Stroke Alters Respiratory Burst in Neutrophils and Monocytes

Johanna Ruhnau*; Karsten Schulze*; Bernadette Gaida, MD; Sönke Langner, MD; Christof Kessler, MD; Barbara Bröker, MD; Alexander Dressel, MD†; Antje Vogelgesang, PhD†

Background and Purpose—Stroke-induced immune alterations predispose patients to infections. Although the relationship between stroke and the adaptive immune system has been investigated in detail, to date it is unknown whether the innate immune system, which forms the first line of antibacterial defense, is also impaired in patients with stroke. Therefore, we investigated whether chemotaxis, phagocytosis, oxidative burst, degranulation of defensins, and NETosis in monocytes and in neutrophil granulocytes are altered in patients with stroke compared with controls.

Methods—Sixty-three patients having acute ischemic stroke were recruited within 12 hours of symptom onset; blood was sampled on admission and on days 1, 3, 5, and 7. Thirty-seven age-matched controls were also recruited. Cell migration, phagocytosis, and oxidative burst of phagocytes were determined in vitro. Human neutrophil peptides 1 to 3 and serum metanephrine levels were measured by enzyme-linked immunosorbent assay, and NETosis was quantified by immunohistochemistry.

Results—The key mechanisms required for bacterial killing, oxidative burst, and NETosis were significantly reduced in samples taken from patients with stroke compared with controls, whereas migration, phagocytic function, and defensin production remained unimpaired in monocytes and granulocytes from patients with stroke.

Conclusions—Stroke-induced immune alterations include impairment of the first-line defense performed by specialized phagocytes against bacteria. The hypothesis that these changes enhance susceptibility to acquired infections is supported by our observation that on admission oxidative burst in monocytes was more impaired in patients with stroke with subsequent stroke-associated infections. (Stroke. 2014;45:794-800.)

Key Words: brain ischemia ■ granulocytes ■ immunosuppression ■ infection ■ phagocytes ■ respiratory burst ■ stroke

In addition to initial stroke severity and brain lesion volume, stroke-associated infections, especially pneumonia, is a well-recognized independent predictor of survival and outcome in patients with stroke.1-3 More recently, stroke-induced immune alterations (SIIAs) have been identified as predisposing factors for stroke-associated bacterial infections (SAIs).4-6 Profound SIIAs include increased granulocyte counts and reduced lymphocyte counts in peripheral blood. T cells isolated from patients with SIIAs, however, seem to be fully functional in vitro.7-9 Monocytes are not reduced in number but are deficient for tumor necrosis factor-α production and HLA-DR expression.5,8-11 This is consistent with the observation that the efficiency of costimulatory cells in patients with stroke is decreased.10 Additionally, the function of innate hepatic invariant natural killer T cells is altered by noradrenergic signals in stroke.12 The majority of SIIAs concern the adaptive immune system or the crosstalk between innate and adaptive immunity. Because SIIAs are associated with the occurrence of SAIs, it is of interest to investigate early bacterial defense mechanisms in stroke.

Granulocytes and monocytes are dedicated phagocytes that are rapidly recruited via chemotaxis to the sites of infection, where phagocytes adhere to and internalize pathogens by endocytosis. Bactericidal effector mechanisms are then initiated. First, within the phagosome, nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase converts oxygen and NADPH to superoxide, generating reactive oxygen species that are required to kill internalized pathogens in a process termed oxidative burst. Mutations in NADPH oxidase cause chronic granulomatosis, a disease characterized by recurrent and persistent bacterial infection.13 Second, as an alternative mechanism, neutrophils can release antimicrobial granula into

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From the Section of Neuroimmunology, Department of Neurology (J.R., K.S., B.G., C.K., A.D., A.V.), Institute of Immunology and Transfusion Medicine (B.B., A.V.), and Diagnostic Radiology and Neuroradiology (S.L.), University Medicine Greifswald, Germany.
+1Ruhnau and K. Schulze are joint first authors contributed equally.
†Drs Dressel and Vogelgesang are senior authors contributed equally.
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Correspondence to Antje Vogelgesang, PhD, Section of Neuroimmunology, Department of Neurology, University Medicine Greifswald, FC3, Fleischmannstr. 41, 17475 Greifswald, Germany, E-mail antje.vogelgesang@uni-greifswald.de

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the extracellular space to fight bacteria. The α-defensin human neutrophil peptides 1 to 3 (HNPs 1–3) are abundant in these granula; these peptides are bactericidal and modulate the production of the chemokine interleukin-8 (IL-8).14 Third, in a process called NETosis, neutrophils fight bacteria by releasing their chromatin into the extracellular space, where the chromatin forms neutrophil extracellular traps (NETs).15

Transient disturbances in phagocytosis and the associated mechanisms for bacterial killing were previously detected in systemic infection and in traumatic brain injury.16,17 Also, reduced phagocytosis has been described in subjects >65 years.18 Little is known about phagocyte function in stroke, but there are hints that it may also be disturbed. One study reported impaired neutrophil burst in a small cohort of patients with intracranial hemorrhage who required neurosurgical intervention,19 whereas an earlier report suggested the occurrence of alterations in neutrophil function in patients with stroke, as indicated by measurement of the granulocyte anti-sedimentation rate.20 To shed light on this issue and to increase our understanding of SIIA, we investigated the key steps of bacterial defense by phagocytes in patients with stroke.

