Influence of Granulocytopenia on Canine Cerebral Ischemia Induced by Air Embolism

Andrew J. Dutka, MD, Patrick M. Kochanek, MD, and J.M. Hallenbeck, MD

We subjected nine dogs with severe granulocytopenia 4 days after the administration of mechlorethamine to 1 hour of cerebral ischemia induced by the controlled, incremental injection of air into the internal carotid artery. Cortical somatosensory evoked responses and cerebral blood flow determined by \([{}^{14}\text{C}]\text{iodoantipyrine autoradiography}\) were compared with those of six control dogs that had received mechlorethamine 1 day previously and were not yet granulocytopenic. Eleven additional control dogs received no mechlorethamine but had identical ischemic insults and were followed for 4 hours after ischemia. Both control groups had identical evoked response outcomes after 1 hour of recovery from ischemia. Granulocytopenic dogs had improved evoked response recoveries compared with either control group after 1 hour of recovery. No areas of very low blood flow were observed 1 hour after ischemia in the granulocytopenic dogs, but three of five dogs in the control group receiving mechlorethamine had such areas. (Stroke 1989;20:390-395)

In previous experiments we have demonstrated that indium-111-labeled granulocytes accumulate in areas of low blood flow following cerebral ischemia induced by air embolism.\textsuperscript{1,2} Granulocytes accumulate during the early reperfusion phase following the end of ischemia (within 4 hours), which is a critical period for the development of posts ischemic hypoperfusion and progressive neuronal cell death.\textsuperscript{3,4} This coincidence of timing and the variety of vasoactive and tissue-damaging effects of activated leukocytes\textsuperscript{5} suggested that improved neuronal function and blood flow should be associated with reduced granulocyte accumulation. Granulocyte accumulation is reduced by treatments that reduce the size of myocardial infarction in experimental animals.\textsuperscript{6,7} In our model of cerebral ischemia, however, modification of leukotriene production improved outcome but did not change the degree of granulocyte accumulation.\textsuperscript{2,8} This result might be explained on the basis of impaired function of the accumulating leukocytes, but it also suggests that leukocytes are not critical in the production of injury after cerebral ischemia. Our present experiment was designed to test directly the hypothesis that leukocytes do participate in the process of ischemic neuronal injury.

Dogs with severe neutropenia induced by mechlorethamine were subjected to 1 hour of controlled ischemia induced by air embolism. Cortical somatosensory evoked response (CSER) amplitudes 1 hour after the end of ischemia were compared with those in dogs that had received mechlorethamine but had not yet developed leukopenia and dogs that had received no mechlorethamine.

Materials and Methods

The experimental protocol was reviewed by the Institutional Animal Use Committee and certified as conforming to the Department of Health and Human Services' "Guide for the Care and Use of Laboratory Animals" from the Institute of Laboratory Animal Resources (National Research Council publication no. NIH 86-23, 1985). We used 26 conditioned male mongrel dogs weighing 9–12 kg. The granulocytopenic group consisted of nine dogs that received 1.5 mg/kg mechlorethamine (nitrogen mustard) 96 hours before the induction of anesthesia for experimental ischemia; the injections were timed so...
Mechlorethamine was prepared as a 1-mg/ml solution in saline within 30 minutes of injection, along with a 6 M thiosulfate solution for use in case of a spill. The dogs were restrained, and 16-gauge forepaw venous catheters were inserted after skin preparation with Betadine. The mechlorethamine was injected after the collection of 3 ml blood for the hemogram and was followed by 150 ml of 0.9% saline. After removal of the catheters, the dogs were returned to a quarantined run and each day after injection received a combination of 33,000 units/kg penicillin G, 66,000 units/kg benzathine penicillin, and 11 mg/kg gentamycin subcutaneously. In preliminary trials of treatment with mechlorethamine, two dogs developed cardiovascular collapse immediately on induction of anesthesia, apparently due to dehydration. Therefore, we estimated the fluid loss in the dogs by their change in body weight and replaced the fluid lost with lactated Ringer's solution as follows: 250–300 ml subcutaneously 48 hours after mechlorethamine, 600 ml 72 and 84 hours after mechlorethamine, and 15 ml/kg just before the induction of anesthesia. The mechlorethamine control dogs began receiving antibiotics 72 hours before the induction of anesthesia, and they received 300 ml Ringer's lactate 48 hours before, 600 ml 12 hours before, and 15 ml/kg just before the induction of anesthesia. These mechlorethamine control dogs received the same dose of mechlorethamine as the granulocytopenic dogs 24 hours before the induction of anesthesia and were maintained without oral intake after the injection of mechlorethamine. The methodologic control group received no antibiotics or fluid replacement. The leukocyte counts, platelet counts, and differential counts were done manually with a hemocytometer and Wright-stained smears.

