Polymorphonuclear Leukocyte Accumulation in Brain Regions with Low Blood Flow During the Early Postischemic Period

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SUMMARY In an anesthetized canine model in which ischemia was induced by incremental air emboli, 16 animals were exposed to 1 hr of ischemia and monitored for 10 min (n = 4), 60 min (n = 6), or 240 min (n = 6). Fourteen animals were observed for corresponding periods without being subjected to ischemia 70 min (n = 4), 120 min (n = 4), or 300 min (n = 6). Autologous granulocytes were labeled with 111In and reinjected just before ischemia. At the conclusion of each experiment, a 14C-iodoantipyrine autoradiographic blood flow study was performed. Granulocyte accumulation measured by gamma scintigraphy (cpm/gm) occurred in the injured hemisphere of ischemic animals at 60 min in anterior brain segments and at 240 min in anterior, middle, and posterior segments. By means of a double-label autoradiography technique, clustering of punctate granulocyte images was detected in regions of low flow or heterogeneous flow in half of the animals at both 60 min and 240 min postischemia. Granulocyte clustering did not occur in the autoradiograms of nonischemic animals. The results implicate granulocyte participation in the acute phase of ischemic brain injury and signal a convergence of hemostatic and inflammatory processes during the immediate postischemic period.

Methods

Thirty conditioned, male mongrel dogs weighing 9–16 kg were premedicated with xylazine (1.1 mg/kg) and atropine (0.05 mg/kg s.c.), followed by an initial intravenous dose of alpha-chloralose (80 mg/kg) and incremental doses as necessary. Anesthetized animals were ventilated mechanically; monitored for aortic blood pressure, temperature, and blood gases; and prepared for the recording of cortical sensory evoked response. The general model was described fully in previous publications.

A 1 h period of focal ischemia was induced in one hemisphere of a dog’s brain by injecting small volumes of air selectively through the ipsilateral internal carotid artery. Blood circulation to the opposite hemisphere remained largely intact as a nonischemic control. The degree of ischemia in the affected hemisphere was regulated by monitoring the cortical sensory evoked response (CSER) as a quantifiable electrophysiologic index of neuronal function. A variable recovery period followed the ischemic period, and a 14C-iodoantipyrine autoradiographic blood flow study concluded the procedure. Granulocytes isolated from arterial blood drawn early during the preparation of the animal were labeled with 111In and reinjected before the start of ischemia. A double-label autoradiography technique permitted correlation of both cerebral blood flow and granulocyte deposition in any given area of brain, and accumulation of granulocytes in brain and other tissues was quantified by gamma scintigraphy.

The animals were divided into six groups defined in table 1. All animals were either observed or exposed to right hemisphere brain ischemia for 60 min. Subsequently, groups of animals were monitored for the periods shown in this table, while blood pressure, blood gases, and body temperature were monitored and maintained at stable levels within physiologic limits.
To obtain granulocytes for these studies, 102 ml of blood were collected from a femoral artery catheter and placed in 18 ml of anticoagulant citrate dextrose solution (ACD-Formula A, Fenwall Laboratories, Deerfield, IL), which yielded a final ratio of 15% v/v. One hundred milliliters of Ringer's lactate solution containing plasma, leukocytes, and platelets was then diluted with sterile dextran 500 (6% in saline, 1 part Dextran to 10 parts saline).  The mixture was allowed to sediment for more than half the height of the tube. The supernatant containing plasma protein, platelets, erythrocytes, and mononuclear cells was removed by increasing gradually the flow rate through the separation chamber until only the remaining plasma remained as described previously. 12 The supernatant was discarded. The granulocyte-enriched pellets were resuspended in 20 ml saline and placed in a small hemolysis tube for counting. In each case the cells underwent a rapid shape change with a resultant 10.4 ± 2.0% increase in volume, which compared favorably with values published previously. 13 Aggregation tracings also compared favorably with reports published previously. 15