**Materials and Methods**

**Patients and Controls**

Patients ≥18 years old having acute media infarct were eligible for this study within 12 hours of disease onset if their National Institute of Health Stroke Scale Score (NIHSS) was ≥26 and no signs of infection were detected. C-reactive protein was ≤50 mg/L, and procalcitonin was ≤0.5 ng/mL. Patients were recruited between June 2011 and February 2013 at the Department of Neurology of the University Medicine Greifswald, Germany. Patients were treated with standard medical care in a dedicated stroke unit and did not receive immune-suppressive drugs. Recombinant tissue plasminogen activator and thrombectomy were administered as clinically indicated.

Age-matched control individuals were either healthy or were recruited from the ophthalmology clinics from patients scheduled to receive cataract surgery. (For patient and control characteristics, see the Table.)

**Definition of SAI**

To define SAI, we used the same stringent criteria applied in our previous studies that identify patients with definite systemic infection and those without any sign of infection. SAI was diagnosed if (1) clinical signs of infection were present; (2) serum concentrations of C-reactive protein were >50 mg/L; and (3) procalcitonin serum concentrations were >0.5 mg/mL. Absence of SAI was assumed if none of the criteria was met. Patients who matched 1 or 2 criteria were not assigned to either cohort. The study protocol was approved by the ethics committee of the Medical Faculty, University of Greifswald (No. III UV 30/01). All patients provided written informed consent directly or through a surrogate where appropriate. Details on patients who are SAI+ and SAI– are given in the Table I in the online-only Data Supplement.

**Cerebral Computed Tomography**

Routine cerebral computed tomographic (cCT) images (sequential cCT native, 4.5 mm layer thickness supra- and infratentorial; mAs=50; kV=120) were acquired on a 16-row multislice CT scanner (Somatom 16, Siemens Medical Systems, Erlangen, Germany). Images were analyzed using OSIRIX 5.6. Regions of interest were defined manually, and the lesion volume was calculated semiautomatically.

**Blood Sampling**

Blood was obtained immediately on admission and between 6:00 and 8:00 AM on days 1, 3, 5, and 7. Investigators were not blinded for control and samples of patients with stroke but were unaware of SAI status.

**Migration**

Cell migration was assayed using the MIGRATEST Kit (Glycotope Biotechnology GmbH, Heidelberg, Germany) according to the manufacturer instructions. Details are given in the online-only Data Supplement.

**Phagocytosis**

The Phagotest Kit (Biotechnology GmbH, Heidelberg, Germany) was used according to the manufacturer instructions to quantify phagocytosis. In brief, heparinized whole blood was incubated with FITC (fluoresceinisothiocyanat) labeled Escherichia coli (E coli) for 10 minutes at 37°C (activated sample). A control sample was kept on ice to determine the background. Thereafter, all cells were transferred on ice; samples were quenched to eliminate the signal of adherent nonphagocytosed bacteria. Erythrocytes were lysed, and cells were washed. Debris and dead cells were excluded by 4’,6-diamidino-2-phenylindole (DAPI) staining. Phagocytes that had internalized E coli were quantified by determining the FITC fluorescence signal by flow cytometry on a BD LSR II. Anti-CD14 antibodies (hCD14 antigen presenting cell; clone M5E2; BD Biosciences) labeling was used to clearly distinguish monocytes from neutrophil granulocytes. The percentage of phagocytosing neutrophils was calculated as activated sample cells (FITC+ CD14– cells×100 / CD14– cells) – control sample cells (FITC+ CD14– cells×100 / CD14– cells)=percentage of phagocytosing cells. The percentage of phagocytosing monocytes was determined accordingly in the CD14+ cell population. The amount of E coli cells phagocytosed by a single

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**Table. Subject Characteristics**