On the experimental day all dogs were tranquilized with 1.1 mg/kg xylazine and 0.05 mg/kg atropine subcutaneously, and 16-gauge forepaw venous catheters were inserted in each. Anesthesia was induced with 80 mg/kg i.v. α-chloralose and maintained with 20 mg/kg i.v. α-chloralose every 20 minutes. The dogs were intubated and mechanically ventilated. End-tidal CO₂ was monitored continuously and maintained between 3.8% and 4.2%, and arterial blood gases were obtained at regular intervals.

In granulocytopenic dogs all skin incision sites were prepared with Betadine, and sterile instruments were used for surgery. Both femoral arteries and veins were cannulated with PE-260 polyethylene catheters, and a PE-50 catheter was inserted into the right internal carotid artery for the injection of air. Mean arterial blood pressures and pulmonary artery pressures were measured continuously. Rectal temperatures were continuously monitored and were maintained at 37.5±0.5°C with heating pads.

A stainless steel screw was fastened into the outer table of the skull over the somatosensory cortex, and a second screw was placed in the nasal bones. The response to electrical stimulation of the left median nerve was averaged using a Nicolet CA 1000 evoked response system (Madison, Wisconsin) with a bandpass of 30–3000 Hz. The average amplitude of the P1–N1 peak in five recordings obtained before the injection of air was used as a baseline.

After obtaining baseline CSERs, blood pressures, hematocrits, and arterial blood gases, the 1-hour ischemic period began with the injection of 50 μl air into the right internal carotid artery. A CSER was obtained immediately following the air injection and approximately every 1.5 minutes for the next 60 minutes. Further injections of air were given as necessary to maintain the CSER below 15% of the baseline amplitude. The timing and amount of air injected were determined by the following protocol: 1) two CSERs were obtained after each bolus of air, before further injections were made; 2) if the second CSER after the first 50 μl air was >50% of baseline, the dog received 40 μl air; if 15–50% the dog received 20 μl air; if <15% no air was given; 3) 20 μl air was given whenever the CSER recovered to 15% of baseline; 4) if the 20-μl bolus of air failed to suppress the CSER to <15% of baseline, 40 μl air was injected. Two granulocytopenic dogs were eliminated from further data analysis at this stage because the CSER amplitude could not be sufficiently reduced despite injection of 700–1000 μl air.

The granulocytopenic and mechlorethamine control dogs were maintained under anesthesia for 1 hour (recovery period) after the end of the 1-hour ischemic period, and CSERs were obtained every 10 minutes. At the end of the recovery period, 60 μCi/kg of [14C]iodoantipyrine (56.1 mCi/mmol; New England Nuclear, Boston, Massachusetts) was injected for the autoradiographic determination of regional cerebral blood flow as previously described.10 One dog in the mechlorethamine control group was eliminated from further data analysis because the autoradiogram contrast was inadequate despite reincubation of the sections.

Data were analyzed using one- and two-way analyses of variance (ANOVA), the Fmax test for homogeneity of variances, and Wilcoxon's rank sum test where appropriate.11 Fisher's exact test of proportions was used to compare the number of dogs in each group with areas of low blood flow. Results were declared significant when p<0.05. All data are reported as mean±SEM.

