Acute Ischemic Stroke
Polymorphonuclear Leukocyte Membrane Fluidity and Cytosolic Ca$^{2+}$ Concentration at Baseline and After Chemotactic Activation

G. Caimi, MD; F. Ferrara, MD; M. Montana, ScD; F. Meli, MD; B. Canino, MD; C. Carollo, MD; R. Lo Presti, MD

Background and Purpose—Several reports have considered the role of systemic leukocytes in acute ischemic stroke (AIS). Initially, greater attention was focused on the leukocyte count and subsequently on their adhesiveness, aggregation, rheology, and activation. The aim of this study was the evaluation of certain polymorphonuclear leukocyte (PMN) parameters, reflecting their rheology and activation, in subjects with AIS.

Methods—In a group of 19 subjects with AIS and in a control group of 18 subjects with asymptomatic vascular atherosclerotic disease, we evaluated the PMN membrane fluidity and cytosolic Ca$^{2+}$ concentration at baseline and after in vitro chemotactic activation with 4-phorbol 12-myristate 13-acetate (PMA) and N-formyl-methionyl-leucyl-phenylalanine (fMLP).

Results—From the obtained data, it is evident that at baseline only PMN membrane fluidity distinguishes control subjects from AIS subjects. After PMN activation with PMA and fMLP, prolonged for 5 and 15 minutes, we found an increase in PMN cytosolic Ca$^{2+}$ concentration and a decrease in PMN membrane fluidity only in subjects with AIS.

Conclusions—These findings emphasize that in subjects with AIS a functional alteration of systemic PMN cells is clearly expressed during chemotactic activation, although the mechanism of this abnormality is not yet explained. (Stroke. 2000;31:1578-1582.)

Key Words: leukocytes ■ neutrophils ■ stroke, acute ■ stroke, ischemic

There are several clinical and experimental reports regarding the role of leukocytes in acute cerebral ischemia. Polymorphonuclear leukocytes (PMN) accumulate in the ischemic tissue from the first hours after the onset of stroke, and the infiltration reaches its maximum after 2 to 4 days.1 After acute ischemic stroke (AIS) leukocytes separated from peripheral blood show an alteration of adhesiveness,2–3 aggregation4–7 and rheology,6–8 and an increase of markers for leukocyte activation9 is also evident.

During acute ischemia and reperfusion, activated leukocytes can favor a progression to irreversible tissue injury through adhesion to endothelium,13,14 microvessel plugging,15 impairment of blood flow, and release of cytotoxic substances such as proteases and oxygen radicals.16–19

Epidemiological studies have shown a direct relation between leukocyte count and risk of AIS7,20–23 and an increased incidence of infections with fever before stroke in young subjects.24 These observations suggest a role of leukocytes not only in the progression of stroke but also in its initiation. Although the primary role of leukocytes in the pathogenesis of stroke has not been demonstrated, they have a likely role in the progression of brain injury. An elevated leukocyte count21 and increased leukocyte aggregation7 are in fact predictors of a poor prognosis.

We have used fluorescence techniques to evaluate PMN membrane fluidity and cytosolic Ca$^{2+}$ concentration in several clinical conditions.25–27 Both parameters influence phagocytosis in PMN cells.28–30 An increase of the cytosolic Ca$^{2+}$ content may be considered a marker of PMN activation,31,32 and it is influenced by membrane fluidity, which regulates the activity of the membrane pumps33,34 and, in general, the membrane protein function. In AIS, plasma markers of PMN activation have been demonstrated,3 as well as an altered trend of the PMN adhesion molecules.35–38 We therefore examined, in a group of AIS subjects, possible alterations of PMN membrane fluidity and cytosolic Ca$^{2+}$ content as markers of altered PMN function. The study was performed at baseline and after in vitro chemotactic activation with 2 stimulating agents. As is known, the activation in vitro can reveal variations not present at rest and simulates what happens in vivo.39–41

Subjects and Methods
Nineteen subjects with AIS (10 men and 9 women; mean age, 68.6±13.3 years) were enrolled. All the subjects were examined 48
to 72 hours after the onset of stroke. Brain CT was performed after admission to exclude hemorrhagic stroke or other clinical conditions (tumors or previous stroke). All subjects had a single lesion, and the site of the stroke was the middle cerebral artery (10 right and 4 left) in 14 patients, the posterior cerebral artery (3 right and 1 left) in 4 patients, and the right mesencephalic artery in 1 patient. Patients were clinically assessed in accordance with the National Institutes of Health Stroke Scale. No patient had cardiac disorders that predisposed him or her to embolic stroke. Five subjects had stable angina, and 6 had peripheral occlusive arterial disease (stage II according to Fontaine’s classification).