<table>
<thead>
<tr>
<th>Total No.</th>
<th>Age*</th>
<th>NIHSS†</th>
<th>Lesion Volume (mm³)‡</th>
<th>Location of Infarction</th>
<th>Thrombolysis/Thrombectomy</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control subjects</td>
<td>37</td>
<td>74.17 (50–88)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>Patients with stroke</td>
<td>63</td>
<td>74.59 (29–93)</td>
<td>12.5 (6–23)</td>
<td>36.6 (0.85–411.49)</td>
<td>60 MCA</td>
<td>37</td>
<td>34</td>
</tr>
<tr>
<td>Noninfected cohort</td>
<td>14</td>
<td>77 (65–93)</td>
<td>7 (6–23)</td>
<td>40 (0.85–103.04)</td>
<td>3 MCA+anterior</td>
<td>14 MCA</td>
<td>7</td>
</tr>
<tr>
<td>Infected cohort</td>
<td>8</td>
<td>70.5 (55–85)</td>
<td>17 (8–19)</td>
<td>275.06 (18.52–411.49)</td>
<td>7 MCA</td>
<td>2</td>
<td>6</td>
</tr>
</tbody>
</table>

MCA indicates middle cerebral artery; NA, not applicable; and NIHSS, National Institute of Health Stroke Scale Score.

*Mean age in years (range).
†Median score (range).
‡Median volume (range).
phagocyte was quantified by the mean fluorescence intensity (MFI) and is referred to as efficacy in this article.

**Oxidative Burst**

Heparinized whole blood was incubated with the fluorogenic substrate dihydrorhodamine 123 and either remained unstimulated or was incubated with unlabeled opsonized *E. coli*, phorbol 12-myristate 13-acetate (PMA), or N-formyl-methionyl-leucyl-phenylalanine (fMLP) as stimulants for 10 minutes at 37°C. Conversion into rhodamine 123 served as a marker for the quantification of reactive oxidants and allowed determination of the percentage of phagocytes that underwent oxidative burst. The enzymatic activity per cell was quantified by MFI and is termed efficacy.

Oxidative burst was measured by flow cytometry on a BD Canto II (BD Biosciences) using the Phagoburst Kit (Glycotope Biotechnology GmbH) according to the manufacturer instructions. Twenty-thousand leukocytes were acquired. Anti-CD14 antibodies (CD14 V450, clone Mφ9; BD Biosciences) were added to clearly distinguish monocyte burst from neutrophil burst. The flow cytometry results were evaluated with FlowJo software 7.6.5 (Tree Star Inc, Ashland, OR).

Hormonal effects on respiratory burst in vitro were assessed by preincubating whole blood for 24 hours at 37°C, 5% CO₂ in the presence of 1×10⁻⁷ mol/l epinephrine or norepinephrine (epinephrine, norepinephrine from Sigma, Deisenhofen, Germany), or 2.5×10⁻⁶ mol/l dexamethasone (MerckPharma GmbH, Darmstadt), followed by a burst test. Additionally, we tested 5×10⁻⁶ mol/l acetylcholine (Sigma, Deisenhofen, Germany) to quantify the influence of this autonomic nervous system neurotransmitter on burst activity. Diluents without hormones or acetylcholine served as controls.

**Serum Hormones**

Metanephrine is the inactive methylation product of epinephrine with longer plasma half-life. In this study, we determined metanephrine to assess the magnitude of the adrenergic response after stroke in patients with and without SAI. Metanephrine levels were determined in serum samples after storage at −80°C by competitive ELISA according to the manufacturer recommendations (MetCombi plasma ELISA; IBL, Hamburg, Germany).

HNPs 1 to 3

The production of HNPs 1 to 3 by neutrophils was determined by standard sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer instructions (Hycult Biotech, Uden, The Netherlands) from serum samples stored at −80°C.

**NETosis Assay**

Neutrophils were separated from hiraparinized whole blood by standard Histopaque/Percoll gradient centrifugation. Neutrophils were resuspended in Hanks’ Balanced Salt Solution (HBSS) and diluted to 5×10⁴ cells/mL. The cell suspension was plated in 24-well plates (1 mL/well) and allowed to rest for 30 minutes at 37°C, 5% CO₂. Thereafter, neutrophils remained unstimulated or were stimulated with either fMLP (0.9 nmol/L) or PMA (1.5 nmol/L) for 2 hours at 37°C, 5% CO₂. DNA was stained with SYTOX Green Nucleic Acid Stain (Invitrogen, Eugene), and fluorescent and phase contrast images were taken of 10 spots per sample dihydrorhodamine 123 and either remained unstimulated or was incubated with unlabeled opsonized *E. coli*, phorbol 12-myristate 13-acetate (PMA), or N-formyl-methionyl-leucyl-phenylalanine (fMLP) as stimulants for 10 minutes at 37°C. Conversion into rhodamine 123 served as a marker for the quantification of reactive oxidants and allowed determination of the percentage of phagocytes that underwent oxidative burst. The enzymatic activity per cell was quantified by MFI and is termed efficacy.