Results

Mechlorethamine produced severe neutropenia 4 days after administration without affecting platelet...
TABLE 1. Hematologic Parameters Before Injection of Air Into Dogs to Induce Cerebral Ischemia

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Leukocytes/mm³</th>
<th>Neutrophils/mm³</th>
<th>Platelets/mm³</th>
<th>Hematocrit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechlorethamine</td>
<td>6</td>
<td>7,666±1,507</td>
<td>6,791±1,273</td>
<td>178,200±24,000</td>
<td>40±1</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methodologic</td>
<td>11</td>
<td>13,600±1,400</td>
<td>7,466±1,265</td>
<td>198,000±12,000</td>
<td>40±6</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granulocytopenic</td>
<td>7</td>
<td>638±126*</td>
<td>606±100*</td>
<td>230,000±21,000</td>
<td>37±2</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

*Significantly different from each control group by Wilcoxon's rank sum test.

counts or hematocrit, which were not different from those in either control group (Table 1). Leukocyte counts and differential counts obtained before the injection of mechlorethamine were within the normal ranges for canines. The mechlorethamine control group had a marked reduction of lymphocytes when subjected to ischemia (total lymphocyte count on Day 1 4524±326, on Day 2 391±80).

The average CSER amplitude at 10-minute intervals during the ischemic and recovery periods are shown in Figure 1. The values are expressed as percent of baseline amplitudes for each dog, and the control values are the average of those in the mechlorethamine and methodologic control groups (there was no difference in the CSERs during the ischemic or recovery periods in these two control groups). A repeated-measures two-way ANOVA revealed a highly significant improvement in CSER amplitude in the granulocytopenic group throughout the recovery period, with no significant effect of time. Repeated-measures two-way ANOVA also revealed significant differences between the granulocytopenic group and each control group tested individually. The recovery at the end of the recovery period was 28.1±3.4% in the granulocytopenic group (n=7), 15.0±3.9% in the mechlorethamine control group (n=6), and 17.5±3.0% in the methodologic control group (n=11). The granulocytopenic group had recovered significantly better (Wilcoxon's rank sum test) than either control group (p<0.05) or the combined control groups (16.5±2.3%, p<0.01). The lack of a significant effect of time suggests that the improved recovery began soon after the end of ischemia. Repeated rank sum tests confirmed significant differences after 20, 30, 40, and 50 minutes of recovery.

The severity of the ischemic insult was compared between groups using several parameters. Each dog had approximately 45 CSERs measured during the ischemic period; the amplitudes of 61±5.8% of these CSERs were <15% of baseline amplitude in the granulocytopenic group compared with 64±4.4% in the combined control groups. CSER amplitude at the end of the ischemic period was 12.7±1.8% of baseline amplitude in the granulocytopenic group and 11.9±1.9% in the combined control groups. The granulocytopenic group required 268±25 µl air for CSER amplitude suppression, while the combined control groups required 240±20 µl. No significant difference between groups existed in any of these parameters (Wilcoxon's rank sum test). There was a difference between groups in the percent of CSERs with amplitudes below 10% of baseline amplitude during the ischemic period (18.6±3.7% in the granulocytopenic group vs. 36±4% in the combined control groups).

Mean arterial blood pressure, heart rate, and arterial blood gases before the injection of air and during the ischemic and recovery periods for the granulocytopenic and combined control groups are presented in Table 2. Differences between groups in arterial pH and PaO₂ before the injection of air were statistically, but not physiologically, significant.

