Monoclonal Leukocyte Antibody Does Not Decrease the Injury of Transient Focal Cerebral Ischemia in Cats

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Background and Purpose: We tested the hypothesis that inhibition of leukocyte function by administration of monoclonal antibody 60.3 (MoAb 60.3) improves electrophysiological recovery and decreases injury volume following transient focal cerebral ischemia in cats.

Methods: Halothane-anesthetized cats underwent 90 minutes of left middle cerebral artery and bilateral common carotid artery occlusion followed by 180 minutes of reperfusion. Cats were assigned to receive either 2 mg/kg MoAb 60.3 (n=8) directed at the CDw18 leukocyte antigen complex or an equal volume of diluent (sterile saline; n=10) at 45 minutes of ischemia in a blinded fashion.

Results: Blood flow to the left temporoparietal cortex decreased to <5 ml/min/100 g with ischemia, but was minimally affected on the right side. Postischemic hyperemia occurred in the left caudate nucleus, whereas blood flow in other brain regions returned to control. No region demonstrated delayed hypoperfusion, and there were no differences between groups. Somatic sensory evoked potential recorded over the left cortex was ablated during ischemia and recovered to <10% of baseline amplitude at 180 minutes of reperfusion in both groups. Left hemispheric injury volume, as assessed by 2,3,5-triphenyltetrazolium chloride staining, was not affected by drug treatment (mean±SE values: MoAb 60.3, 37±5%; placebo, 38±7% of hemisphere).

Conclusions: Inhibition of leukocyte function with MoAb 60.3 does not afford protection from severe focal ischemia and reperfusion in cats. (Stroke 1992;23:247-252)

Leukocyte accumulation can occur in brain regions with low blood flow produced by air embolism. During ischemia, activation of leukocytes may result in release of chemotaxis factors. During reperfusion, interaction of leukocytes with platelets may result in metabolism of arachidonic acid through lipoxygenase and cyclooxygenase pathways producing prostanoids and oxygen radicals. Leukocyte depletion before transient forebrain ischemia leads to amelioration of hypoperfusion during reperfusion. However, administration of antineutrophil serum at the time of reperfusion is not associated with improved postischemic blood flow in rats or improved neurological recovery in dogs, despite severe leukopenia. Nevertheless, the small number of remaining leukocytes may be sufficient to contribute to ischemia-reperfusion injury in the brain.

An alternative strategy successfully used for evaluating leukocyte involvement in ischemia and reperfusion injury in the spinal cord as well as in other organs such as intestine, skin, and heart involves the administration of an antibody that inhibits leukocyte function. The human leukocyte differentiation antigen Mo1 (CD11b/CD18) is a glycoprotein expressed on the plasma membrane of neutrophils and monocytes, but is not normally present in T and B lymphocytes. Patients deficient in this antigen have leukocytes which are defective in adherence-dependent function, and, therefore, these patients suffer from recurrent bacterial infections. Monoclonal antibody 60.3 (MoAb 60.3) binds to the specific membrane-associated glycoprotein (β-chain of CD18 complex) and thereby inhibits neutrophil aggregation, adhesion, and spreading on natural and artificial substrates, and chemotaxis in vitro. The finding that
neurological recovery is improved by administration of CD18 antibody before or at 30 minutes of reperfusion from transient spinal cord ischemia suggests that the CD18 glycoprotein complex may be involved in leukocyte adhesion in the cerebral circulation exposed to ischemia and reperfusion.

We tested the hypothesis that inhibiting neutrophil function during reperfusion with MoAb 60.3 results in decreased volume of cerebral injury and improved evoked potential recovery after transient focal cerebral ischemia in cats. Regional cerebral blood flow (CBF) was measured during ischemia and reperfusion to determine if blood flow changes could account for any beneficial effect detected.

Materials and Methods

Eighteen female adult cats weighing 2.7–3.9 kg were studied. Anesthesia was induced with halothane in oxygen with spontaneous ventilation. After induction of anesthesia, cats were orally intubated and mechanically ventilated (model 661, Harvard Apparatus, South Natick, Mass.) to maintain arterial partial pressure of carbon dioxide (Paco 2) at approximately 35–40 mm Hg. Anesthesia was maintained with halothane (0.5–1.5%) in oxygen-enriched air. Halothane concentration was increased for signs of cardiovascular stimulation during surgery, but was not changed throughout the experimental protocol.

Catheters were placed in both femoral veins for infusion of fluids (lactated Ringer's solution) and drugs and in the descending aorta for arterial blood pressure recording, arterial blood gas sampling, and for withdrawal of reference blood samples during injection of radiolabeled microspheres. A catheter placed in the left atrium through a left thoracotomy was used for injection of radiolabeled microspheres. A catheter placed in the left atrium through a left thoracotomy was used for injection of radiolabeled microspheres. Moistened ligatures were loosely applied to both common carotid arteries for later occlusion.

The cat was turned prone and its head positioned in a stereotaxic frame approximately 4 cm higher than its heart. A thermistor placed in the right temporal epidural space acted as an estimate of brain temperature. Epidural temperature was maintained at 38.0±0.5°C using a heating pad and a heating lamp. Halothane concentration was increased for signs of cardiovascular stimulation during surgery, but was not changed throughout the experimental protocol.