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**Statistical Analyses**

All data sets were tested for adherence to the Gaussian distribution with the Kolmogorov–Smirnov test. Multiple comparisons of Gaussian-distributed data were performed using analysis of variance with the Kolmogorov–Smirnov test. Multiple comparisons of nonparametric data were performed using the Kruskal–Wallis test, with Dunn’s multiple comparison test as a post-test or the Mann–Whitney test were used as appropriate. Post-tests were only performed when initial testing revealed significant differences between groups. Correlations were determined by Spearman analysis. GraphPad-PRISM 5.0 (GraphPad Software Inc, San Diego, CA) was used for all analyses. A p value <0.05 was regarded as significant.

**Results**

**Cellular Alterations in the Peripheral Blood of Patients With Stroke**

Compared with healthy controls, samples from patients with stroke indicated that stroke induced a mild leukocytosis (median 9.3 Gpt/l; range 3.3 Gpt/l−25.6 Gpt/l; P=0.0036) dominated by neutrophils, which reached a maximum on day 1 (median 75.20%; range 46.0%–90.4%; P<0.0001). Monocytes remained unchanged, whereas the percentage of lymphocytes declined (P<0.0001), reaching its lowest value on day 1 (median 14.8%; range 2.6%–39.3%; P<0.001). Data are not shown. These findings confirm previous observations.

**Migration and Phagocytosis**

To evaluate the early steps in the defense of the innate immune system against bacteria after stroke, the chemotactic and phagocytic capacities of blood cells were determined. Ex vivo migration toward the chemotaxin fMLP was assessed in a standard transwell assay in granulocytes and monocytes; this migration was unaltered in patients with stroke on day 1 (data not shown). The phagocytosis of fluorescently labeled *E. coli* by granulocytes and monocytes was quantified ex vivo. We analyzed the percentages of phagocytosing cells and the number of phagocytosed bacteria per cell as reflected by MFI. In samples from control subjects, 80% of granulocytes and 70% of monocytes phagocytosed *E. coli* (median values); the percentage of phagocytosing cells did not significantly differ between patients with stroke and controls (Figure 1A and 1C). The efficacy of phagocytosis (MFI) also remained unaltered in both cell types after stroke (Figure 1B and 1D). Taken together, these ex vivo data show that during the acute phase

![Figure 1](http://stroke.ahajournals.org/)

**Figure 1.** Percentage and efficacy of phagocytosis. The percentage of phagocytosing cells (A and C) and the efficacy of phagocytosis defined as mean fluorescence intensity (MFI) (B and D) for control subjects (white) and patients with stroke (gray). Granulocytes (dark gray, A and B) and monocytes (light gray, C and D) were investigated. Patient samples were obtained on admission (d0), the next morning (d1), and on days 3, 5, and 7 (d3, d5, d7). P<0.0001; A-D. Kruskal–Wallis test. A, P=0.1230; B, P=0.9682; C, P=0.9657; and D, P=0.3637. Medians and interquartile ranges are shown.

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*p* values <0.05 were regarded as significant.
Oxidative Burst

Oxidative burst is an efficient means used by phagocytes to kill internalized bacteria. Therefore, we determined whether stroke altered the percentage or efficacy of oxidative burst in our samples. In granulocytes, spontaneous oxidative burst was observed in 3.9% of cells in the control samples. The baseline oxidative burst activity of the granulocytes of patients with stroke was significantly reduced throughout the first week compared with controls (P=0.0011). The strongest effect was seen on day 5, when spontaneous oxidative burst only occurred in 1.0% of granulocytes (Figure 2A). This observation was paralleled by a highly significant reduction in the oxidative burst efficacy of granulocytes (P=0.0001), which persisted throughout the observation period (Figure 2C).

fMLP acts as weak stimulant of oxidative burst; it induced burst in 7.2% of granulocytes derived from age-matched controls. In patients with stroke, the percentage of bursting granulocytes was lower at all time points, but this difference did not achieve statistical significance (Figure 2B). However, the efficacy of oxidative burst induced in granulocytes was strongly reduced in patients with stroke compared with controls (P=0.0001; Figure 2D). E coli and PMA strongly stimulated oxidative burst in virtually all granulocytes of controls. Under the influence of these supramaximal stimuli, granulocytes from patients with stroke were as likely to burst as those from controls (Figure 1A and IB in the online-only Data Supplement). The efficacy of bursting was also unaltered (Figure IC and ID in the online-only Data Supplement).

In monocytes, spontaneous oxidative burst was observed in 3.0% of the cells in control samples. After stroke, the percentage of fMLP–stimulated monocytes remained intact in granulocytes and monocytes.

