Flow Cytometric Analysis of Inflammatory Cells in Ischemic Rat Brain

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Background and Purpose—Inflammation plays a key role in cerebral ischemia through activation of microglia and infiltration by leukocytes. Flow cytometry is a well-established method for quantitative and qualitative analysis of inflammatory cells. However, this technique has not been applied to the study of cerebral ischemia inflammation. The aim of this study was to establish a flow cytometric method to measure inflammatory cells in ischemic brain.

Methods—To perform flow cytometry on brain tissue, we developed 2 cell-isolation methods based on different mechanical dissociation and Percoll gradient separation techniques. The methods were tested on a rat model of permanent middle cerebral artery occlusion. Morphological and immunophenotypic analyses, with the use of anti-CD11b, anti-CD45, and αβ T-cell receptor antibodies, were employed to identify and quantify inflammatory cells.

Results—Both methods gave consistent results in terms of yield and reproducibility. The cell suspension contained granulocytes, macrophages, lymphocytes, and neural cells. Morphological and immunophenotypic analyses enabled the identification of a cell-scatter gate (R1a) enriched in inflammatory cells. With both methods, a higher number of events in R1a were recorded in the ischemic hemisphere than in the nonischemic hemisphere (P<0.001). CD11b, CD45, and αβ T-cell receptor staining confirmed that this augmentation was a reflection of the increase in the number of granulocytes, cells of the monocytic lineage, and lymphocytes.

Conclusions—Quantitative flow cytometric analysis of ischemic rat brain is feasible and provides a reliable and rapid assay to assess neuroinflammation in experimental models of brain ischemia. (Stroke. 2002;33:586-592.)

Key Words: cerebral ischemia • flow cytometry • granulocytes • inflammation • microglia • rats

Inflammation plays a central role in the pathogenesis of several brain disorders, such as viral meningoencephalitis, cerebral malaria, multiple sclerosis, and cerebral ischemia. After cerebral ischemia, inflammation develops as a consequence of 2 processes: activation of microglia and resident perivascular/parenchymal macrophages, and mobilization and infiltration of peripheral inflammatory cells.

Inflammatory response in focal cerebral ischemia has been studied in animal models by histological, immunohistochemical, and biochemical methods.1–5 These studies prompted researchers to test the neuroprotective effect of anti-inflammatory approaches6 despite the time-consuming methods required to evaluate the effective inhibition of inflammatory cell infiltration (ie, cell count in histological sections).7,8 The efficacy of these strategies in reducing stroke damage9 was tested in clinical trials with the use of antibodies against adhesion molecules (intercellular adhesion molecule-1 [Enlimomab trial] and CD11/CD18 [Hu23F2G trial]).

Flow cytometric assays have been developed to study immune cells involved in nervous system inflammatory diseases such as experimental autoimmune encephalomyelitis,10–12 multiple sclerosis,13 and experimental neuritis.14 Despite the quantitative analysis advantages over histological and immunohistochemical approaches, the use of flow cytometry to study cerebral ischemia has been extremely limited. It was used to detect apoptosis, by analysis of DNA content in cortical cells,15 and to evaluate the expression of adhesion molecules in blood samples of stroke patients.16,17

The main difficulties encountered in the application of flow cytometry to study cerebral ischemia reside in cell suspension preparation, in preserving intact antigen determinants, and in identifying the different cell populations present in the suspension. The aims of this study were to identify a method to overcome these problems and to apply it to the evaluation of infiltrating cells in a rat model of permanent middle cerebral artery occlusion (pMCAO). The choice of both model and species was dictated by the following considerations. Even though reperfusion is considered a major factor in inflammation after MCAO, evidence exists that in the pMCAO model an important inflammatory reaction involving cell infiltration occurs as well. When we considered that a relevant proportion of human strokes could be assimilated to a permanent occlusion, the characterization of inflammatory reaction in a pMCAO model seemed relevant from a therapeutic perspective. With respect to species choice, the mouse is clearly the reference animal for immu-
Materials and Methods

Animals and Surgical Procedures

Procedures involving animals were conducted in conformity with institutional guidelines and in compliance with European Community Council policy. Rats (Sprague-Dawley; weight, 250 to 275 g; Charles River) had free access to food (until 12 hours before surgery) and water and were maintained on a 12-hour light/dark cycle.