Table 3 presents mean blood flows for the granulocytopenic and mechlorethamine control groups subdivided into left and right cortical and white matter regions. The methodologic control group was followed for 4 hours after ischemia to allow time for platelet accumulation; blood flow data at 1 hour is therefore not available for this group. Blood flow from brain gray matter is an unweighted average of seven cortical sites throughout the hemispheres. White matter flow is an average of those in five areas. The Fₚ₀₉₀ test indicated that the raw blood flow data was significantly heteroscedastic. Therefore, we transformed the data by taking the loga-
TABLE 2. Physiologic Parameters at Three Times During Experiment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Combined control group (n=17)</th>
<th>Granulocytopenic group (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (min⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before air injection</td>
<td>103±4</td>
<td>97±8</td>
</tr>
<tr>
<td>Ischemia</td>
<td>110±5</td>
<td>106±11</td>
</tr>
<tr>
<td>Recovery</td>
<td>116±6</td>
<td>117±12</td>
</tr>
<tr>
<td>Mean arterial blood pressure (mm Hg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before air injection</td>
<td>128±5</td>
<td>116±7</td>
</tr>
<tr>
<td>Ischemia</td>
<td>115±4</td>
<td>118±7</td>
</tr>
<tr>
<td>Recovery</td>
<td>121±4</td>
<td>116±6</td>
</tr>
<tr>
<td>Arterial pH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before air injection</td>
<td>7.36±0.01</td>
<td>7.43±0.01*</td>
</tr>
<tr>
<td>Ischemia</td>
<td>7.35±0.01</td>
<td>7.39±0.02</td>
</tr>
<tr>
<td>Recovery</td>
<td>7.35±0.01</td>
<td>7.38±0.01</td>
</tr>
<tr>
<td>PaO₂ (mm Hg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before air injection</td>
<td>37±0.6</td>
<td>36±1.3</td>
</tr>
<tr>
<td>Ischemia</td>
<td>36±0.7</td>
<td>36±1.2</td>
</tr>
<tr>
<td>Recovery</td>
<td>34±0.7</td>
<td>36±1.1</td>
</tr>
<tr>
<td>PaO₂ (mm Hg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before air injection</td>
<td>89±1</td>
<td>97±3*</td>
</tr>
<tr>
<td>Ischemia</td>
<td>92±1</td>
<td>97±3</td>
</tr>
<tr>
<td>Recovery</td>
<td>95±2</td>
<td>99±1</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Combined control groups, mechlorethamine controls plus methodologic controls.

*Significantly different from combined control groups by Wilcoxon's rank sum test.

Aithm of each value before application of a t test. No significant differences were found.

The number of dogs in each group that had cortical blood flows of <15 ml/100 g/min and white matter blood flows of <6 ml/100 g/min was determined. Cerebral blood flow below these levels is usually associated with irreversible cellular damage.9 None of the seven granulocytopenic dogs had flows in this severely ischemic range, but three of the five mechlorethamine control dogs had one to three areas each of very low flows. These proportions are significantly different by Fisher's exact test.

TABLE 3. Average Blood Flows From Seven Cortical and Five White Matter Areas 1 Hour After 1 Hour of Cerebral Ischemia Induced by Injection of Air Into Dogs

<table>
<thead>
<tr>
<th>Area</th>
<th>Mechlorethamine control group (n=5)</th>
<th>Granulocytopenic group (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortical Right</td>
<td>33.6±17.2</td>
<td>66.8±18.1</td>
</tr>
<tr>
<td>Left</td>
<td>35.2±14.2</td>
<td>62.1±16.9</td>
</tr>
<tr>
<td>White matter Right</td>
<td>15.4±4.3</td>
<td>15.4±2.4</td>
</tr>
<tr>
<td>Left</td>
<td>15.5±3.2</td>
<td>14.1±2.4</td>
</tr>
</tbody>
</table>

Values are mean±SEM ml/100 g/min.

Discussion

We find that severe granulocytopenia improved outcome after cerebral ischemia produced by air embolism in dogs. The improved outcome in CSER was associated with the elimination of areas of very low blood flow after 1 hour of recovery in the granulocytopenic group, in contrast to the occurrence of these low-flow areas in a large proportion of the mechlorethamine control dogs.