![Image](https://stroke.ahajournals.org/)

Figure 2. Granulocyte oxidative burst under different stimuli. Oxidative burst analysis was performed for samples from healthy control subjects (white bars) and patients with stroke (gray bars) taken on admission (d0), the next morning (d1), and on days 3, 5, and 7 (d3, d5, d7). We evaluated the percentage of bursting cells (A and B) and the efficacy (defined as mean fluorescence intensity, MFI) of oxidative burst (C and D). Unstimulated samples (A and C; ncontrol, d0, 1, 3, 5, 7=15, 36, 43, 39, 36, 27) and samples stimulated by N-formyl-methionyl-leucyl-phenylalanine (fMLP) (B and D; ncontrol, d0, 1, 3, 5, 7=15, 37, 45, 41, 33, 26) were used to quantify monocyte oxidative burst. A-D, Kruskal–Wallis test. A, P=0.0011; B, P=0.0318; C, P=0.0001; and D, P=0.0001; Dunn’s Multiple Comparison Test; *P<0.05; **P<0.01; ***P<0.005. Medians and interquartile ranges are given.

Figure 3. Monocyte oxidative burst under different stimuli. Oxidative burst analysis was performed for samples from healthy control subjects (white bars) and patients with stroke (gray bars) taken on admission (d0), the next morning (d1), and on days 3, 5, and 7 (d3, d5, d7). We evaluated the percentage of bursting cells (A and B) and the efficacy (defined as mean fluorescence intensity, MFI) of oxidative burst (C and D). Unstimulated samples (A and C; ncontrol, d0, 1, 3, 5, 7=15, 36, 43, 39, 36, 27) and samples stimulated by N-formyl-methionyl-leucyl-phenylalanine (fMLP) (B and D; ncontrol, d0, 1, 3, 5, 7=15, 37, 45, 41, 33, 26) were used to quantify monocyte oxidative burst. A-D, Kruskal–Wallis test. A, P=0.0318; B, P<0.0001; C, P<0.0001; and D, P<0.0001; Dunn’s Multiple Comparison Test; *P<0.05; **P<0.01; ***P<0.005. Medians and interquartile ranges are given.
As expected, patients with subsequent infections had more severe disease, with much larger lesions (Table I in the online-only Data Supplement). In good agreement with previous observations by us and by others,7,11,25 granulocytes were increased in patients with SAI+ (median, SAI+ d0,1,3,5,7=53.3%; 84.3%, 76.0%, 79.0%, 82.2%; median, SAI− d0,1,3,5,7=56.8%, 70.3%, 67.5%, 55.7%, 64.1%; P=0.0004); whereas monocytes remained unaltered. Metanephrine serum concentrations did not differ significantly between patients with and without SAI (Table I in the online-only Data Supplement).

**In Vitro Effects of Stress Hormones and Acetylcholine on Phagocyte Function**

Stress hormones have been suggested to act as central regulators of SIIAs6,25,26; some characteristics of SIIAs can be mimicked in cell culture by adding catecholamines.7 Here we investigated, whether the alterations observed in patients with stroke derived whether the alterations observed in patients with stroke derived from in vitro exposure to the catecholamines epinephrine and norepinephrine, the glucocorticoid dexamethasone or acetylcholine. Phagocytosis remained unaltered in monocytes and granulocytes in all conditions investigated (data not shown).

In contrast, the fraction of phagocytes spontaneously generating an oxidative burst was decreased after treatment with norepinephrine (Figures IIIA and IVA in the online-only Data Supplement). Dexamethasone inhibited burst in all stimulated conditions (Figures IIIB, IIIC, IVB, and IVC in the online-only Data Supplement); whereas the effect of catecholamines was most pronounced in E coli–stimulated monocytes (Figures IIIC and IVC in the online-only Data Supplement).

Because other cell functions remained intact, this observation is not attributable to toxicity.

**HNPs 1 to 3**

The serum concentrations of HNPs 1 to 3 were increased in patients with stroke but, when the data were corrected for granulocyte counts, no differences were detected (data not shown).

**NETs in Stroke**

After NETosis by neutrophils, pathogens are trapped within nets of condensed chromatin. We quantified the percentage (median) of NETosing cells and assessed the area (median) covered by a cell undergoing NETosis (efficacy; Figure 5A–C).

