Cerebral Neutrophil Recruitment, Histology, and Outcome in Acute Ischemic Stroke
An Imaging-Based Study

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Background and Purpose—Evidence now exists for a pathogenic role for neutrophils in acute cerebral ischemia. We have studied the patterns and temporal profile of cerebral neutrophil recruitment to areas of acute ischemic stroke (IS) and have attempted to correlate this with neurological status and outcome.

Methods—Patients with cortical middle cerebral artery (MCA) IS were recruited within 24 hours of clinical onset. Neutrophil recruitment was studied using indium-111 (111 In) troponolate-labeled neutrophils, planar imaging, and single-photon emission computed tomography (SPECT). Volume of brain infarction was calculated from concurrent computed tomography (CT). Hematoxylin and eosin sections were obtained postmortem (n/H11005 2). Outcome was measured using Barthel, Rankin, and National Institute of Health Stroke (NIHSS) scales.

Results—Fifteen patients were studied. Significant 111 In–neutrophil recruitment to ipsilateral hemisphere, as measured by asymmetry index (AI), was demonstrated within 24 hours of onset in 9 patients; this response was heterogeneous between patients and on repeated measurement attenuated over time. Histologically, recruitment was confirmed within intravascular, intramural, and intraparenchymal compartments. Interindividual heterogeneity in neutrophil response did not correlate with infarct volume or outcome. In an exploratory analysis, neutrophil accumulation appeared to correlate significantly with infarct expansion (Spearman rho = 0.66; P = 0.03, n = 12).

Conclusions—Neutrophils recruit to areas of ischemic brain within 24 hours of symptom onset. This recruitment attenuates over time and is confirmed histologically. While neutrophil accumulation may be associated with either the magnitude or the rate of infarct growth, these results require confirmation in future studies. (Stroke. 2004;35:1659-1664.)

Key Words: stroke ■ ischemia ■ neutrophils ■ SPECT ■ histology

Despite decreases in mortality, ischemic stroke (IS) remains a leading cause of death and disability,¹ and the need for novel effective therapies remains imperative. Evidence from experimental studies suggests a direct role of leukocytes in the pathogenesis of ischemic injury. This evidence goes beyond histological accumulation² to a more direct role in ischemic pathophysiology.³ Neutrophils have been implicated in the development of the “no-reflow” phenomenon in a primate model,⁴ whereas selective experimental interventional studies have strengthened the case for a causative role.⁵ Despite such studies, this remains a controversial area.⁶

In the clinical arena, evidence for such processes is less robust. The failure of clinical trials⁷⁻⁸ has prompted a need for precise definition of human pathophysiology and this has partly been addressed by imaging studies. Single-photon emission computed tomography (SPECT) studies have localized mixed populations of leukocytes to ischemic hemisphere using indium-111 (111 In) and technetium-99m (⁹⁹m Tc).⁹,¹⁰ While the use of mixed cells offers limited pathological insights, such studies are confounded because ⁹⁹m Tc-hexamethylpropylenamine oxime (HMPAO) elutes from leukocytes in hydrophilic-form that may undergo reuptake in the brain as a result of blood-brain barrier (BBB) disruption.¹¹ Furthermore, they provide little information about acute pathophysiology or outcome. Regions of cerebral infarction do attract selective populations of ⁹⁹m Tc-HMPAO–labeled leukocytes, but such studies may offer only limited insights into recovery measured on a single scale.¹² None of these studies has provided histological evidence to support the imaging data.

Received November 24, 2003; final revision received March 9, 2004; accepted March 25, 2004.
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Stroke is available at http://www.strokeaha.org
DOI: 10.1161/01.STR.0000130592.71028.92
In this article, we seek to study quantitatively the spatial and temporal profiles of cerebral recruitment of $^{111}$In-labeled neutrophils using planar imaging and SPECT. We have attempted to underpin these imaging studies with postmortem histology from a small subset of patients and explored the relationship between neutrophil accumulation, infarct evolution, and clinical outcome.