For granulocyte labeling, the 50 ml tubes containing the granulocyte suspension were spun at 185 × g for 10 min. The pellets were resuspended in 3 ml of saline and this constituted the incubating mixture. One mCi of 111In-oxine in 50 μl pure ethanol was diluted with 1 ml saline and added to the 3 ml incubating mixture drop by drop, with a gentle rotation of the tube to rapidly disperse the isotope solution in the granulocyte concentrate. After 30 min incubation at room temperature, the labeling was stopped by diluting the incubating medium with saline to a final volume of 20 ml. Before pelleting the labeled granulocytes, an aliquot of the granulocyte suspension (GRS) was removed to evaluate the labeling efficiency as described below. The labeled granulocytes were spun at 185 × g for 10 min, and the radioactive granulocyte-poor supernatant was discarded. The granulocytes were gently resuspended in 20 ml saline, and a small aliquot (GRS,) was withdrawn to determine the fraction of the 111In remaining unbound in the suspension of labeled granulocytes that was to be injected. Twenty microliters of GRS, and GRS2 were diluted 10 times with saline and placed in a small hemolysis tube for counting. The remainder of the undiluted GRS and GRS2 was centrifuged at 1200 × g for 10 min, and 20 μl of granulocyte-free supernatant from each (GRS, and GRS, respectively) was diluted 10 times with saline. Twenty microliters of the diluted GRS, and GRS2 were placed in a hemolysis tube for counting. After dilution GRS, GRS, GRS2, and GRS2 were analyzed by gamma scintigraphy. The labeling efficiency was calculated from the following gamma activity ratio: (GRS, - GRS2)/GRS2. This ratio is a reasonable approximation of the efficiency because the volume of the granulocytes is small in comparison to the total volume of the granulocyte suspension. The ratio of GRS, gamma activity to that in GRS2 indicated the fraction of 111In remaining unbound in the infused suspension of granulocytes.

After reinjection of the labeled granulocytes, the activity of 111In that was free in plasma and the activity of 111In that was bound to cells were measured at some or all of the following time points in 15 dogs: 5 min, 15 min, 1 h, 2 h, 3 h, 4 h, and 5 h. Blood samples (4.5 ml blood diluted 1:10 in ACD) were drawn at each time point. A hematocrit level was measured for each sample and 20 μl aliquots were analyzed by gamma scintigraphy. Each remaining sample was centrifuged at 1200 × g, and 20 μl of the supernatant plasma were analyzed for 111In in concentrate. Free 111In in plasma (111In-free) was calculated as: (1-Hct) × plasma counts/ml. Cell-bound 111In (111In-cell bound) was calculated as: whole blood counts/ml × 111In-free. The percentage of circulating leukocytes was calculated as:

\[
\text{circulating leukocytes (\%)} = \frac{111\text{In-cell bound cpm/ml} \times \text{blood vol (ml)} \times 100}{\text{Total cell bound CPM injected}}
\]

Relative plasma activity was computed as:

\[
\text{Relative plasma activity =}
\]
For these calculations, blood volume was estimated as 86 ml/kg and plasma volume as 50 ml/kg.16

The brain was removed after the 14C-iodantipyrine autoradiographic blood flow study9 and frozen at -50 to -60°C in liquid freon suspended over liquid nitrogen. Later, the brain was divided coronally into three segments, each containing symmetrical portions of the right and left hemispheres and termed anterior (containing the thalamus), and posterior (containing the head of the caudate nucleus), middle (containing the hippocampal formation). Each segment was then mounted on a chuck and sectioned in a freezing microtome for autoradiography. Blood flow was calculated from 20-μm sections,9 and 40-μm sections enhanced the detection of granulocyte accumulation in the tissue.7 Immediately after the microtome sectioning, samples of the cortex with subjacent white matter attached were excised from homologous water-housed areas of the superolateral right and left hemispheres of each segment. In ischemic animals, the right hemisphere constituted the injured side and the left hemisphere constituted the noninjured side. The samples were then weighed and counted in a Packard autogamma scintillation spectrometer with a 100-600 Kev energy window.