Ischemia was induced by permanent occlusion of the left MCA in rats anesthetized with chloral hydrate (400 mg/kg IP) (BDH). The pMCAO was performed according to the method described by Shigeno et al with minor changes. Briefly, the main trunk of the MCA was exposed, electrocoagulated, and resected close to its origin at the junction with the olfactory branch. Rats were allowed to breathe spontaneously, and rectal temperature was maintained at 37 ± 0.5°C. Sham surgery was performed with omission of the electrocoagulation and resection steps.

Infarct volume was performed in parallel experiments according to the method described by Rabuffetti et al (infarct volume, 90.0 ± 5.7 mm³; data are expressed as mean ± SEM; n = 20).

Preparation of Infiltrating Cells

Twenty-four hours after pMCAO, animal ischemic status was evaluated with the use of a behavioral scale ranging from 0 (no neurological deficit) to 3 (severe neurological deficit). Only animals showing both a score of 3 and brain with morphological signs of ischemia were included in the study. The brain was rapidly removed, and hemispheres were separated, freed from meninges, placed into 15 mL of ice-cold PBS containing 0.2% bovine serum albumin (BSA) (Sigma), 0.01 mol/L EDTA (BDH), and 10 mg/mL deoxyribonuclease I (Sigma), and processed according to 2 different methods (Figure 1).

Method 1

This procedure combines 2 previously published methods. The hemispheres were disrupted in a glass homogenizer and passed through a 40-μm nylon cell strainer (Becton Dickinson). The suspension was centrifuged at 400g for 10 minutes at room temperature; the pellet was resuspended in 4 mL of 30% Percoll (Amersham Pharmacia Biotech) and overlaid on the top of a gradient containing 3.5 mL of 37% and 3.5 mL of 70% Percoll solution. Percoll was prepared by dilution in Hanks’ balanced salt solution (HBSS) (Bio-Whittaker). The gradient was centrifuged at 500g for 20 minutes at room temperature; cells were collected from the 37% to 70% interface (approximately 5 mL) and washed once with HBSS containing 10% fetal bovine serum (FBS) (GibcoBRL).

Method 2

This method is a modification of the isolation protocol of Ford et al. The hemispheres were mechanically dissociated through a 40-mesh stainless sieve (Sigma) and passed through a 40-μm nylon cell strainer. The suspension was centrifuged (10 minutes at 400g at room temperature in absence of brake), and the pellet was resuspended in 5 mL of isotonic Percoll brought to a density (ρ) of 1.030 g/mL. This solution was underlayered with 2.5 mL of Percoll (ρ 1.095 g/mL), overlayed with 2.5 mL of HBSS, and centrifuged for 20 minutes at 1000g at room temperature in absence of brake. Cells were collected from the top of the p 1.095 g/mL layer (approximately 5 mL) and washed once (400g, 10 minutes) with 10 mL of HBSS containing 10% FBS.

Cells obtained by both methods were resuspended in 400 μL of PBS with 0.2% BSA, counted with the use of a Bürker’s chamber, and divided into 4 aliquots collected in round-bottom tubes (Becton Dickinson) for flow cytometric analysis.
in HBSS (200 μL), cytocentrifuged (45g, 10 minutes) onto slides, air dried, and stained with May-Grünwald-Giemsa.

Statistical Analysis
Results are expressed as mean±SEM. Statistical analysis was performed with the use of either paired or unpaired Student’s t test (Sigma-Plot version 4.01). Differences between the 2 methods were assessed by 2-way ANOVA followed by the Bonferroni posttest (SigmaStat 2.03). P < 0.05 was considered statistically significant.

Results

Inflammatory Cell Isolation
Cell number estimation with the use of the Bürker’s chamber yielded similar results for both methods: ischemic hemisphere: method 1, 180 000±15 000; method 2, 151 000±17 000; nonischemic hemisphere: method 1, 77 000±15 000; method 2, 67 000±17 000. Both methods allowed collection of more cells from the ischemic than from the nonischemic hemisphere (method 1: P < 0.001, n=15; method 2: P =0.001, n=11). Saline intracardial perfusion, to eliminate cells from the cerebral vasculature, did not significantly modify the number of harvested cells (data not shown).