Five patients had a history of diabetes mellitus and were treated with sulfonylureas. The mean fasting blood glucose level in the whole group of AIS patients was 5.6±1.0 mmol/L. Three patients had a history of elevated blood pressure levels but had not taken any drug for hypertension in the 2 months preceding the stroke. At the time of the study the mean systolic blood pressure in the AIS patients was 147.1±21.7 mmHg and the mean diastolic blood pressure was 82.9±9.3 mmHg. No patient had dyslipidemia. On admission, total serum cholesterol was 5.4±0.9 mmol/L and serum triglycerides were 1.5±0.4 mmol/L. Eight patients were smokers, 11 nonsmokers. Patients with bacterial infections were excluded. Mean white blood cell count was 7.76±1.910^3/µL; mean erythrocyte sedimentation rate was 18.2±8.5 mm. No subject was treated with aspirin, antibiotics, calcium antagonists, or pentoxifylline.

The control group included 18 subjects (10 men and 8 women; mean age, 70.5±8.8 years) with asymptomatic carotid atherosclerotic plaques, demonstrated by echo-Doppler ultrasonography. No subject had history of cerebral ischemia or other acute ischemic diseases (such as myocardial infarction or critical limb ischemia). Seven subjects had stable angina, and 8 had peripheral occlusive arterial disease (stage II according to Fontaine’s classification).

No subject had arterial hypertension or dyslipidemia, 4 were diabetics, and 9 were smokers. In this group the mean systolic blood pressure was 144.4±15.2 mmHg, mean diastolic blood pressure was 83.9±7.6 mmHg, mean fasting blood glucose level was 5.3±0.7 mmol/L, serum total cholesterol was 5.0±0.8 mmol/L, and serum triglycerides were 1.6±0.7 mmol/L. No subject had bacterial infections, was treated with antibiotics, or was taking calcium antagonists, aspirin, or pentoxifylline. Mean white blood cell count was 6.930±1.05610^3/µL, and mean erythrocyte sedimentation rate was 16.3±5.7 mm.

Venous blood samples were drawn from arms of patients in a fasting state and anticoagulated with EDTA-K3 (1.5 mg/mL). An unfraccionated leucocyte suspension was prepared according to the method described by Mikita et al.42 In the final preparation, leucocytes were separated into Dulbecco’s phosphate-buffered saline containing EDTA-K3 (1 mg/mL). Leucocytes were separated into mononuclear and PMN cells43 with the use of a Ficoll-Hypaque medium with a density of 1.114 g/mL (Mono-Poly Resolving Medium, Flow Laboratories).

PMN Membrane Fluidity
PMN cells were suspended in Dulbecco’s buffer at a concentration of 4x10^6 cells per milliliter and labeled with 1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) (Molecular Probes), previously dissolved in acetone. The labeling was performed as follows: 4 minutes of preincubation at 4°C followed by incubation for 30 minutes at 37°C, with a final probe concentration of 1.0 µmol/L and a final DMSO concentration of 0.5%. This procedure is sufficient to inhibit fura 2-AM uptake in endocytic vesicles.47 Fluorescence measurement was performed with the Perkin-Elmer LS5 spectrophotofluorimeter. The excitation wavelength was 335 nm for the fura 2–Ca^{2+} complex and 385 nm for the unchelated fura 2, while the emission wavelength was 505 nm. In accordance with Roe et al.47 and Davíduffítho et al.,48 we considered the ratio between the fura 2–Ca^{2+} complex and the fluorescence intensity of the unchelated fura 2 (335 nm/385 nm).