At the conclusion of some experiments, tissue samples weighing 102 ± 65 mg (mean ± SD) from the brain, liver, spleen, lung, muscle, and buccal mucosa were excised, frozen, and analyzed by gamma scintigraphy. The frozen tissue was weighed and counted for 111In activity in the same manner as the brain samples. Radioactivity expressed as counts/min/gm (cpm/gm) of tissue provided an index of granulocyte sequestration by the various tissues.

**Results**

Heart rate, mean aortic pressure (AoP mean), hematocrit, pH, and blood gases were comparable among groups as shown in table 2. The final CSER expressed as percent of baseline P–N, amplitude is displayed for the various groups in table 3. Animals subjected to ischemia in Groups 2, 4, and 6 clustered around 20% CSER recovery, a figure observed repeatedly in untreated animals in the past.6 The quantity of air required to induce an ischemic reduction of CSER to 10–20% of baseline values during the ischemic interval was 260 ± 30 μl (mean ± SEM) for Group 2, 158 ± 36 μl for Group 4, and 215 ± 25 μl for Group 6. These values did not differ significantly by one-way analysis of variance (ANOVA).

The efficiency of granulocyte labeling was 97 ± 3% (mean ± SD), and the percentage of 111In activity in the injected granulocyte suspension that was not in cells was 2 ± 1%. Despite anesthesia and indwelling catheters, the percentage of circulating granulocytes had not reached half its initial value by 5 h, suggesting

### TABLE 2 Comparison of Several Physiologic Variables Among Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Heart rate (min⁻¹)</th>
<th>AoP mean (mm Hg)</th>
<th>Hematocrit (%)</th>
<th>pH</th>
<th>Po2 (mm Hg)</th>
<th>PCO2 (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (a)</td>
<td>105 ± 15</td>
<td>108 ± 7</td>
<td>40 ± 2</td>
<td>7.41 ± 0.07</td>
<td>101 ± 11</td>
<td>34 ± 8</td>
</tr>
<tr>
<td>1 (b)</td>
<td>119 ± 33</td>
<td>101 ± 12</td>
<td>38 ± 2</td>
<td>7.36 ± 0.04</td>
<td>92 ± 7</td>
<td>37 ± 5</td>
</tr>
<tr>
<td>1 (c)</td>
<td>135 ± 34</td>
<td>99 ± 21</td>
<td>36 ± 4</td>
<td>7.35 ± 0.03</td>
<td>92.6 ± 6</td>
<td>36.4 ± 2</td>
</tr>
<tr>
<td>2 (a)</td>
<td>128 ± 24</td>
<td>108 ± 15</td>
<td>40 ± 2</td>
<td>7.38 ± 0.05</td>
<td>82 ± 5</td>
<td>36.4 ± 2</td>
</tr>
<tr>
<td>2 (b)</td>
<td>146 ± 27</td>
<td>116 ± 19</td>
<td>40 ± 2</td>
<td>7.34 ± 0.03</td>
<td>93 ± 3</td>
<td>36.3 ± 2</td>
</tr>
<tr>
<td>2 (c)</td>
<td>166 ± 14</td>
<td>123 ± 26</td>
<td>41 ± 4</td>
<td>7.35 ± 0.04</td>
<td>90 ± 5</td>
<td>35.4 ± 2</td>
</tr>
<tr>
<td>3 (a)</td>
<td>132 ± 40</td>
<td>121 ± 13</td>
<td>41 ± 2</td>
<td>7.43 ± 0.04</td>
<td>99 ± 6</td>
<td>31 ± 2</td>
</tr>
<tr>
<td>3 (b)</td>
<td>143 ± 12</td>
<td>106 ± 10</td>
<td>41 ± 5</td>
<td>7.38 ± 0.02</td>
<td>103 ± 10</td>
<td>33.2 ± 2</td>
</tr>
<tr>
<td>3 (c)</td>
<td>141 ± 28</td>
<td>110 ± 17</td>
<td>40 ± 5</td>
<td>7.37 ± 0.02</td>
<td>101 ± 6</td>
<td>32.1 ± 2</td>
</tr>
<tr>
<td>4 (a)</td>
<td>106 ± 12</td>
<td>134 ± 28</td>
<td>38 ± 4</td>
<td>7.37 ± 0.05</td>
<td>93 ± 12</td>
<td>36.4 ± 2</td>
</tr>
<tr>
<td>4 (b)</td>
<td>118 ± 26</td>
<td>127 ± 26</td>
<td>38 ± 5</td>
<td>7.36 ± 0.05</td>
<td>90 ± 2</td>
<td>36.3 ± 2</td>
</tr>
<tr>
<td>4 (c)</td>
<td>142 ± 40</td>
<td>132 ± 28</td>
<td>39 ± 7</td>
<td>7.35 ± 0.02</td>
<td>86 ± 5</td>
<td>34.2 ± 2</td>
</tr>
<tr>
<td>5 (a)</td>
<td>131 ± 12</td>
<td>130 ± 35</td>
<td>40 ± 4</td>
<td>7.40 ± 0.05</td>
<td>95 ± 11</td>
<td>33 ± 2</td>
</tr>
<tr>
<td>5 (b)</td>
<td>152 ± 15</td>
<td>117 ± 40</td>
<td>41 ± 8</td>
<td>7.42 ± 0.03</td>
<td>90 ± 5</td>
<td>30.2 ± 2</td>
</tr>
<tr>
<td>5 (c)</td>
<td>153 ± 6</td>
<td>96 ± 14</td>
<td>38 ± 5</td>
<td>7.36 ± 0.02</td>
<td>91 ± 6</td>
<td>34.2 ± 2</td>
</tr>
<tr>
<td>6 (a)</td>
<td>113 ± 28</td>
<td>111 ± 18</td>
<td>37 ± 1</td>
<td>7.40 ± 0.03</td>
<td>88 ± 7</td>
<td>34.3 ± 2</td>
</tr>
<tr>
<td>6 (b)</td>
<td>128 ± 5</td>
<td>107 ± 23</td>
<td>34 ± 3</td>
<td>7.38 ± 0.05</td>
<td>97 ± 8</td>
<td>34.2 ± 2</td>
</tr>
<tr>
<td>6 (c)</td>
<td>118 ± 11</td>
<td>109 ± 26</td>
<td>33 ± 3</td>
<td>7.38 ± 0.02</td>
<td>92 ± 8</td>
<td>36.3 ± 3</td>
</tr>
</tbody>
</table>