Cytospin Evaluation
Viable populations of monocytes, polymorphonuclear cells, and lymphocytes were identified by cytospin analysis (Figure 2). No qualitative differences were observed in the cell population isolated by the 2 methods, whereas a higher number of inflammatory cells were consistently present in the ischemic hemisphere regardless of the method used (Figure 2).

Identification of Inflammatory Cells by Flow Cytometry
In the FSC and SSC plots (Figure 3C and 3F), different cell populations and cell debris were identified. Dead cells stained by PI (Figure 3A and 3D) were excluded by designing a region (R1), used for further analysis (Figure 3C and 3F), in which they represent <1% of the population (Figure 3B and 3E). Analysis of the data gathered with method 2 resulted in a region with identical characteristics (data not shown).

Two populations showing clearly different SSC could be identified in R1. To better characterize these populations, we performed immunophenotyping using either FITC-conjugated anti-CD11b and phycoerythrin-conjugated anti-CD45 antibodies or FITC-conjugated anti-CD11b and phycoerythrin-conjugated anti-TCR. CD11b antibody recognizes monocytes/macrophages, granulocytes, and microglia; CD45 antibody identifies the rat leukocyte common antigen expressed in all leukocytes and, at lower levels, in resting microglia.24 TCR antibody recognizes the αβ TCR. Two-color analysis with the use of anti-CD11b and anti-CD45 antibodies revealed the presence of 2 populations characterized by different CD45 expression level (Figure 4A and 4E, labeled R2 and R3). On the basis of nonischemic hemisphere data, a threshold was set at 900 to separate CD45high+ from CD45low+ cells. The CD45high+ population showed predominantly a high granularity (Figure 4B and 4F) compared with

Figure 2. Photomicrographs from nonischemic (A) and ischemic (B) hemispheres (method 1) after cytospin. The cells were stained by the May-Grünwald-Giemsa method. In both nonischemic and ischemic hemispheres, we identified cells with morphological characteristics of macrophages/monocytes (arrowhead) (ie, large nucleus surrounded by a thin cytoplasmic halo). In the ischemic hemisphere, a second cellular type was identified corresponding to polymorphonuclear cells (probably neutrophils) (arrows). Bar=50 μm.

Figure 3. Representative dot-plot scatter analysis of cells obtained from nonischemic (A, B, and C) and ischemic (D, E, and F) hemispheres (method 1). A population of events strongly labeled with propidium iodide (PI, dead cells) was identified in both hemispheres (A and D). On the basis of physical parameters, a gate was drawn to exclude PI+ cells from further analysis (B and E, R1 region). C and F show the R1 region superimposed on the dot plot used for morphological analysis. FL1, fluorescence channel 1; FL2, fluorescence channel 2.
According to these data, polymorphonuclear leukocytes were matched with the population showing higher SSC, macrophage-like cells and activated microglia had intermediate scatter parameters, while resting microglia were matched with cells showing lower SSC. Thus, we designed a new region (R1a), used to perform quantitative analysis (Figure 4D and 4H), containing mainly cells bearing a combination of immunophenotypic and morphological parameters typical of infiltrating cells. A similar analysis was conducted on preparations stained for CD11b and TCR (Figure 5).

Infiltrating Cell Quantification

Cells in R1a were significantly more abundant in the ischemic than in the nonischemic hemisphere (method 1: \( P < 0.01 \), \( n = 8 \); method 2: \( P < 0.01 \), \( n = 11 \)) (Table 1).

With the use of CD11b and CD45 antibodies, 3 different populations were identified in R1a: CD11b\(^+/\)CD45\(^{high^+} \) (granulocytes and macrophage-like cells, R2), CD11b\(^+/\)CD45\(^{low^+} \) (microglia, R3), and CD11b\(^+/\)CD45\(^+ \) (presumably lymphocytes, R4). Morphological analysis of the R2 and R3 cell populations indicated that they are distinct in regard to physical characteristics (B, C, and F, G) as well. The CD11b\(^+/\)CD45\(^{low^+} \) showed a lower side scatter (C and G) compared with CD11b\(^+/\)CD45\(^{high^+} \) (B and F). According to these data, a new region (R1a) was identified (D and H) containing CD11b\(^+/\)CD45\(^{high^+} \) cells and mainly excluding CD11b\(^+/\)CD45\(^{low^+} \) cells.