**PMN Activation**

PMN membrane fluidity and Ca^{2+} calcium concentration were also evaluated, following the methods described above, after activation with chemotactic agents. After separation, part of the PMN cells were subdivided into several fractions, each of which had a concentration of 5x10^6 cells per milliliter. Each fraction was treated with 2 activating agents: 4-phorbol 12-myristate 13-acetate (PMA) and N-formyl-methionyl-leucyl-phenylalanine (fMLP; Sigma Chemical). The activation was performed in vitro, in accordance with the methods described by Yasui et al.49 and Masuda et al.,50 modified in agreement with the techniques used by us for the evaluation of the PMN parameters, as follows: the fractions of PMN suspension were treated, in separate experiments, with 4.5 µmol/L of PMA or with 10 µmol/L of fMLP and incubated for 5 minutes at 37°C; additional PMN suspensions, submitted to the same treatment, were incubated for 15 minutes at 37°C. At the end of incubation the activation was stopped by plunging the tubes into melting ice for a few minutes and, soon after, the PMN suspensions were centrifuged at 200g for 10 minutes at 20°C and resuspended in 1 mL of Dulbecco’s buffer containing EDTA-K3 (1 mg/mL).

**Statistical Analysis**

The results were expressed as mean±SD. The mean difference between controls and stroke subjects was evaluated according to the Student t test for unpaired data. The null hypothesis was rejected for probability value <0.05. The difference between the means of PMN parameters at baseline and after activation was investigated following the repeated-measures 1-way ANOVA. The relationships between PMN parameters and the neurological scores were evaluated by means of linear regression.

**Results**

At baseline (Table 1), there was a significant difference in PMN membrane fluidity between controls and subjects with AIS. In the latter, PMN membrane fluidity was significantly reduced. No significant difference was evident for PMN cytosolic Ca^{2+} concentration.

| TABLE 1. PMN Leukocyte Filtration Parameter, PMN Membrane Fluidity (Expressed as TMA-DPH Polarization Degree), and Cytosolic Ca^{2+} Content (Expressed as Ratio Between Fura 2–Ca^{2+} and Fura 2 Fluorescence Intensity) in Control Group and in Subjects With AIS |
|---------------------------------|-----------------|-----------------|
| TMA-DPH                         | 0.340±0.028     | 0.356±0.008*    |
| Ca^{2+}                         | 0.853±0.046     | 0.852±0.014     |

Values are mean±SD. *P<0.05 vs controls (Student’s t test).
in PMN cytosolic Ca\(^{2+}\) concentration and a significant decrease in PMN membrane fluidity.

No significant relationship was observed between the PMN parameters and the neurological scores.

**Discussion**

From our data it is evident that in AIS subjects a decrease in PMN membrane fluidity is present at baseline, and during activation with PMA (not receptor mediated) and fMLP (receptor mediated) there is a significant difference between control subjects and subjects with AIS, ie, the PMN of AIS patients is more susceptible to activation. These data reflect a systemic functional alteration of PMN cells, probably secondary to acute ischemia. Increased susceptibility to activation in peripheral blood PMN cells probably indicates a trend toward their further accumulation and activation in the infarcted brain, with an adverse effect on clinical outcome.

After AIS, circulating PMN cells have impaired rheological properties, demonstrated by filtration studies, and this alteration seems to be due to an increased adhesiveness. PMN deformability, related to the viscoelastic properties of cells, appeared to be normal in a micropipette aspiration study. Our research focused on PMN membrane lipid fluidity, which is only one aspect of cell deformability and is not directly related (as is the cytosolic Ca\(^{2+}\) content) to the findings obtained by other techniques that explore cell membrane dynamic properties.

PMN membrane fluidity and cytosolic Ca\(^{2+}\) content did not change significantly in control subjects with the technique for in vitro activation adopted by us, although the concentration of the activators was rather elevated. We obtained similar results by activating PMN from healthy subjects. This behavior might be explained, at least in part, by considering the observation times (5 and 15 minutes) chosen by us. Some modifications of membrane dynamics and Ca\(^{2+}\) concentrations may appear very early and are rapidly reversible under our experimental conditions.