*All values are mean ± SD.
†Before ischemia.
‡Before treatment or observation period.
§Before blood flow study.
TABLE 4

<table>
<thead>
<tr>
<th>Group</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CSER</td>
<td>101 ± 17*</td>
<td>19 ± 14</td>
<td>113 ± 12</td>
<td>23 ± 9</td>
<td>93 ± 6</td>
<td>23 ± 16</td>
</tr>
</tbody>
</table>

*All values are mean ± SD.

Figure 1. Evolution of right-left differences in 111In-labeled granulocyte accumulation at 10, 60, and 240 min after 1 h period of observation or ischemia. Graphs depict activity differences in cpm/gm of tissue from anterior segments. Solid line connecting the triangles indicates values from control groups not exposed to ischemia; broken line connecting the squares indicates values from groups exposed to ischemia. The 10 min value in the control group is elevated by a single deviant value as signified by the enormous standard error bar.
EFFECT OF ISCHEMIA ON LEUKOCYTE ACCUMULATION

FIGURE 2. Evolution of right-left differences in $^{111}$In-labeled granulocyte accumulation at 10, 60, and 240 min after 1 h period of observation or ischemia. Graphs depict activity differences in cpm/gm of tissue from middle segments. Solid line connecting the triangles indicates values from control groups not exposed to ischemia; broken line connecting the squares indicates values from groups exposed to ischemia.

same time points were: $-465 \pm 1.438$, $321 \pm 276$, and $224 \pm 786$, respectively. Right-left hemispheric differences in ischemic animals from corresponding brain levels in the posterior segment at the same time points were: $508 \pm 321$, $2,843 \pm 1,232$, and $6,497 \pm 2,327$, respectively. These group and time relationships are depicted in fig. 3. The excess accumulation of $^{111}$In-labeled leukocytes in the injured right hemisphere of animals subjected to unilateral ischemia reached significance in the posterior segment at the 240 min time point ($p < 0.03$) as compared to animals not subjected to ischemia by two-way ANOVA and Bonferroni testing.