**Subjects and Methods**

**Patients**

After informed consent, patients with a clear time of onset and ischemic cortical middle cerebral artery (MCA) territory syndromes fitting appropriate criteria (Table 1) were recruited. The Local Regional Ethics Committee (LREC) and the Administration of Radioactive Substances Advisory Committee (ARSAC) of the United Kingdom approved the study.

**Cell Labeling**

Separation and labeling of neutrophils was performed with the assistance of the Nuclear Medicine Department using a standard protocol as previously described. Briefly, venous blood is mixed with acid citrate dextrose (ACD) (NIH Formula A) before centrifugation and resuspension of cells in cell free plasma (CFP). Neutrophils were separated using an iso-osmotic discontinuous gradient of Percoll (Pharmacia) and labeled with $^{111}$In troponolate. Autologous-labeled neutrophils were returned to the patient within 24 hours and thereafter at follow-up studies at 4 to 7 and 8 to 15 days. Administered radiation dose aimed for a target of 16 MBq equating to 7.2 mSv effective dose equivalent (ED). Cell recovery was calculated from a 40-minute venous sample and extrapolated to a blood volume of 5 L to allow comparison with previous studies. Recovery provides an indication of the percentage of labeled cells that remain within the circulation. In follow-up studies, recovery figures were corrected for residual radioactivity in a presample. Low recovery figure (< 10%) may indicate impaired cell viability or removal of cells into the reticuloendothelial system as a result of ex vivo activation. Previous studies for $^{111}$In troponolate human granulocyte cell recovery are ~40%. Labeling efficiency was calculated by measuring radioactivity in labeled cells versus that in washes, expressed as a percentage of radioactivity incorporated into cells. Labeling efficiency may vary with cell number, and incubation time and figure from previous studies are ~72% for $^{111}$In troponolate.

**Exclusion**

Premorbid cognitive impairment
Previous stroke
Malignant disease
Other ongoing organ failure
Myocardial infarction within 2 mo
Posterior circulation or noncortical stroke
Systemic or intraarterial thrombolysis

**Table 1. Inclusion and Exclusion Criteria**

<table>
<thead>
<tr>
<th>Inclusion</th>
<th>Exclusion</th>
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<tr>
<td>Cortical IS attributable to the MCA</td>
<td>Premorbid cognitive impairment</td>
</tr>
<tr>
<td>Male or females, aged 50 – 85 y</td>
<td>Previous stroke</td>
</tr>
<tr>
<td>Informed consent</td>
<td>Malignant disease</td>
</tr>
<tr>
<td>Patients are able to undergo neutrophil labeling and injection within 24 h of onset</td>
<td>Other ongoing organ failure</td>
</tr>
<tr>
<td>CT has excluded hemorrhage or other intracranial pathology</td>
<td>Myocardial infarction within 2 mo</td>
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**Imaging**

Planar gamma camera and SPECT imaging were performed on an Elscint dual-headed gamma camera fitted with medium energy collimators. The targeted interval between injection of labeled cells and onset of clinical symptoms for these 3 time points were 24 hours, 4 to 7 days, and 8 to 15 days. Planar images (anterior and posterior) and SPECT were performed within 24 hours after injection. All quantitative studies were performed on regions of interest (ROI) defined manually from the planar hemispheric images (Figure 1C) and outside the head as a background region. Counts were measured from ipsilateral and contralateral hemispheres anteriorly and posteriorly, and decay corrected and adjusted for administered dose to give counts 10 minutes $^{111}$In. Baseline images were acquired before each follow-up study when decay-corrected counts were subtracted. SPECT data were acquired to give a slice thickness of ~8 mm. Data were reconstructed into transaxial, coronal, and sagittal planes using filtered back projection.

For coregistration, X-ray computed tomography (CT) and SPECT images were downloaded in digital format and coregistered using mutual information software. CT was performed within 24 hours of follow-up nuclear imaging. Axial images were reconstructed with a slice thickness of 5 mm. Volumetric analysis of CT determined infarct volume was performed using manually defined regions of interest using Analyze (Analyze 5.0; Mayo Clinic Biomedical Imaging Resource). Differences in infarct volume between the initial and subsequent studies were calculated from these volumes and denoted, $\Delta$CT$_{1-2}$ and $\Delta$CT$_{2-3}$, for differences between the first and second and first and third studies, respectively.

