GLIA 7:183–191 (1993)

Detection of Lysosomal Cysteine Proteinases in Microglia: Flow Cytometric Measurement and Histochemical Localization of Cathepsin B and L

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KEY WORDS Brain macrophages, Peritoneal macrophages, (Z-Phe-Arg)$_2$-rhodamine 110, Alzheimer's disease, AIDS

ABSTRACT The activation and differentiation of microglia is a prominent pathophysiological process in numerous inflammatory and demyelinating diseases of the central nervous system, including Alzheimer's disease and the AIDS encephalopathy. The tissue damage during these diseases has partly been attributed to lipid peroxidating reactive oxygen intermediates for which activated microglia are a major source. The destruction of tissue may also involve the release of proteolytic enzymes, such as the lysosomal cysteine proteinases cathepsin B and L, which are present notably in phagocytic cells. The cathepsins B and L are endopeptidases with a substrate specificity including important proteins, like myelin basic protein, extracellular matrix components, or the class II major histocompatibility complex. Because of this pathophysiological relevance the cathepsins B and L were chosen for histochemical demonstration in isolated and cultured rat microglia and measurement by a new flow cytometric method.

Cathepsin B/L activity was measured flow cytometrically in single viable cells by the intracellular cleavage of non-fluorescent (Z-Phe-Arg)$_2$-rhodamine 110 to the green fluorescent monoamide Z-Phe-Arg-rhodamine 110 and rhodamine 110. In microglia we measured a cathepsin B/L activity that was 2.5 times higher than in thioglycolate-elicited, i.e., inflammatory peritoneal rat macrophages. In elicited peritoneal macrophages the formation of fluorescent product was 6.2 times higher than in unstimulated resident peritoneal macrophages, demonstrating that the activation and differentiation of mononuclear phagocytes is accompanied by an increased cathepsin B/L enzyme activity.

The subcellular localization of cathepsin B/L activity in plated viable microglia was demonstrated histochemically by the use of Z-Ala-Arg-Arg-4-methoxy-2-naphthylamide. Its blue fluorescent cleavage product 4-methoxy-2-naphthylamide was found in lysosomes.

Our study shows that activated microglia are an important potential source of cathepsin B/L. This is particularly interesting as enzymatically active cathepsins have recently been found extracellularly at high levels in the senile plaques of Alzheimer's disease, which are known to contain many activated microglia. The release of proteinases by microglia may play a crucial role in the pathomechanism of tissue-destructing diseases in the brain.

Received October 11, 1991; accepted June 2, 1992.

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INTRODUCTION

Microglia are activated under various pathological conditions of the central nervous system (CNS) showing proliferation, changes into a macrophage-like morphology, migration to the site of tissue damage, and phagocytosis (Graeber et al., 1988; Guilian, 1987; Ling, 1981; McGeer et al., 1988a,b; Oehmichen, 1982; Perry and Gordon, 1988; Streit et al., 1988).

During differentiation and activation macrophages have an increased activity of the lysosomal cysteine proteinases cathepsin B and cathepsin L (Kominami et al., 1985; Lesser et al., 1985; Morland, 1985; Morland and Pedersen, 1979; Reilly et al., 1989). Cathepsin L has recently been identified as a ras oncogene product (Joseph et al., 1987; Mason et al., 1987). The priming, activation, and differentiation of microglia might also be accompanied by an increase of the activity of lysosomal cysteine proteinases.

A number of important proteins can serve as substrate for cathepsin B/L: Extracellularly the cathepsins B and L are potent degradative enzymes for extracellular matrix proteins and their activity is therefore seen as the pathophysiological basis for the invasive behavior of, e.g., cancerous cells (Chauhan et al., 1991). The extracellular release of cathepsin B was demonstrated in the amyloid deposits in Alzheimer brains (Cataldo and Nixon, 1990), where microglial activation is a prominent neuropathological feature (McGeer et al., 1988a,b; Styren et al., 1990). Intracellularly cathepsin B/L also has the capacity to participate in the metabolism of neuropeptides (Marks et al., 1986; Marks and Berg, 1987). A possible role in immunological processes can be suspected as cathepsin B/L cleaves the invariant chain of the class II major histocompatibility complex (Reyes et al., 1991; Roche and Cresswell, 1991) and CNS-specific proteins such as the highly immunogenic myelin basic protein (Barrett and Kirschke, 1981; Berlet and Ilzenhöfer, 1985; Kopitar et al., 1983; Whitaker et al., 1982; Yanagisawa et al., 1984). In addition to the high amount of reactive oxygen intermediates produced by microglia (Banati et al., 1991) these enzymatic reactions may contribute to the tissue damage and repair processes in which microglia are activated.