CD45\(^{low^+} \) cells (Figure 4C and 4G). According to these data, polymorphonuclear leukocytes were matched with the population showing higher SSC, macrophage-like cells and activated microglia had intermediate scatter parameters, while resting microglia were matched with cells showing lower SSC. Thus, we designed a new region (R1a), used to perform quantitative analysis (Figure 4D and 4H), containing mainly cells bearing a combination of immunophenotypic and morphological parameters typical of infiltrating cells. A similar analysis was conducted on preparations stained for CD11b and TCR (Figure 5).

Morphological Evaluation of Inflammatory Cells From Peripheral Blood

Population size and physical parameters of peripheral blood from naive, sham-operated, and ischemic rats differed considerably. In the naive rat R1a region there was a relatively small population with well-defined physical parameters, corresponding to granulocytes. Conversely, the R1a region in sham-operated and ischemic rats contained a larger population with a more scattered profile (data not shown). No clear difference was observed between sham-operated and ischemic rats (data not shown).

Discussion

Methodological Considerations

Flow cytometry was originally developed to study blood cells. The remarkable potential of this technique encouraged researchers to develop reliable methods to study cells from solid tissues. The main problem encountered is to obtain a cell suspension without harming the cells. Published cell isolation methods use enzymatic and/or mechanical dissociation followed by separation via Ficoll-paque or Percoll density gradient. We examined these methods and identified 2 protocols suitable for flow cytometric analysis of inflammatory cells in ischemic brain. The procedures developed avoid enzymatic digestions, thereby preventing 2 poten-
bronectin.27 Thus, it may be suggested that a reduction of and B, which degrade type IV collagen, laminin, and fibronectin, is likely due to cell infiltration from the periphery. However, compared with the nonischemic hemisphere. This difference is likely due to cell infiltration and is not related to a method bias.

Cytospin confirmed the presence of leukocytes in cell suspensions obtained with both methods and showed the presence of scanty glial cells, endothelial cells, neurons, dead cells, and cellular debris. Further studies using antibodies against CD11b (Ox-42), ED-1 (a myeloid cell lysosomal glycoprotein), CD-3 (a TCR-associated antigen), and myeloperoxidase (a marker of polymorphonuclear leukocytes) confirmed the presence of inflammatory cells in pMCAO ischemic brain sections (G. Tarozzo, PhD, 2001).

An advantage of the flow cytometric technique resides in the possibility of discriminating at least some of these different populations without introducing further purification steps that may result in a reduced yield. By setting the FSC threshold at 200 and through PI staining, cell debris and dead cells were excluded from further analysis.

Qualitative and quantitative data obtained with both methods were generally superimposable; therefore, unless indicated otherwise, in the following discussion results will not be considered separately.

### Ischemia and Inflammation

Quantitative data regarding leukocyte infiltration after cerebral ischemia were obtained by histochemistry, immunohistochemistry, or biochemistry.1,2,3,5,28 However, these approaches have some limitations. Cell quantification in histological sections is subject to a number of artifacts that can lead to biased conclusions.29,30 This problem can be overcome with the use of unbiased stereological methods, which are, however, rather tedious and time consuming.29,30 Conversely, the use of biochemical parameters such as protein expression and activity evaluation (eg, myeloperoxidase) or molecular biology methods (RNA and gene expression measurements) allows only an indirect quantification of the cell number. In fact, the activation state of the cell may indeed induce changes in mRNA and protein levels that will affect the results.

Although information about cell localization is lost after dissociation, the use of flow cytometry has several advantages. It allows a rapid multiparameter evaluation of inflammatory cells and permits qualitative and quantitative analysis by measuring morphological parameters and marker expression.23 Furthermore, different inflammatory cells may be analyzed simultaneously by gating specific subpopulations. The present study is the first attempt to apply the flow cytometric technique to the quantification of inflammatory cells in ischemic brain tissue.

The most simplistic flow cytometric approach would be to analyze peripheral blood to infer, from changes in the cell composition, the different cell types infiltrating into the brain after ischemia. When compared with naive rats, ischemic rats showed strongly reduced blood monocyte number, possibly as a result of migration to the injury site and slow replacement.