Analysis by comparison of right-left differences between ischemic animals and control animals tended to underestimate the true differences between these groups. This systematic bias was introduced by a consistent small increase in granulocyte accumulation in the noninjured left hemisphere of ischemic animals as compared to the left hemisphere of control animals. A pooled mean (cpm/gm) from the left hemisphere of ischemic animals was $3,385$ at 60 min and $6,117$ at 240 min (mean ± SEM). The corresponding values from the control group were $2,621$ at 60 min and $3,039$ at 240 min. Analysis by interhemispheric differences in each animal, however, was more sensitive than comparison of ipsilateral hemispheres between ischemic animals and control animals due to the scatter introduced by the variable $^{111}$In-labeled granulocyte activities in blood among the animals.

Mean blood flow rates (± SD) calculated for each of the six groups ranged from $94$ ml/100 g/min (± 49) to $46$ ml/100 g/min (± 23) in areas of right hemisphere gray matter and from $30$ ml/100 g/min (± 26) to $8$ ml/100 g/min (± 6) in right hemisphere white matter. The flow rates of left hemisphere gray matter ranged from $93$ ml/100 g/min (± 53) to $44$ ml/100 g/min (± 119).
of images could be discerned on the autoradiograms and none of the control animals demonstrated clustering at any time point. Examples of autoradiograms from ischemic and control animals at 10, 60 and 240 min postischemia are shown in figures 4–6.

**Discussion**

"Indium-oxine is an excellent label for granulocytes. When granulocytes are suspended in saline rather than plasma so that the potential for competitive binding to transferrin is removed, indium-oxine diffuses rapidly across their cell membranes. The indium then dissociates from the oxine and binds to various cytoplasmic components. The concentrations of indium reached in these studies appears to be nontoxic to the cells and the isotope remains firmly bound to the cell with no evidence of elution or reutilization. The T½ of "Ind (67 h) is ample to process the tissue and assay its activity and the emission characteristics of the isotope (two gamma photons 173 and 247 Kev in high abundance, i.e., 84% and 94%, respectively) permit external body scanning and gamma scintigraphy as well as autoradiography.

16), and flow rates of left hemisphere white matter went from a high of 31 ml/100 g/min (± 25) to a low of 12 ml/100 g/min (± 8). One-way ANOVA testing, applied individually to areas of right and left hemisphere gray and white matter, showed no significant differences among any of the group means. Neuron-disabling blood flows defined previously as 15 ml/100 gm/min or less in gray matter and 6 ml/100 gm/min or less in white matter occurred in three of four animals at 10 min postischemia (Group 6), three of six animals at 60 min postischemia (Group 4), and three of six animals at 240 min postischemia (Group 2). Neuron-disabling flow rates did not occur in the control groups not subjected to ischemia. One animal in Group 3, a control animal for 60 min of postischemic follow-up, inadvertently received too little "C-iodoantipyrine precluding accurate blood flow measurement; this animal was not included in the blood flow analyses.