**Histology**

Consent was obtained for brain postmortem. Brains were fixed in buffered 10% formal saline before sectioning. Sections were taken from peri-infarct areas in transaxial or coronal planes, stained with hematoxylin and eosin (H&E), and examined by an independent neuropathologist blinded to the imaging studies.

**Statistical Methods**

Asymmetry indices (AI) were calculated for each SPECT study as previously described:

$$ AI = (A - B)/(A + B), $$

where A = ipsilateral and B = contralateral summed count rates. AI values from the 3 imaging studies were referred to as Al1, Al2, and Al3. A and B were modeled as being Poisson variables with a common parameter, Lambda ($\lambda$), and the posterior distribution (having observed A and B) of $(A - B)/(A + B)$ was used to generate a 95% confidence interval for the AI. A and B were observed for all patients. If the observation of AI was not within the confidence interval, then this was deemed significant evidence that the assumption of a common parameter, ie, the assumption of symmetry, did not hold. This analysis was conducted with WinBUGS version 1.4 (MRC Biostatistics Unit). Because of the simplicity of the model, achieving convergence was not apparently problematic, nor did varying the previous distribution of $\lambda$ (within reasonable choices)
seriously affect the results. Correlation coefficients presented are Spearman rank rho ($\rho$) with $P$ values for the 2-tailed test. Comparison of groups was conducted using the Mann–Whitney $U$ test. The Friedman test was applied as a measure of decrement of AI over time when AI was measured repeatedly.

### Outcome

Clinical assessment on admission, at 4 to 7 days, 8 to 15 days, and 3 months included functional rating on Barthel and Rankin scales and neurological rating on the National Institute of Health Stroke Scale (NIHSS). These scales have been validated in large-scale trials. Scales were measured throughout the study by a single physician (C.J.S.P.). When subjects died before follow-up assessments, they were allocated maximal deficit Barthel, Rankin, and NIHSS scores of 0, 5, and 36, respectively. For the purposes of correlation, corresponding indices were plotted against each scale at each time point. Patients were divided into groups using 3 stratification approaches to address separate questions. In the first instance, patients were classified into 2 groups (group 1 and group 2), based on whether the AI was significantly abnormal, to assess the relationship of hemispheric neutrophil localization to clinical presentation.

In the second instance, patients were classified into 2 groups (group A and group B), based on their stroke severity as either mild/ moderate (NIHSS $\leq$ 15) or severe (NIHSS $>15$). This stratification allowed us to assess the relationship of clinical stroke severity to hemispheric neutrophil recruitment. Finally, patients were stratified into 2 groups (group X and group Y) depending on clinical progression of deficit: those with a reduction in NIHSS scores over the first 3 months (improving) or those with an increase in NIHSS over the first 3 months (deteriorating). This stratification allowed us to analyze whether patients who exhibited clinical deterioration showed greater hemispheric neutrophil localization. Finally, we undertook an exploratory analysis to determine whether the degree of neutrophil recruitment to the damaged hemisphere was related to the extent and speed of infarct expansion (as defined by $\Delta CT_{1-2}$ and $\Delta CT_{1.5-3}$).

### Results

#### Imaging

Fifteen patients were entered into the study. Administered activity for each study ranged from 7.5 to 18.5 MBq with a calculated radiation burden range of 3.4 to 8.3 mSv ED. All patients were imaged with CT within 24 hours of symptom onset, except patient 10 who was imaged with magnetic resonance imaging and died after the first $^{111}$In SPECT study. Mean time from clinical onset (and ranges) to respective SPECT scanning sessions were 38.7 hours (23 to 49), 5.9 days (4 to 7), and 12.1 days (10 to 14), and corresponding intervals for CT were 10.9 hours (2 to 24), 4.9 days (3 to 7), and 11 days (8 to 12). Primary cerebral hemorrhage was excluded in all subjects. Quality of images varied according to movement and the clinical state of the patient.