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**Table 1. Number of Cells Gated in R1a in Nonischemic and Ischemic Hemispheres**

<table>
<thead>
<tr>
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<th>Method 1 (n=8)</th>
<th>Method 2 (n=11)</th>
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<tr>
<td>Nonischemic hemisphere</td>
<td>41 445±7058</td>
<td>15 623±3631</td>
</tr>
<tr>
<td>Ischemic hemisphere</td>
<td>107 930±18 022*</td>
<td>95 525±25 583*</td>
</tr>
</tbody>
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*Values are mean±SEM. Cells were isolated by either method 1 or 2. *P<0.01, nonischemic vs ischemic hemisphere (paired Student’s t test).
rate, and an increased FSC and SSC of the granulocyte population. However, not surprisingly, blood from sham-operated rats closely resembled that observed in ischemic animals, and no difference between ischemic and sham-operated animals was detectable. A more compelling approach would be to compare the brain of ischemic and nonischemic animals. However, the better-suited approach, which also takes into consideration interindividual variability, is to compare the ischemic with the nonischemic hemisphere of the same animal, as we did in the present study.

Studies of the time course of inflammation\textsuperscript{1,2,28} suggest an early (24 hours) accumulation of granulocytes after ischemic brain damage. Macrophages show a different kinetic: they are first observed at 12 hours after ischemia but increase in number for several days before reaching a plateau.\textsuperscript{1,3,31}

By using CD45, a panleukocyte marker, and CD11b, a protein expressed on granulocytes and macrophages, it was possible to label a sizable cell population present in the ischemic hemisphere. CD11b and CD45 are also expressed in macrophages and granulocytes.\textsuperscript{23,24,32} In the experimental autoimmune encephalomyelitis model, it was possible, with the use of double labeling, to sort resting microglia (CD11b\textsuperscript{+/}H11001\textsuperscript{-}/CD45\textsuperscript{-}) characteristic of lymphocytes, but showing physical parameters distinct from that of typical lymphocytes, were observed. The combination of TCR and CD11b markers revealed that the majority of these cells were TCR\textsuperscript{+}. Quantitative analysis, in agreement with that performed on CD11b\textsuperscript{-}/CD45\textsuperscript{-} cells, showed a significant increase of CD11b\textsuperscript{-}/TCR\textsuperscript{+} cells in samples obtained with method 1 and a trend that, however, did not reach significance (\(P=0.07\)) on samples obtained with method 2. Thus, method 2, originally designed to isolate microglia, is probably less appropriate to study lymphocytes.

Both methods indicated a significant increase of microglia (CD11b\textsuperscript{+}/CD45\textsuperscript{low\textsuperscript{-}}\textsuperscript{-}) in R1a.Activation of microglia after ischemia results in functional and morphological modifications\textsuperscript{33} that have not yet been completely described because of the difficulties in clearly distinguishing all the intermediate states between microglia and macrophages. A macrophagic population with unusual phenotypic traits (CD8\textsuperscript{+}) was observed after cerebral ischemia but not in other neurodegenerative conditions (wallerian degeneration).\textsuperscript{34} This suggests the possibility that undescribed microglia/macrophage populations with unusual phenotypes, such as the microglial population with high FSC and SSC that we observed, could exist.

Finally, our data showed that at 24 hours the CD11b\textsuperscript{-}/CD45\textsuperscript{low\textsuperscript{-}} population (mainly granulocyte) is dramatically increased in the ischemic hemisphere. This result is in agreement with previous findings indicating an increase in the granulocyte population with a peak at approximately 24 hours after ischemia.\textsuperscript{36}

In conclusion, we have implemented a new technical approach to study brain ischemia via inflammatory cell isolation and flow cytometric analysis. The 2 methods developed for this purpose provided satisfactory and comparable results. However, because of a more gentle dissociation procedure, method 2 would probably be more suitable for viable cell studies, whereas method 1 seems more suitable to perform research on lymphocytes. These methods, which allow a rapid and reproducible quantitative and qualitative evaluation of different cell populations, may find application in the preclinical testing of new neuroprotective drugs possessing anti-inflammatory properties.

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References


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