Double isotope autoradiography with "In-labeled granulocytes and "C-iodoantipyrine permitted assessment of any relationship between blood flow and granulocyte accumulation within a single brain section. Some degree of clustering of punctate granulocyte images was noted in regions of low flow or heterogeneous flow in three of six animals at 60 min postischemia and in three of six animals at 240 min postischemia. At 10 min postischemia, no clustering
The major finding in the present study is that during the first few hours of the postischemic period, granulocytes accumulate in regions of brain that have been subjected to ischemia. Furthermore, there is a tendency for this accumulation to conform to areas of low blood flow or heterogeneous blood flow in patterns that resemble those noted for platelet accumulation in studies published previously.10,17

The accumulation of granulocytes in regions of the brain during the first few hours after an ischemic insult presupposes a series of foregoing reactions that are not generally emphasized in concepts of the pathophysiology of stroke. Granulocytes will collect in a tissue in response to any of a number of chemotactic factors. Such factors include fragments of complement system derivatives C5a and C5b, the C5b9 complex, leukotrienes, kallikrein, and plasminogen activator plus in addition to several products of fibrinolysis such as fibrinopeptide B, fragment D, and fragment E.24 These findings, therefore, signal a convergence of hemostatic and inflammatory processes during the immediate postischemic period.

Models of inflammation provide some insight into the possible modes of interaction of these processes. After intradermal injection of a variety of inflammatory stimuli (FMLP, E. Coli, C5a, desarginine, and zymosan-activated plasma) in rabbits, granulocytes and platelets accumulate concurrently at the injection site.23 The maximum rate of accumulation occurs at 1.5–2 h and falls off by 7 h. Activation of granulocytes by chemotactic factors increases their adhesiveness and causes them to marginate in the first capillary bed they encounter,23 and platelets tend to deposit and coassociate with the granulocytes when they marginate in capillaries and postcapillary venules.25 Rabbits rendered neutropenic by Nitrogen Mustard do not accumulate platelets in response to inflammatory stimuli but do accumulate platelets in response to thrombin. Also, rabbits that experience a secondary hemorrhage in the inflammatory skin site develop a second wave of platelet accumulation 4–7 h after the initial inflammatory stimulus. Reconstitution of the skin sites with granulocytes does not cause platelet accumulation in rabbits pretreated with Nitrogen Mustard, indicating that the interaction of platelets and granulocytes occurs at an intravascular location.25

Potential forms of interaction among platelets, granulocytes, and the endothelium have features suggestive of feedback regulation with both positive and negative characters. Platelets produce 12-HETE from arachidonic acid, which is chemotactic for leukocytes.27 Platelet-derived arachidonic acid can be utilized by granulocytes in addition to their endogenous arachidonate to produce such lipoxygenation products as 5HETE, DHETE, and leukotrienes.28 The products DHETE and 5HETE are chemotactic and DHETE may regulate leukotriene formation.30 Leukotriene B4 (LTB4) is a chemoattractant and complete secretagogue for neutrophils. Platelet-derived endoperoxides can also be utilized by endothelial cells to synthesize PGI2 in quantities sufficient to inhibit platelet aggrega-

tion.31 PGI2 inhibits formation of thromboxane A2 by platelets by raising levels of platelet cyclic adenosine monophosphate (cAMP).32 A rise in cAMP in endothelium as a consequence of PGI2 could diminish further PGI2 production.33 Neutrophils elaborate a platelet activating factor, acetyl glyceryl ether phosphorylcholine (AGEPC), which causes local accumulation of platelets and is a very potent agent for increasing vascular permeability. It produces both the early, transient and the delayed, prolonged phases of vascular leakage.34 The delayed phase, however, may be dependent on leukocytes that are also activated by AGEPC. Additionally, AGEPC is a vasoconstrictor of arterioles. The neutrophils may also extend the tissue damage by generating free radicals,35 releasing intracellular constituents,36 or tunneling under endothelium.37 Some authors regard several platelet functions as components of the inflammatory response.38 Thus, events at the blood-endothelial interface in the acute postischemic period are potentially complex, multifactorial, and cybernetic, leading to edema and heterogeneous microcirculatory blood flow rates.

Authors’ Notes
The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or as reflecting the views of the Navy Department or the Naval Service at large.

References
14. O’Flaherty JT, Kreutzer DL, Ward PA: Chemotactic factor influ-
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J M Hallenbeck, A J Dutka, T Tanishima, P M Kochanek, K K Kumaroo, C B Thompson, T P Obrenovitch and T J Contreras

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