MATERIALS AND METHODS

Cultivation of Microglia

Cultures of newborn Wistar rat brain were prepared as described previously (Frei et al., 1987; Giulian and Baker, 1986). 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 to 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.

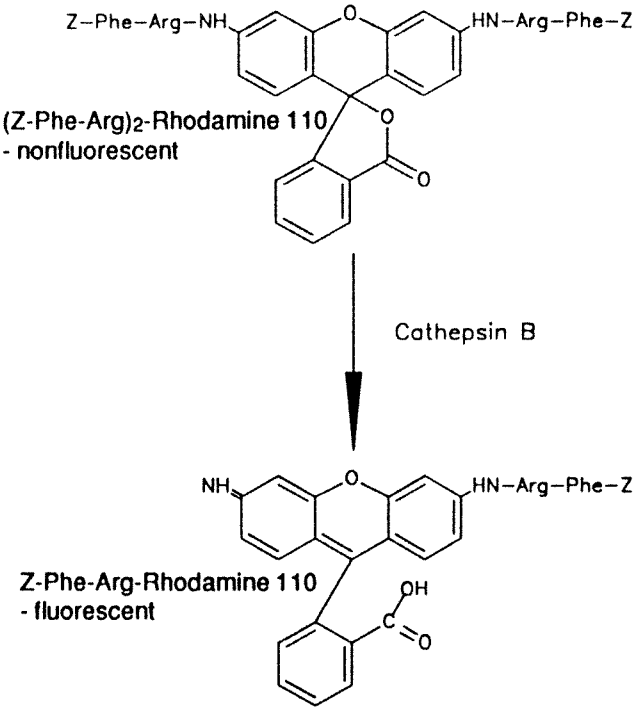

Fig. 1. $(Z\text{-Phe-Arg})_2$-rhodamine 110. Cathepsin B/L specifically converts the nonfluorescent $(Z\text{-Phe-Arg})_2$-rhodamine 110 into the monoamide Z-Phe-Arg-rhodamine 110 and rhodamine 110, the green fluorescence of which is measured by flow cytometry.

Peritoneal Macrophages

Peritoneal macrophages were obtained from 12 week old male white Wistar rats by peritoneal lavage with 10 ml HBS-EDTA either as resident or as elicited cells 4 days after intraperitoneal injection with 3 ml thioglycolate (Serva Feinbiochemica, Heidelberg, FRG). 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.

Flow Cytometry

The substrate $(Z\text{-Phe-Arg})_2$-R110 (Fig. 1) (Rothe et al., 1990) was synthesized according to Leytus et al. (1983) and dissolved to a 5 mM stock solution in N,N-dimethylformamide (DMF; Merck, Darmstadt, FRG). For the flow cytometric measurements microglia harvested from primary culture were 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).

The macrophage suspensions (10 μl) were further diluted with 1 ml HBS-EDTA. The diluted cell suspensions (1 ml total volume) were split into 4 aliquots of 250 μl and incubated for 5 min at 37°C either as a control or in the presence of either 10 μM Z-Phe-Ala-CHN_2 (stock 5 mM in DMSO, Bachem Biochemica, Heidelberg, FRG) (Green and Shaw, 1981), or 200 μM E-64 (stock 100 mM in DMSO; Sigma, Deisenhofen, FRG) (Barrett et al., 1982) or 0.2% (v/v) DMSO. This preincubation was followed by staining for 20 min at 37°C with 10 μM $(Z\text{-Phe-Arg})_2$-R110. The DNA of dead cells was counterstained with 5 μl of 3-mM propidium iodide (PI) (Serva Feinbiochemica) solution in HBS 3 min prior to the flow cytometric measurement.