Without stimulation, 2.1% of granulocytes from samples from healthy controls released NETs with an area of 472 m2, similar to granulocytes from patients with stroke on days 1 (2.0%; 504 m2) and 5 (2.3%; 447 m2). Stimulation with fMLP or PMA induced strong NETosis in granulocytes from control subjects (fMLP: 5.6% [range 1.7%–41.2%]; PMA: 17.3% [range 3.7%–49.8%]). In stroke-derived cells, the fraction of cells undergoing NETosis in response to fMLP or PMA stimulation was unaftered on days 1 and 5 (fMLP: d1: 7.6% [range 0.5%–15.4%]; d5: 7.9% [0.9%–19.9%]; PMA: d1: 12.7% [range 1.6%–33.1%], d5: 17.7% [range 8.7%–52.6%]). However, the efficacy of NETosis was significantly reduced on day 1 after stroke (fMLP: P=0.0128; PMA: P=0.0004; Figure 5B and 5C; Figures 5A, VB, and IVC in the online-only Data Supplement). On day 5, the efficacy of NETosis in stroke was again indistinguishable from controls (Figure 5B and 5C; Figures VA, VB, VIA, and VIC in the online-only Data Supplement).
Discussion

Whether the innate immune system is impaired in patients with stroke is unknown. Therefore, we investigated monocyte and granulocyte functions involved in bacterial defense.

Although migration and phagocytic functions remained intact in monocytes and granulocytes in the present investigation, the key mechanisms required for bacterial killing, oxidative burst, and NETosis were significantly impaired in patients with stroke. These findings are in good agreement with a study describing impaired burst in 17 patients with hemorrhagic stroke.19

Stroke localization and stroke lateralization have been suggested to contribute to the extent of immunologic changes seen in patients with stroke.27–29 This could explain our finding that oxidative burst was not correlated to stroke severity in our cohort.

The alterations in NETosis were transient and had normalized on day 5, inhibition of oxidative burst persisted throughout the study period. Because these impaired phagocyte responses were present on admission and on day 1, they could contribute to susceptibility for SAI, which typically manifest around day 3.

To explore whether the changes seen in the bactericidal functions in cells of the innate immune system correlate with SAI in our patient cohort, we compared patients who remained completely free from infection (SAI–) and those with subsequent severe systemic infection (SAI+) according to predefined criteria. There were no significant differences between patients who are SAI+ and SAI– with respect to granulocyte oxidative burst function. In monocytes, E coli–induced oxidative burst was reduced on admission in patients with SAI. This was not observed for other stimuli. It is tempting to speculate that the in vitro response to a bacterial stimulus would be most closely related to the in vivo situation.

Stress hormones and especially catecholamines have been identified because important mediators of several SIIAs and catecholamine serum concentrations have been correlated with SAI.3,28 We observed that in vitro exposure of granulocytes and monocytes to catecholamines did not alter phagocytosis but inhibited oxidative burst. Thus, the results of our cell-culture experiments parallel our ex vivo findings and strengthen the case for a causal role of catecholamines in SIIAs.

We are aware of the limitations of the current study: The in vitro testing of key steps of bacterial defense mechanism was performed in cells derived from the peripheral blood of healthy donors and patients with stroke. Although this approach is often used in patient studies, it does not extend to cells with intact systemic infection. Also, it does not address the clearance of bacteria from the host. Experimental stroke models will be required to clarify the causal relationship between impairment of phagocyte functions and SAI and to determine bacterial clearance from the host.

To further delineate the relationship of phagocyte function, stroke localization and infection larger patient cohorts will have to be studied; such a study will also reveal whether our in vitro finding that stress hormones impair phagocyte function is reflected in patients with stroke.

Stroke-induced alterations in adaptive immune function, especially the strong reduction of blood lymphocyte counts, are closely associated with early bacterial infection.5,24 Although this correlation is robust, it is difficult to explain how the adaptive immune system affects the early defense against bacteria. Considering that catecholamines, which suppress phagocyte function in vitro and have been shown to induce lymphocytopenia, the stress hormones could be the missing link in this mechanism. Reducing oxidative burst and NET formation impairs the defense against bacteria and thus increases the risk of infection. We propose that peripheral blood lymphopenia can serve as a biomarker of such innate immune defects, which are newly recognized features of SIIA. Whether the persistent, profound lymphopenia observed in some patients with stroke affects early innate defense mechanisms merits further study.

Conclusions

We have demonstrated the impairment of bactericidal phagocyte function in patients with stroke. This impairment is a novel aspect of SIIA. We hypothesize that the suppression of bacterial killing is causally related to the enhanced infection risk of patients with stroke.

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Disclosures

None.

References

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/content/45/4/e68.full.pdf

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2014/02/13/STROKEAHA.113.003342.DC1
The version of the article, “Stroke Alters Respiratory Burst in Neutrophils and Monocytes” by Ruhnau et al. (Stroke. 2014;45:794–800) that published online ahead-of-print on February 12, 2014, a correction was needed.

The published article was a prior version.