Neutrophils were separated, labeled with $^{111}$In, and injected into all patients initially. Cell recovery, labeling efficiency data, and AIs derived from a total of 40 studies are given in Table 2. Assuming a blood volume of 5 L, our figures are comparable to those in established literature. AIs showed a trend toward decrement over the time period (Figure 1); the Friedman test for the 12 patients with repeated measures gives $P=0.038$, with the mean rank monotonically decreasing with time point. Significant AIs were seen in 9 patients (range: 0.07 to 0.72) (Figures 2 and 3 and Table 2). Volumetric CT data did not correlate with AI across all time points ($\rho=0.11, P=0.53, n=35$).

#### Histology

Consent for histopathological study was obtained for patients 10 and 7 who died at 3 and 11 days after ictus from brain stem herniation and cardiac failure, respectively.
For patient 10 (Figure 4A through 4C), a macroscopic examination of a coronal slice revealed left hemisphere infarction throughout the entire territory of the MCA cerebral artery with involvement of subcortical structures. Microscopically, a brisk inflammatory infiltrate was noted in both meninges and superficial cortex in peri-infarct zones with evidence of perivascular neutrophil aggregation in underlying white matter. Macrophages were also present in this cellular reaction.

For patient 7 (Figure 4D through 4F), macroscopic examination of coronal sections revealed infarction of the right MCA cortex with involvement of subcortical structures. Microscopic examination revealed cellular infiltrate, consisting of neutrophils and macrophages in the cerebral cortex extending into the Virchow–Robin spaces.

Clinical Progression and Outcome
AI did not correlate with any outcome measure at any time point. Although high AIs were associated with worse neurological scores, these were not predictive. No significant differences in NIHSS were detected between groups 1 and 2 (P=0.5) or in AI between groups A and B or X and Y, (P=0.5 and 0.4, respectively). The ΔCT1,2 correlated significantly with AI1 (rho=0.66; P=0.03) but not with AI1 or AI1. There were no significant relationships detected between ΔCT1,3 and AI at any time point. The ΔCT1,2 in groups 1 and 2 did not differ significantly (P=0.26).

Discussion
Using robust, quality-controlled methods, we have demonstrated that within the first 24 hours of IS, autologous neutrophils may be separated, labeled with 111In troponolate, and used for quantitative and sequential studies of ipsilateral cerebral hemispheric recruitment. This is the first study to our knowledge to use histological confirmation in this context. The labeling process does not activate neutrophils and hence provides a measure of in vivo recruitment, presumably triggered by ischemia. Neutrophils appear early in regions of ischemic cerebral hemisphere defined structurally by coregistration of SPECT images and CT, and may be present from as early as 19 hours. Consistent with previous studies,12 recruitment shows a trend toward ipsilateral attenuation over time. Late or persisting neutrophil recruitment could not be
attributed to hemorrhagic transformation. AI values appear to be associated with the rate at which stroke volume expands as defined by CT. However, the extent of eventual (presumably penumbral) brain recruitment into the stroke was unrelated to AI in our study. Further AI values did not discriminate between patients with different clinical progression patterns or outcome.

Methodological Issues
Although previous studies have addressed leukocyte recruitment after stroke, these results are confounded by the use of mixed cells or 99mTc-based techniques, because label elution is a significant problem. In contrast to earlier studies, we can be confident that ipsilateral cerebral signal relates not to eluted complexes, but specifically to the neutrophil component of the inflammatory response. 99mTc-HMPAO decomposition products are hydrophilic and are unable to cross an intact BBB. Where BBB is disrupted, as is likely in IS patients, 99mTc-HMPAO-labeled leukocyte studies remain uninterpretable. The imaging data do not discriminate between intravascular and intraparenchymal neutrophils when histology remains key. Cell recovery and labeling efficiency were comparable to optimal figures in the literature, suggesting that variations in AI were unlikely to be caused by deficiencies in labeling methodology.