The electrical cell volume and the red and green fluorescences of more than 2,000 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}. Z-Phe-Arg-R110 and R110 green fluorescence (500–530 nm) and PI red fluorescence (590–700 nm) were measured after excitation (470–500 nm) with an HBO-100 high pressure mercury arc lamp (Osram, Augsburg, FRG). The flow cytometer was calibrated with standardized yellow-green fluorescent microspheres of 4.7 μm diameter (Polysciences, St. Goar, FRG). The three signals of each cell were amplified logarithmically and digitized at maximum pulse height by a 4,096-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 et al., 1987).

Histochemistry

For the histochemical localization of cathepsin B/L activity, microglia were seeded onto glass coverslips with a mean plating density of 20 (±4.4 SD) × 10^3 cells/cm^2. Non-adherent cells were removed after 30 min. Remaining cells were kept in the same medium overnight. For the detection of cathepsin B/L-like activity a modified protocol of Dolbeare and Vanderlaan (1979) and Van Noorden et al. (1987), which omits 5-nitrosalicylaldehyde coupling, was used. The coverslips with the adherent microglia were incubated in a medium containing 100 mM phosphate buffer (pH 6.0), 10% (w/v) polyvinyl alcohol (PVA type III, Sigma, Deisenhofen, FRG) as tissue stabilizer, 2 mg/ml Z-Ala-Arg-Arg-4-methoxy-2-naphthylamide (stock 1 mg/10 μl in dimethylformamide, Bachem Biochemica, Heidelberg, FRG), 1.3 mM EDTA, 10 mM dithiothreitol (Sigma), and 2.7 mM L-cysteine (Sigma). After an incubation time of 20 min at 37°C the coverslips were rinsed for 10 min in 100 mM phosphate buffer (pH 8.0) containing 10 mM N-ethylmaleimide (Sigma) to stop the enzymatic reaction. To ensure the specificity of the fluorescence the incubation was either performed in the absence of substrate or presence of inhibitors of cysteine proteinases such as 5 μM E-64 (Barrett et al., 1982) or 5 μM leupeptin (Sigma). To test whether the site of the histochemical localization of the fluorescent product was identical with the actual site of the enzymatic reaction or was due to intracellular redistribution of the fluorescent product the cells were incubated in the same buffers following the protocol described above with the product 4-methoxy-2-naphthylamide (2 mg/ml MNA, Serva Feinbiochemica) in place of the substrate.

The photomicroscopic detection of the fluorescent product MNA (maximal excitation wavelength 340 nm, maximal emission wavelength 425 nm) (Dolbeare and Vanderlaan, 1979) was performed with a ×100/1.30 oil Leitz Neofluar objective using an excitation wavelength of 340–380 nm from an HBO-200 high pressure mercury arc lamp (Osram) and an emission filter set of 418–443 nm.

Statistical Analysis

The significance of the differences in the enzymatic activity of the various cell types was tested by analysis of variance (SPSS/PC+, SPSS, Inc., Chicago, IL).

RESULTS

Flow Cytometry

In order to obtain optimal measurements, a number of conditions had to be tested. Microglial cells showed significant autofluorescence at an excitation of 400–500 nm. The level of autofluorescence was drastically reduced when an excitation wavelength of 470–500 nm was chosen.

After 20 min incubation with 10 μM of the substrate $(Z\text{-Phe-Arg})_2$-R110, strong green fluorescence developed, indicating the formation of the fluorescent products Z-Phe-Arg-R110 and R110 (Figs. 1, 2). The specificity was shown by the almost complete inhibition of the product formation by the cathepsin B/L-specific inhibitors E-64 (200 μM) or Z-Phe-Ala-CHN_2 (10 μM). Concentrations higher than 10 μM $(Z\text{-Phe-Arg})_2$-R110 led to an increase in the number of dead cells as indicated by a rise in PI staining. An increase of dead cells did not occur in the simultaneous presence of $(Z\text{-Phe-Arg})_2$-R110 and one of the inhibitors Z-Phe-Ala-CHN_2 (10 μM) or E-64 (200 μM), showing that the cellular toxicity was caused by the intracellularly accumulated products Z-Phe-Art-R110 and R110.