This has been corrected in the online and print version of the article.
**SUPPLEMENTAL MATERIAL**

**Supplemental methods**

**Migration**

Cell migration was assayed using the MIGRATEST Kit® (Glycotope Biotechnology GmbH, Heidelberg, Germany) according to the manufacturer’s instructions. Briefly, leukocyte-rich plasma was extracted from whole blood. The bottom chamber of the test equipment was filled with either 350 μL of incubation buffer (control conditions) or incubation buffer enriched with the chemotactic peptide N-formyl-Met-Leu-Phe (fMLP; stimulated conditions, 50 nM fMLP final concentration). One hundred microliters of leukocyte-rich plasma were added into the top chamber. Cells were allowed to migrate for 30 min at 37 °C, 5% CO₂, before the number of migrated cells was counted. Fluorescein isothiocyanate (FITC)-labeled antibody against L-selectin, propidium iodide for vital staining, and counting beads were added (5000 beads were counted). Propidium iodide staining was used to exclude dead cells. Since cell activation is inversely correlated with L-selectin expression #1 downregulation of L-Selectin can be used as an activation marker. The effect of fMLP on cell activation was therefore assessed by calculating (percentage selectin-negative cells<sub>stimulated</sub> - percentage selectin-negative cells<sub>unstimulated</sub>) = fMLP-induced activation.

The migratory activity of the cells was determined as the ratio of cells to beads under stimulated and unstimulated conditions. Fluorescence-activated cell sorting (FACS) analysis was performed on a BD LSR II (BD Biosciences, San Jose, CA, USA).
### Characteristics of Patients that fullfilled all criteria of SAI (SAI+ patients) and those that did not match any criteria of SAI (SAI-patients)

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>NIHSS on admission</th>
<th>1. day of infection</th>
<th>Type of infection</th>
<th>Bacterial culture</th>
<th>Nasogastric tubes/dysphagia</th>
<th>rtPA tx</th>
<th>Thrombectomy</th>
<th>Additional risk factors</th>
<th>Metanephrine (ng/l)</th>
<th>SAI status</th>
</tr>
</thead>
<tbody>
<tr>
<td>55</td>
<td>m</td>
<td>16</td>
<td>Day 1</td>
<td>Pneumonia</td>
<td>Negative</td>
<td>yes, day1</td>
<td>Thrombectomy/ i.a. rtPA</td>
<td>Craniotomy</td>
<td>n.d.</td>
<td>SAI +</td>
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<tr>
<td>60</td>
<td>m</td>
<td>19</td>
<td>Day 3</td>
<td>Pneumonia</td>
<td>Negative</td>
<td>yes day 1</td>
<td>no</td>
<td>Craniotomy</td>
<td>&lt;15</td>
<td>SAI +</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>f</td>
<td>17</td>
<td>Day 5</td>
<td>Pneumonia</td>
<td>Klebsiella pneumoniae</td>
<td>Yes</td>
<td>i.v. rtPA</td>
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<td>35</td>
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<tr>
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<td>f</td>
<td>18</td>
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<td>MRSA colonisation</td>
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<td>Ulcus cruris</td>
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<tr>
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<td>m</td>
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<td>None</td>
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<tr>
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<td>m</td>
<td>13</td>
<td>Day 3</td>
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<td>Craniotomy</td>
<td>n.d.</td>
<td>SAI +</td>
<td></td>
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<td>m</td>
<td>8</td>
<td>Day 1</td>
<td>Pneumonia</td>
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<td>no</td>
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<td>&lt;15</td>
<td>SAI +</td>
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<td>no</td>
<td>None</td>
<td>91</td>
<td>SAI -</td>
<td></td>
</tr>
<tr>
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<td>f</td>
<td>7</td>
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<td>n.a.</td>
<td>n.a.</td>
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<tr>
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<td></td>
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<td>n.a.</td>
<td>no</td>
<td>no</td>
<td>None</td>
<td>&lt;15</td>
<td>SAI -</td>
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</tr>
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<td>f</td>
<td>8</td>
<td>n.a.</td>
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<td>n.a.</td>
<td>no</td>
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<td>None</td>
<td>44</td>
<td>SAI -</td>
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<td>m</td>
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<tr>
<td>92</td>
<td>m</td>
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<td>None</td>
<td>39</td>
<td>SAI -</td>
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<tr>
<td>72</td>
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<td>no</td>
<td>i.v. rtPA</td>
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<td>NA</td>
<td>SAI -</td>
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<td>i.v. rtPa</td>
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<tr>
<td>79</td>
<td>m</td>
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<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>yes</td>
<td>i.v. rtPA</td>
<td>None</td>
<td>NA</td>
<td>SAI -</td>
<td></td>
</tr>
</tbody>
</table>

SAI status was determined as stated in the methods section, thrombectomy and local (i.a.) and systemic (i.v.) rtPA treatment was performed as clinical indicated. Bacterial cultures were obtained on the basis of clinical suspicion, therefore no cultures were obtained in patients without clinical signs of infection; craniotomy was performed in patients with malignant infarction. Metanephrine levels as determined on admission.