In previous studies, we have demonstrated that air embolism produces a transient, but severe, reduction in blood flow in cortical areas that generate the somatosensory evoked response.12-9,10 The CSER amplitude decreases with increasing ischemia.12-14 The injection of air to maintain the CSER amplitude at 10-15% of baseline amplitude for 1 hour is therefore equivalent to maintaining the population of cells that is used to assess outcome in a critically ischemic state. Variability in collateral circulation can cause regional variation in cerebral blood flow in models of arterial ligation.15 Our model avoids the problem of electrically sampling insufficiently ischemic neurons, and a difference in outcome measured by CSER amplitude has been accepted as evidence of efficacy in controlled settings.16

We chose mechlorethamine to induce granulocytopenia because we found that hydroxyurea produced immediate and severe anemia in dogs. Mechlorethamine is very rapidly degraded by contact with body fluids so it is unlikely to have a pharmacologic effect 4 days after injection, and effects on hepatic function are minimal after a single dose.17 However, mechlorethamine produces lymphocyte depletion within 24 hours. The similarity in CSER outcome between the mechlorethamine and the methodologic control groups therefore suggests that the observed beneficial effect on recovery in the granulocytopenic group is due primarily to a lack of granulocytes.

Granulocytopenia may have increased the resistance of the dogs to air embolism. In two granulocytopenic dogs, injection of three times the usual amount of air failed to affect the CSER, and these dogs were not studied further. The granulocytopenic group had fewer CSERs during ischemia with amplitudes below 10% of baseline amplitude than the combined control groups, despite receiving as much or more air. The granulocytopenic group had just as many CSERs below the 15% threshold established for repeated air injection during ischemia as the combined control groups, thus suggesting that the control dogs were more sensitive to each air bolus, their CSER amplitudes often decreasing below 10% of baseline with 20-μl air boluses.

The combined control group dogs had slightly lower PaO₂ throughout the experiment than the granulocytopenic dogs. Intubation and mechanical ventilation alone causes accumulation of leukocytes in the lungs of experimental dogs,18 and leukocyte...
accumulation in pathologic lung conditions impairs oxygenation. Further granulocyte accumulation in the lungs might occur because of activation of leukocytes by the tissue damage incurred during surgery. It is therefore possible that the observed difference is due to leukopenia, but the hemoglobin oxygen saturation was well above the range in which worsening of ischemic damage would occur. The mild alkalosis before injection of air in the granulocytopenic group was corrected by the end of the experiment and might have been due to vomiting on Days 2 and 3 after mechlorethamine injection.

There is some evidence that leukocytes are essential to the production of lung damage by air embolism; leukocytes have been seen adhering to the blood–bubble interface under electron microscopy. The adhesion of leukocytes to the air bubble might have the effect of increasing the size of the plug formed by the embolus and possibly increasing the adhesion of the embolus to the vessel wall. We cannot, therefore, be certain that leukocyte depletion would similarly influence other forms of ischemia. Postischemic administration of antineutrophil serum, however, has been shown to reduce myocardial damage.

We found a significant reduction in the number of dogs with areas of very low blood flow in the granulocytopenic group, suggesting that leukocytes damage ischemic tissue by decreasing microcirculatory blood flow. Granulocytes are relatively large and stiff and, therefore, may mechanically block capillaries in areas of low blood flow. This has been shown to occur in muscle capillaries following hypotension and in myocardial ischemia. Leukocyte depletion improves blood flow in the gastric mucosa following shock and in the heart muscle after ischemia. The progressive increase in cerebrovascular resistance that occurs when the isolated dog brain is perfused with whole blood is prevented by removing the buffy coat. The formation of aggregates and adhesion to endothelium by activated leukocytes will impair blood flow further. There is evidence that leukocytes in peripheral blood are less filterable after stroke in humans, suggesting that activation occurs. Activated leukocytes can also synthesize and release thromboxane and PAF-acether, both of which cause vasoconstriction and activate platelet aggregation. The removal of leukocytes reduces platelet accumulation in models of inflammation and myocardial ischemia. Chemo- tactic and vasoconstrictive leukotrienes are also produced by activated leukocytes.

Severe leukocyte depletion cannot be regarded as a possible treatment for stroke. Our current results, however, do indicate a role for leukocytes in the mediation of postischemic injury and suggest that treatments that modify the accumulation or activity of leukocytes may be useful in ameliorating the effects of cerebral ischemia.

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References


**Key Words** • agranulocytosis • embolism • cerebral ischemia • dogs
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