With the established protocol the following results were obtained (Figs. 2, 3). The microglial cell population was homogeneous, with an average cell volume 2.8-fold higher than that of the peritoneal macrophages. At the working concentration of 10 μM $(Z\text{-Phe-Arg})_2$-R110, the total cathepsin B/L activity of microglia was 15.5 times higher than in resident ($P < 0.001$) and

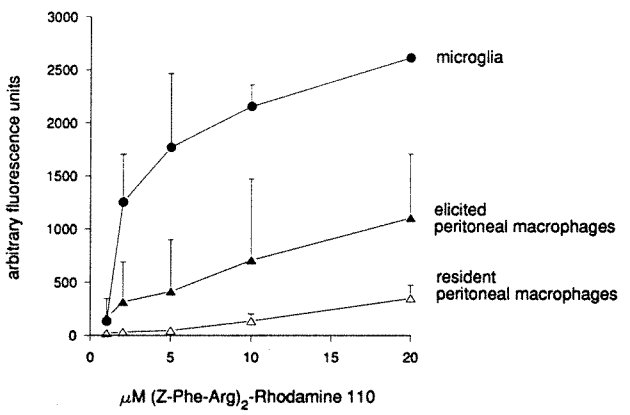

Fig. 2. Cathepsin B/L activity in microglia compared to peritoneal macrophages. Cathepsin B/L activity of microglia was significantly higher ($P < 0.001$) than in both elicited or resident peritoneal macrophages. Compared to elicited peritoneal macrophages, resident peritoneal macrophages had only low cathepsin B/L activity. The significantly higher ($P = 0.029$) fluorescence of thioglycolate-elicited versus resident rat peritoneal macrophages demonstrated that the activation of macrophages is accompanied by an increase in cathepsin B/L expression. The diagram contains at least two to four measurements of more than 2,000 living cells per measurement for each substrate concentration. Data are represented in arbitrary fluorescence units (y-axis); error bar shows the standard deviation.

2.5 times higher than in elicited peritoneal macrophages ($P < 0.001$). Compared to elicited peritoneal macrophages, resident peritoneal macrophages had only low cathepsin B/L activity ($P = 0.029$). The 6.2 times higher fluorescence of thioglycolate-elicited rat peritoneal macrophages demonstrated that the activation of mononuclear phagocytes was associated with an increase of the lysosomal cysteine proteinases cathepsin B and L.

Histochemistry

After a 20 min incubation with the substrate Z-Ala-Arg-Arg-MNA, the blue fluorescent product MNA was found in cultured rat microglia in granules of various size consistent with the lysosomal occurrence of cathepsin B/L (Fig. 4). Cathepsin B/L-containing vesicles were also seen at the tip of long cytoplasmic protrusions (Fig. 4A, open arrow). No such blue granules were seen either in the absence of substrate or in the presence of the inhibitors E-64 (5 μM) or leupeptin (5 μM). Incubation with the product MNA alone resulted in a slight and diffuse blue fluorescent background staining, demonstrating that the granular cathepsin B/L-dependent product localization was not the result of a re-distribution phenomenon (Fig. 4D).

DISCUSSION

Microglia are cells of mononuclear-phagocyte lineage ubiquitously spread in the CNS. As part of the immune system of the brain (Perry and Gordon, 1988; Streit et al., 1988), they are involved in the human immune deficiency virus infections (Gendelman et al., 1989), demyelinating diseases (Konno et al., 1989; McGeer et al., 1988a), peripheral nerve injury, direct brain trauma (Streit et al., 1988), and other neurological disease states (Haga et al., 1989; McGeer et al., 1988b). Under these conditions an activation of microglia can be observed, i.e., proliferation, migration, and eventually changes into a macrophage-like phenotype. A similar differentiation into macrophages is observed in cultures of microglia from neonatal rat brain as used in this study. Activation and differentiation of mononuclear phagocytes is accompanied by the expression of lysosomal cysteine proteinases, particularly the cathepsins B and L. The secretion of the cathepsins B and L (Ishii et al., 1991) may play an important role in tissue destruction and repair.