This study used validated, neurological, and functional stroke scales, shown to be reproducible in larger trials; this contrasts with the Mathew scale used in one study.27 The Mathew scale is dominated by consciousness, combines impairments and disabilities, and its validity and reliability have not been proven.21

Pathophysiological Implications
In our small sample of patients, a statistically significant relationship between AI and outcome could not be established. In a larger sample, AI may predict outcome but this remains speculative. Given the available data, the contribution of neutrophils to pathophysiology also remains speculative. Instead, neutrophils may act as biological markers of disease. A pathophysiological role of neutrophils in clinical stroke may only be put beyond doubt within the context of clinical interventional studies or by satisfying more stringent etiological criteria.6 Such criteria, as set out in this article, are not met in our study, and the results of such interventions have, to date, been disappointing.22,23 Significant AI values were observed at later time points, but only in those patients with higher values of AI initially. Our data are consistent with animal models in which polymorphonuclear leukocytes (PMNL) were observed histologically 24 hours after MCA occlusion in rats.24 The data presented further serve to illustrate the heterogeneity of cellular inflammatory pathophysiological responses to IS.

Insignificant AI values could not be attributed to variations in cell recovery and labeling efficiency. However, a number of possibilities could account for these negative results. First, we did not know if patients spontaneously recanalized. Complete and persistent MCA occlusion, accompanied by a concurrent lack of collateral circulation, may have prohibited neutrophil recruitment to densely ischemic areas. Hence, it is possible that significant neutrophil recruitment may be not only dependent on the ischemic process per se but also a marker of reperfusion injury. This is consistent with experimental models in which temporary MCA occlusion results in a rapid and dense pattern of cerebral leukocyte recruitment.25 Opportunities for assessment of recanalization are provided by modern magnetic resonance angiography techniques or transcranial Doppler. This type of data was not available in this study. Second, an early time window of neutrophil recruitment to ischemic brain may be being missed. Granulocytes have been documented as early as 6 hours after experimental ischemia,26 whereas clinical studies suggest 15 hours as the earliest time point at which cerebral neutrophils appear.27 Signal derived from contralateral hemisphere may itself represent pathological invasion and hence may not provide an adequate control ROI for comparison. In contrast to other studies, in which many ROIs from ipsilateral hemisphere were selected (and in which the corresponding control ROIs are not explicitly defined), we have tested activity on a hemispheric basis as defined on planar images, with individual AI values tested statistically for significance. Given the spatial resolution of SPECT, it is currently not possible to precisely coregister areas of maximal neutrophil density with areas of infarction as defined by CT. The use of appropriate fiducial markers would improve anatomical localization within this context. Furthermore, we can only speculate as to whether such signal relates to ischemic penumbral areas, ie, areas that may represent a neuroprotective target or to core infarction. The limited histological evidence that we have would suggest that the former was indeed the case.

The 111In neutrophil SPECT provides evidence of acute neutrophil localization to infarcted hemispheres after IS. Extent of hemispheric neutrophil recruitment appeared unrelated to stroke severity at onset, clinical progression, or outcome, but may be related to early infarct expansion. Whether such findings represent a causal relationship remains to be established. The 111In neutrophil SPECT may be a useful tool for the initial assessment of the efficacy of antiinflammatory interventions aimed at reducing neutrophil-mediated injury in stroke. Failure to improve clinical outcomes with such interventions may be because of the fact that the agent is not effective in preventing neutrophil recruitment at the time points or dosages used. However, the agent may be efficacious in reducing neutrophil recruitment but fail to improve outcome because these cells may not cause pathology in IS. Labeled leukocyte studies may be useful in making the important distinction between these two scenarios.

Acknowledgments
C.J.S.P. and this work is funded by the Medical Research Council and the Sackler Fund. A.G.L. is supported by a grant from the NHS (RCC57117). D.K.M. is supported by a grant from the MRC (grant G9439390 ID 56833). E.A.W. is supported by a PPP Mid Career Fellowship and by the Stroke Association.
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Stroke. 2004;35:1659-1664; originally published online May 20, 2004;
doi: 10.1161/01.STR.0000130592.71028.92
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:
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