat microglia was measured by flow cytometry through the intracellular oxidation of DHR to R123 as shown in Figure 2**a** and **b**. Each value represents the mean and standard deviation of four to six experiments. Cellular autofluorescence is shown as a control.

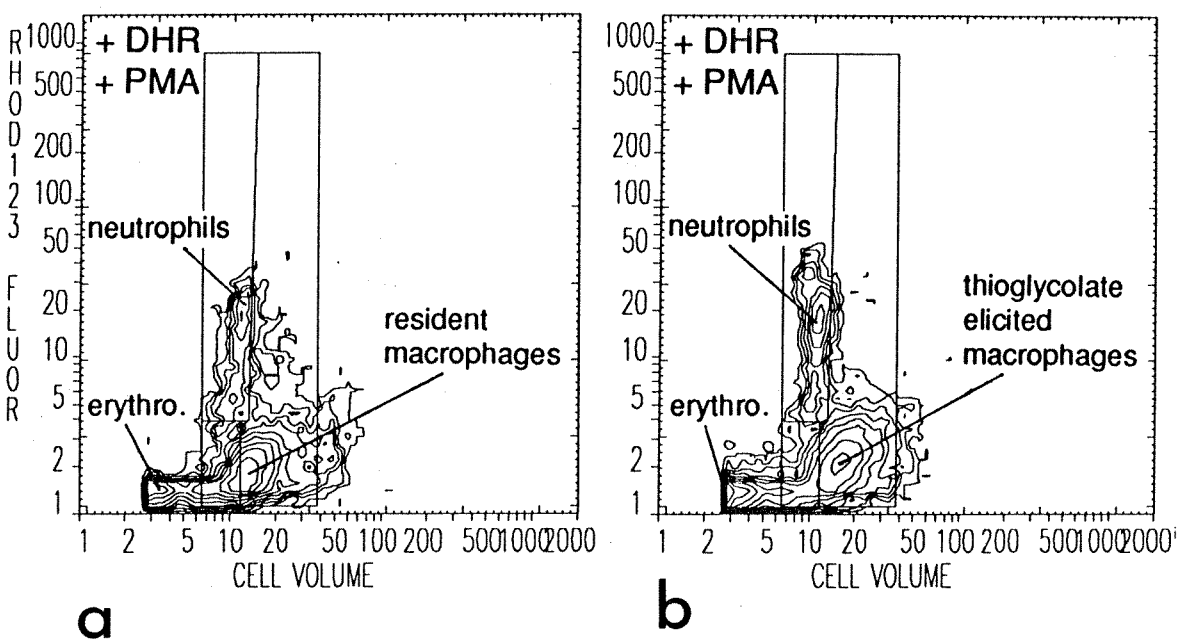

Figure 4. Rat peritoneal macrophages. Flow cytometric measurement of the cell volume and respiratory burst activity of **a**, PMA-stimulated resident and **b**, thioglycolate-elicited rat peritoneal macrophages (data presented as in Figure 2). Small numbers of contaminating neutrophils in the haemorrhagic peritoneal exudates are readily detected by their characteristically smaller volume and higher respiratory burst activity. Neutrophils can therefore be separately evaluated.

viral infections (Gendelman *et al.*, 1989), demyelinating diseases (McGeer, Itagaki & McGeer, 1988a; Konno *et al.* 1989), peripheral nerve injury, direct brain trauma (Streit *et al.* 1988) and other neurological diseases (McGeer *et al.*, 1988b; Haga, Akai & Ishii, 1989). The activation of microglia in these diseases is apparent through changes into a macrophage-like morphology, and by migration and proliferation. Although the precise function of microglia in the brain is not fully understood, a wide range of functional properties, e.g. phagocytosis, participation in lipid turnover or release of mediators in inflammation and repair, has been ascribed to microglia (Ling, 1981; Oehmichen, 1982; Perry & Gordon, 1988; Guilian, 1987). Microglia can indeed be regarded as the intrinsic source of brain macrophages as demonstrated by experimental brain damage without disturbance of the blood–brain barrier (Graeber *et al.*, 1988). Irrespective of the mechanism of microglia activation (Wong *et al.*, 1985; Merrill, 1987; Frei *et al.*, 1987) the transition from resting over activated microglia into fully competent brain macrophages should be of major importance for the severity of neuropathologic changes like demyelination. The activation may result in the production of potentially harmful reactive oxygen intermediates to which the brain, by virtue of its abundant lipids, is particularly susceptible (Halliwell & Gutteridge, 1985; Halliwell, Hoult & Blake, 1988; Konat & Wiggins, 1985). Immune-complex induced generation of reactive oxygen radicals by brain macrophages has been reported as an important feature of the canine distemper encephalitis (Griot *et al.*, 1989; Kumar *et al.*, 1989). This mechanism for demyelination may also be important for other inflammatory demyelinating diseases.

We present a new and highly sensitive flow cytometric method for the analysis of respiratory burst activity which is based on the simultaneous measurement of cell volume and the oxidative formation of a fluorescent product in single living cells. Thus heterogeneous cell populations with low or high respiratory burst activity can be analysed individually and simultaneously in the same sample. Dead cells are discriminated from living cells by DNA counterstaining of dead cells with propidium iodide. The H_2O_2 and peroxidase dependent oxidation of the substrate, DHR, and thus the production of a specific fluorescence signal is exclusively found in phagocytic cells indicating specificity of the respiratory burst activity.

The capacity of microglia to secrete spontaneously reactive oxygen intermediates and the higher respiratory burst activity following stimulation with PMA has been investigated earlier by the ferricytochrome C reduction assay or chemiluminescence methods mostly on mixed or microglia enriched cell cultures (Colton & Gilbert, 1987; Giulian & Baker, 1986; Sonderer *et al.*, 1987; Woodroofe *et al.*, 1989). Unlike the SOD-inhibitable cytochrome C reduction assay which only detects the amount of oxidants released extracellularly, the new flow cytometric method measures the total oxidant production inside stimulated macrophages through the intracellular oxidation of DHR and thus is an accurate indicator for the stimulation of these cells.

The flow cytometric method can be combined with immunocytochemical epitope analysis for cellular subtype classification using as little as 2000 to 3000 cells per sample. The high fluorescence yield makes it also applicable for fluorescence microscopy. The flow cytometric method for the study of the respiratory burst activity in microglia, the resident macrophage of the brain, is of further interest as it can be applied to determine and quantify the cellular effects of cytokines such as interferon-gamma, growth factors, or interleukins.

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