Both cathepsin B and cathepsin L are powerful degradative enzymes for extracellular matrix proteins such as collagen (Burleigh et al., 1974; Etherington and Evans, 1977; Mason et al., 1987) and basal membrane constituents like laminin (Lah et al., 1989). The role of cathepsin B/L in proteolytic tissue destruction has therefore led to therapeutic concepts aiming at the inhibition of elevated cysteine proteinase activity during inflammatory processes (Baici and Lang, 1990; Martel-Pelletier et al., 1990; Van Noorden and Vogels, 1987).

Since cathepsins B and L also have the capacity to degrade myelin (Berlet and Ilzenhöfer, 1985; Kopitar et al., 1983, Whitaker et al., 1982; Yanagisawa et al., 1984), their presence in the brain (Kominami et al., 1985) might be especially important in demyelinating processes like multiple sclerosis or experimental allergic encephalomyelitis (EAE). A potential role for cathepsin B as an immune-regulating enzyme is suggested by its ability to process class II major histocompatibility complex (Reyes et al., 1991; Roche and Cresswell, 1991).

Enzymatically active lysosomal proteinases, in particular cathepsin B, were recently found to be major

Fig. 3. Flow cytometric measurement of cathepsin B/L activity. Cathepsin B/L activity was measured by the intracellular conversion of non-fluorescent $(Z\text{-Phe-Arg})_2$-rhodamine 110 (10 μM) to the green fluorescent monoamide Z-Phe-Arg-rhodamine 110 and rhodamine 110. All graphs are standardized to the maximum logarithmic channel content, which lies in the center of the contour plot (arrow) 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 2,000 living cells. A: Microglia. Microglial cells have a high cathepsin B/L activity (left). The enzymatic activity of cathepsin B/L is specifically inhibited by 10 μM Z-Phe-Ala-CHN_2 (right). B: Rat peritoneal macrophages. Resident (left) and thioglycolate-elicited (right) peritoneal macrophages were compared. Thioglycolate-elicited, i.e., inflammatory, rat peritoneal macrophages have a higher cathepsin B/L activity, demonstrating that cathepsin B/L is a sensitive marker for macrophage activation. C: Thioglycolate-elicited peritoneal macrophages. The high activity of enzymatic cleavage of $(Z\text{-Phe-Arg})_2$-rhodamine 110 in elicited peritoneal macrophages is almost completely blocked by 10 μM Z-Phe-Ala-CHN_2. This shows that in fact the proteolytic activity of cathepsin B/L was measured.