This research sought to identify alterations due to AIS. We excluded from the study subjects with stable, treated arterial hypertension, because in previous research we had demonstrated an increase of PMN cytosolic Ca\(^{2+}\) content in hypertensives, and some antihypertensive drugs, such as calcium antagonists, can influence the same parameter. For a similar reason, since it is obviously impossible to exclude patients with atherosclerotic disease from the AIS group, we compared AIS patients with subjects with chronic, stable atherosclerotic disease, in whom we had already demonstrated an increase of PMN Ca\(^{2+}\) concentration in comparison with healthy subjects. Thus, in our study the increase in PMN membrane fluidity and the alteration of the same parameter and of cytosolic Ca\(^{2+}\) content induced by chemotactic activation seem to be markers of acute brain ischemia.

The absence of a significant correlation between our findings and the severity of stroke is perhaps due to the

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**TABLE 2. PMN Membrane Fluidity (Expressed as TMA-DPH Polarization Degree) and PMN Cytosolic Ca\(^{2+}\) Content (Expressed as Ratio Between Fura 2–Ca\(^{2+}\) and Fura 2 Fluorescence Intensity) in Control Subjects and in Subjects With AIS at Baseline and After Activation With PMA**

<table>
<thead>
<tr>
<th></th>
<th>At Baseline</th>
<th>After 5 min</th>
<th>After 15 min</th>
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<tbody>
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<td>Control group</td>
<td></td>
<td></td>
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<tr>
<td>TMA-DPH</td>
<td>0.340±0.028</td>
<td>0.349±0.027</td>
<td>0.352±0.028</td>
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<td>Ca(^{2+})</td>
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<tr>
<td>AIS group</td>
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<tr>
<td>TMA-DPH</td>
<td>0.356±0.008</td>
<td>0.368±0.010</td>
<td>0.369±0.007</td>
<td>64.0*</td>
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<tr>
<td>Ca(^{2+})</td>
<td>0.852±0.014</td>
<td>0.872±0.016</td>
<td>0.877±0.014</td>
<td>141.8*</td>
</tr>
</tbody>
</table>

Values are mean±SD.

*P<0.001 (repeated-measures ANOVA).

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**TABLE 3. PMN Membrane Fluidity (Expressed as TMA-DPH Polarization Degree) and PMN Cytosolic Ca\(^{2+}\) Content (Expressed as Ratio Between Fura 2–Ca\(^{2+}\) and Fura 2 Fluorescence Intensity) in Control Subjects and in Subjects With AIS at Baseline and After Activation With fMLP**

<table>
<thead>
<tr>
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<th>At Baseline</th>
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<tr>
<td>Control group</td>
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<tr>
<td>TMA-DPH</td>
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<td>0.349±0.021</td>
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<tr>
<td>Ca(^{2+})</td>
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<tr>
<td>AIS group</td>
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<tr>
<td>TMA-DPH</td>
<td>0.356±0.008</td>
<td>0.365±0.009</td>
<td>0.368±0.009</td>
<td>29.3*</td>
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<tr>
<td>Ca(^{2+})</td>
<td>0.852±0.014</td>
<td>0.872±0.014</td>
<td>0.875±0.013</td>
<td>89.4*</td>
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</tbody>
</table>

Values are mean±SD.

*P<0.001 (repeated-measures ANOVA).
relatively small number of subjects studied, and further investigation is required to definitively rule out a prognostic role for these parameters.

After the onset of AIS, an intervention directed at inhibiting leukocyte accumulation and activation in the ischemic brain might have great therapeutic importance, possibly combined with reperfusion but not limited by a similarly narrow therapeutic window. Encouraging results emerged from experiments in animal models, but the results of a human trial using an anti–intercellular adhesion molecule-1 murine monoclonal antibody have been disappointing.

Further investigation is needed to clarify the role of leukocytes in the pathophysiology of stroke to improve the therapeutic intervention and possibly to monitor the effectiveness of therapy.

References

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Stroke. 2000;31:1578-1582
doi: 10.1161/01.STR.31.7.1578

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/31/7/1578

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