Abbreviations: NIHSS: NIH stroke scale score; MRSA: Methicillin-resistant Staphylococcus aureus; rtPA tx : treatment with recombinant tissue plasminogen activator; ICU: intensive care unit; i.v. intravenous; i.a. intraarterial; n.a.: not applicable; n.d. not determined
Suppl. Fig. I: Granulocytic oxidative burst under different stimuli.

Oxidative burst analysis was performed for samples from healthy control subjects (white bars) and stroke patients (grey bars) taken on days on admission (d0), the next morning (d1), and on days 3, 5, and 7 (d3, d5, d7). We evaluated the percentage of bursting cells (A, B) and the efficacy (MFI) of oxidative burst (C, D). *E. coli* (A, C; ncontrol, d0, 1, 3, 5, 7 = 15, 36, 45, 40, 36, 25), or PMA (B, D; ncontrol, d0, 1, 3, 5, 7 = 15, 36, 44, 40, 35, 27) stimulated samples were used to quantify granulocytic oxidative burst. A-D: Kruskal-Wallis test (A: \( p = 0.1640 \)), (B: \( p = 0.4156 \)), (C: \( p = 0.4958 \)), (D: \( p = 0.8522 \)). Medians and interquartile ranges are given.
Suppl. Fig.II: Monocytic oxidative burst under different stimuli.

Oxidative burst analysis was performed for samples from healthy control subjects (white bars) and stroke patients (grey bars) taken on days on admission (d0), the next morning (d1), and on days 3, 5, and 7 (d3, d5, d7). We evaluated the percentage of bursting cells (A, B) and the efficacy (MFI) of oxidative burst (C, D). *E. coli* (A, C; n_{control, d0, 1, 3, 5, 7} = 15, 36, 45, 36, 25), or PMA (B, D; n_{control, d0, 1, 3, 5, 7} = 15, 36, 44, 40, 35, 27) stimulated samples were used to quantify monocytic oxidative burst. A-H: Kruskal-Wallis test (A: p = 0.0137), (B: p = 0.0110), (C: p = 0.5293), (D: p = 0.2967); Dunn's Multiple Comparison Test; *p < 0.05; **p < 0.01. Medians and interquartile ranges are given.
Suppl. Fig. III: In vitro stress hormone effects on granulocyte function.

Effect of in vitro administration of $1 \times 10^{-7}$ M epinephrine (E) and norepinephrine (NE), $2.5 \times 10^{-6}$ M dexamethasone (D) or $5.5 \times 10^{-6}$ M acetylcholine (A) compared to the untreated condition (U) on oxidative burst in granulocytes in unstimulated cells (A) or in cell stimulated with fMLP (B) or *E. coli* (C). n = 5; A-C: Kruskal-Wallis test (A: $p = 0.0837$), (B: $p = 0.0490$), (C: $p = 0.0333$), Dunn's Multiple Comparison Test; *$p < 0.05$. Whiskers depict maximal and minimal values.

Suppl. Fig. IV: In vitro stress hormone effects on monocyte function.

Effect of in vitro administration of $1 \times 10^{-7}$ M epinephrine (E) and norepinephrine (NE), $2.5 \times 10^{-6}$ M dexamethasone (D) or $5.5 \times 10^{-6}$ M acetylcholine (A) compared to the untreated condition (U) on oxidative burst in monocytes in unstimulated cells (A) or in cell stimulated with fMLP (B) or *E. coli* (C). n = 5; A-C: Kruskal-Wallis test (A: $p = 0.0292$), (B: $p = 0.0275$), (C: $p = 0.0040$), Dunn's Multiple Comparison Test; *$p < 0.05$; **$p < 0.01$. Whiskers depict maximal and minimal values.
Suppl. Fig.V: NET formation following fMLP stimulation.

SYTOX Green-stained cells illustrate NET formation in samples from controls (A) or stroke patients on day 1 (B) and day 5 (C) following fMLP stimulation. Scale bar = 63 µm; exposure time = 170 ms.

Suppl. Fig.VI: NET formation following PMA stimulation.

SYTOX Green-stained cells illustrate NET formation in samples from controls (A) or stroke patients on day 1 (B) and day 5 (C) following PMA stimulation. Scale bar = 63 µm; exposure time = 170 ms.

Supplemental References