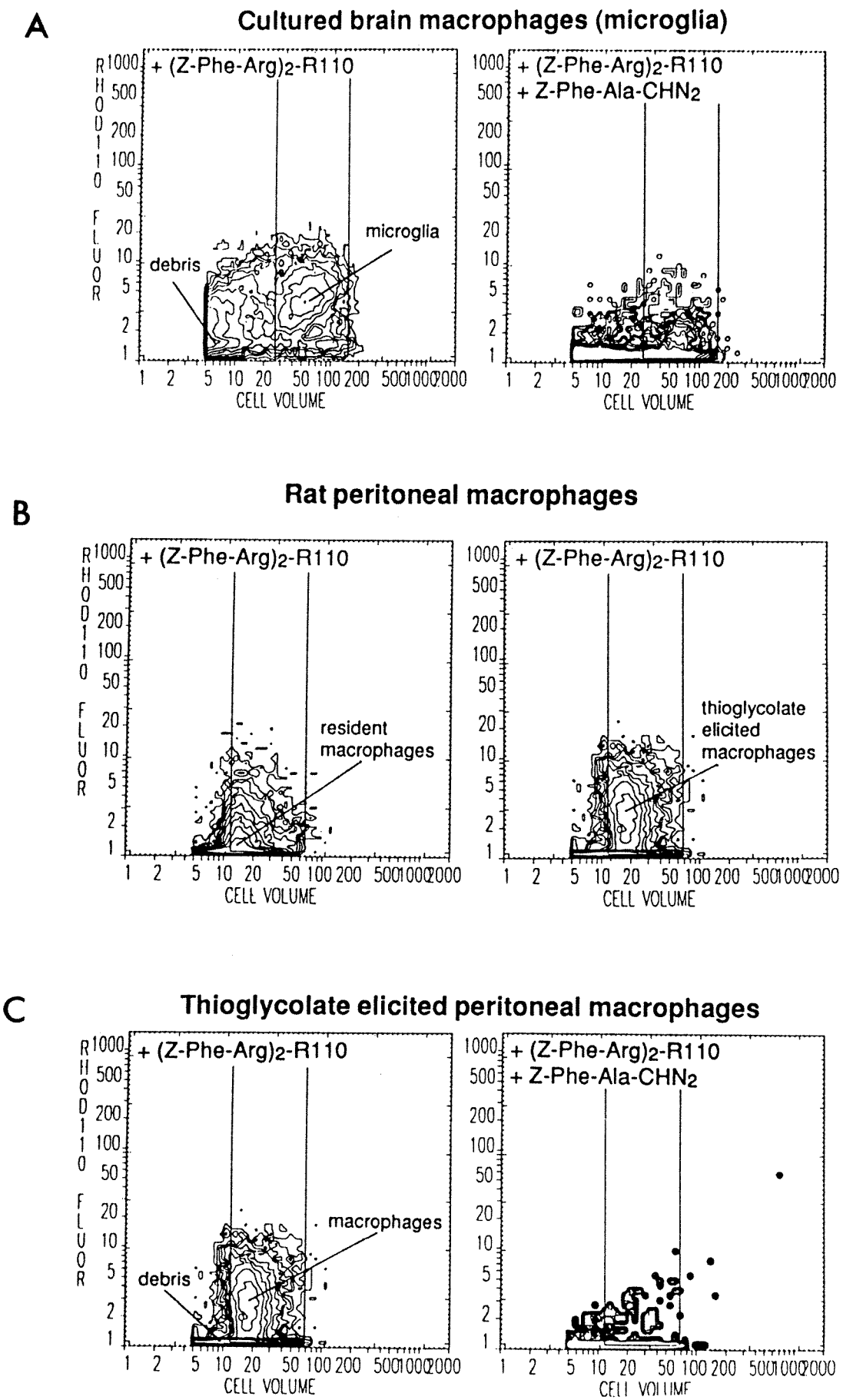
A
Cultured brain macrophages (microglia)
+ (Z-Phe-Arg)2-R110
+ (Z-Phe-Arg)2-R110
+ Z-Phe-Ala-CHN2
RHODO110 FLUOR
CELL VOLUME
debris
microglia
B
Rat peritoneal macrophages
+ (Z-Phe-Arg)2-R110
resident macrophages
thioglycolate elicited macrophages
C
Thioglycolate elicited peritoneal macrophages
+ (Z-Phe-Arg)2-R110
+ Z-Phe-Ala-CHN2
macrophages
debris

Fig. 3.

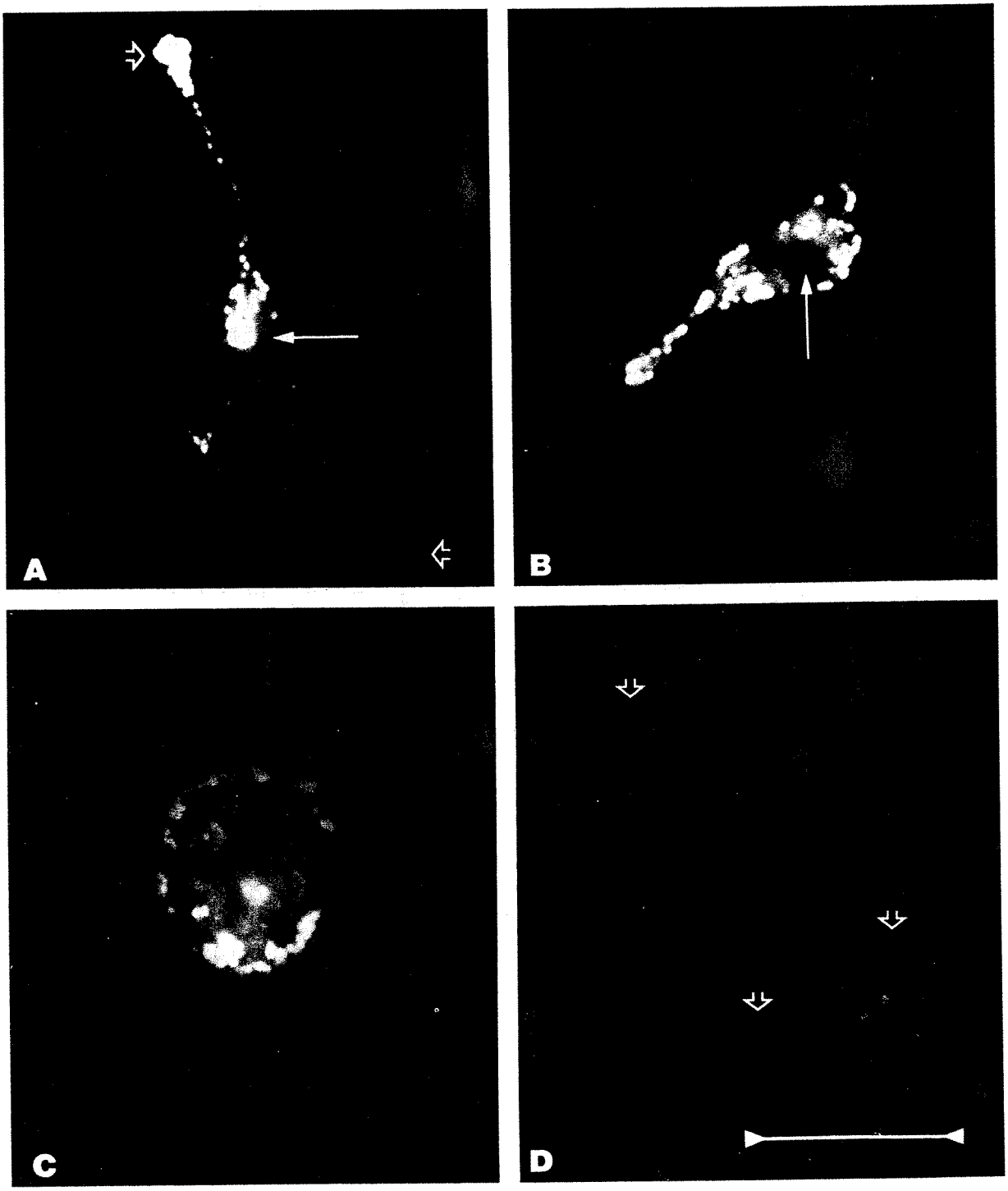

Fig. 4. Histochemical localization of cathepsin B. The pictures show the typical morphology of microglia: ramified (A, B) and round forms (C). The fluorescent reaction product is localized in lysosomes. The arrow points to the nucleus, which is non-fluorescent. The open arrow (A) points to cytoplasmic protrusions. The control incubation (D) with the fluorescent reaction product MNA shows only a slight and diffuse background fluorescence (open arrows). Scale bar = 40 μm.

constituents of the senile plaques in Alzheimer's disease, possibly resulting in an abnormal cleavage of accumulating amyloid precursor protein (Cataldo and Nixon, 1990). With the high number of reactive microglia around amyloid fibrils (McGeer et al., 1988a,b; Styren et al., 1990), they must be considered as a potential

source for lysosomal proteinases. It has been reported that cells of the mononuclear phagocytic lineage may play a direct role in the formation of amyloid (Fuks and Zucker-Franklin, 1985; Durie et al., 1982). However, it is at present highly controversial whether microglia are not only in intimate contact with the amyloid of senile plaques but also take part in its production (Itagaki et al., 1989; Wisniewski et al., 1989). Further, microglia have been shown to contain cystatin C, a very potent inhibitor of cathepsin B, which is by localization and partial sequence homology with glucagon and corticotrophin related to the peptidergic neuroendocrine system (Grubb and Lofberg, 1982; Zucker-Franklin et al., 1987). This inhibitor is downregulated in monocytes and macrophages after stimulation (Chapman et al., 1990; Warfel et al., 1987). The microglial cells of senile plaques are clearly stimulated, as indicated by the production and secretion of factors like interleukin 1 (Griffin et al., 1989). This allows the hypothesis that with the downregulation of important inhibitors unopposed and abberant cleavage of proteins including the amyloid precursor protein (Cataldo and Nixon, 1990), possibly by microglial proteases, could take place and lead to amyloid plaque formation. A deficiency of cystatin C has been confirmed to be crucial for the amyloidogenesis in hereditary cerebral hemorrhage with amyloidosis (Ghiso et al., 1986).

However, since a simple and fast detection method for cathepsin B/L was hitherto not available, this part of the proteolytic enzyme system has only rarely been analyzed and so far not described for microglia.

A number of highly sensitive fluorogenic peptide-derived substrates are available to study the enzymatic activity of lysosomal cysteine proteinases, i.e., cathepsin B and L. In our study Z-Ala-Arg-Arg-4-methoxy-2-naphthylamide, which is cleaved by cathepsin B and L, proved satisfactory for histochemistry. For flow cytometry, however, the new substrate $(Z\text{-Phe-Arg})_2$-R110 was used, in contrast to the substrate originally employed by Dolbeare and Smith (1977). $(Z\text{-Phe-Arg})_2$-R110 has a number of advantages. It can be used at physiological pH and has less background than the previously used naphthylamine derivates. Further, $(Z\text{-Phe-Arg})_2$-R110 has a higher sensitivity because its optimal excitation wavelength lies at 488 nm and thus reduces the level of cellular autofluescence. As cathepsin B and L show virtually the same substrate specificity, we did not further differentiate between them (Barrett and Kirschke, 1981).

We have shown the presence of cathepsin B/L in lysosomes of microglia histochemically using Z-Ala-Arg-Arg-4-methoxy-2-naphthylamide as substrate (Dolbeare and Vanderlaan, 1979; Van Noorden et al., 1987). The product 4-methoxy-2-naphthylamide (MNA) could be viewed directly in plated microglia by its blue fluorescence. The direct detection of MNA circumvented the formation of background by unspecific coupling of nitrosalcylaldehyde to proteins (Van Noorden et al., 1987). Due to insufficient focal resolution for detecting the fluorescent product in the lysosomes of microglia in situ, the method was applied to tissue sections.

The activity of the cysteine proteinase cathepsin B/L was further quantitatively determined in single viable cells by a new flow cytometric method. In principle, the formation of the green fluorescent cleavage products Z-Phe-Arg-rhodamine 110 and rhodamine 110 from the nonfluorescent substrate $(Z\text{-Phe-Arg})_2$-rhodamine 110 was measured simultaneously with the cellular volume. The specificity of product formation was shown by inhibition with the specific inhibitors, E-64 (Barrett et al., 1982) and Z-Phe-Ala-CHN_2 (Green and Shaw, 1981). The method also allows the study of single cells within heterogeneous cell populations. Thus cells with low or high cathepsin B/L activity can be analyzed individually in the same sample. Dead cells are discriminated from the population of living cells by DNA counter-staining of dead cells with PI. This highly sensitive flow cytometric measurement can be combined with immunocytochemical epitope analysis for cellular subtype classification. The new flow cytometric method for the study of cathepsin B/L activity in microglia, the macrophages of the brain, is particularly interesting for the quantification of the cellular effects of factors such as interferon-gamma, growth factors, or interleukins.

In summary, we have detected a high content of cathepsin B/L in activated microglia compared with other inflammatory cells. Our results demonstrate that microglia differentiating into macrophages, as can be seen in vivo and in vitro, constitute a potent source of cathepsin B/L. The differentiation and activation of mononuclear phagocytes during inflammation, a process that is induced in peritoneal macrophages by intraperitoneal thioglycolate injection, accompanied an increased expression of cathepsin B/L. The high cathepsin B/L activity of cultured microglia suggests that these cells are differentiated and activated. Thus an increased formation of cathepsin B/L in microglia in vivo might bear some importance with respect to disease states in which a microglial activation is known to take place (Cataldo and Nixon, 1990; Haga et al., 1989; McGeer et al., 1988a,b; Polman et al., 1986).

ACKNOWLEDGMENTS

We would like to thank R. Gleich for excellent technical assistance in cell culture and A. Oser for the synthesis of $(Z\text{-Phe-Arg})_2$-R110. This work was supported in part by the Forschungsverbundprojekt BGA 2/89.

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