At the end of the experiment, the animal was killed with potassium chloride. The brain was removed, and coronal sections 2.5 mm thick were cut immediately. The slices were then incubated in a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) for 40 minutes at 37°C. Regions that failed to demonstrate dark red staining with TTC were considered to represent areas of brain injury. Each slice was photographed in color, and the area of injury was determined on both surfaces of 12 sections by digital planimetry. Injury volume was calculated by averaging the anterior and posterior infarction areas, multiplying by section thickness, and summing these volumes over the entire hemisphere. Caudate injury volume was calculated separately. The slices of brain were placed in 10% buffered formalin for 1–2 days.

Ipsilateral and contralateral temporal and parietal lobe slices were subsequently sectioned into three cortical gray matter regions (temporal, temporoparietal, and parietal) and one white matter region. Brain tissue specimens were counted in a Packard multichannel autogamma scintillation spectrometer (Model 5310, Downers Grove, Ill.). The energy levels of the window settings for the six isotopes were as follows: 153Gd, 68–170; 114mIn, 174–230; 115Sn, 360–440; 103mRu, 450–560; 95Nb, 690–820; 46Sc, 830–1200 KeV. The overlap of activity among isotopes was corrected by differential spectroscopy, and blood flow was calculated by the reference sample technique.

Somatosensory evoked potentials (SEPs) were measured using a multichannel signal averager (Nicolet Med-80, Nicolet Instruments Corp., Madison, Wis.). After the scalp, skin, and musculature were dissected laterally, silver ball electrodes with shielded cables were placed into the holes drilled approximately 1 cm lateral to the midline and just posterior to the coronal suture on both sides of the cranium. A reference electrode was placed in the nose. Another needle electrode was inserted into the muscle over the second cervical vertebra to ensure that peripheral nerve transmission to the spinal cord remained intact. Stimulatory needle electrodes were
placed percutaneously on the volar surface of both forelegs. A needle electrode ground was secured to the animal's tongue. Stimuli at a voltage of twice motor threshold and duration of 150 μsec were delivered at a rate of 5.9/sec. Bandpass filters were set at 30–1,500 Hz cutoff frequencies, and 256 evoked responses were averaged. The amplitude of the first negative wave occurring at approximately 12–14 msec was measured.

Each cat was randomized to receive either placebo (n=10) or MoAb 60.3 (n=8). Investigators remained blinded to treatment group until data from all cats in the study were analyzed. The microvascular clip was placed on the left MCA and SEPs were recorded to document a decrease in amplitude of the major cortical negative wave to <25% of preischemic value. Cats (n=3) were excluded from the study if SEP amplitude did not decrease below this level by 5 minutes of occlusion. The ligatures around the common carotid arteries were tightened when adequate reduction of SEP amplitude was documented. At 40 minutes of left MCA occlusion and bilateral common carotid artery ligation, 2 ml placebo (phosphate buffered saline; pH 7.4) or MoAb 60.3 (2 mg/kg in saline) was administered over 10 minutes by continuous intravenous infusion. After 90 minutes of ischemia, the carotid ligatures were loosened and the microvascular clip on the MCA was removed. Reperfusion lasted 180 minutes. Evoked potentials were measured before ischemia, at 5, 15, 30, 60, and 90 minutes of ischemia, and at 15, 30, 60, 90, 120, and 180 minutes of reperfusion. Arterial blood gas and CBF measurements were made before ischemia, at 30 and 90 minutes of ischemia, and at 10, 60, and 180 minutes of reperfusion.

Values are expressed as mean±SEM. Analysis between groups for physiological variables, blood flow, and SEP amplitude were made with planned orthogonal comparisons. To assess changes in blood flow over time within groups, paired t test with Bonferroni correction was performed. Differences in injury volumes between groups were determined with Student’s t test. Statistical differences were considered significant at p<0.05.

Results

There were no physiologically significant differences between groups for average blood gas data (pH 7.30–7.35; PacO₂ 38–44 mm Hg; PacO₂ 110–160 mm Hg), hemoglobin (9–12 g/dl), glucose (90–130 mg/dl), or mean arterial blood pressure (100–125 mm Hg) during 90 minutes ischemia and 180 minutes reperfusion. Circulating white blood cell count increased in both groups (placebo, from 3,565±1,088/cc to 15,334±6,823/cc; MoAb 60.3, from 4,251±2,002/cc to 17,065±4,309/cc) during ischemia, and there was no difference between groups.

During left MCA and bilateral common carotid artery occlusion, both placebo- and MoAb 60.3–treated cats had a sustained decrease in blood flow to all left-sided structures except the hippocampus (Figures 1 and 2). The greatest decrease was in the left temporoparietal cortex (Figure 1). At 10 minutes of reperfusion, hyperemia was demonstrated to the left caudate nucleus in both groups (placebo, 162±20% of preischemic values; MoAb 60.3, 176±24% of preischemic values) and left temporoparietal white matter in MoAb 60.3–treated cats (143±12% of preischemic values). No left-sided regions demonstrated delayed hypoperfusion at the time points measured. Furthermore, there were no differences between groups in blood flow to any region during either ischemia or reperfusion (Figures 1 and 2).

However, in placebo-treated but not MoAb 60.3–treated cats, there was a transient decrease in blood flow to all regions within the right hemisphere (e.g.,
FIGURE 2. Regional cerebral blood flow to subcortical structures ipsilateral to occlusion of the middle cerebral artery during control, 30 and 90 minutes of ischemia, and 10, 60, and 180 minutes of reperfusion in cats treated with either placebo or monoclonal antibody 60.3 (MoAb 60.3). Temporoparietal white matter blood flow is expressed as a percentage of control because absolute white matter blood flow values determined by microspheres in cats may be overestimated, whereas relative changes are accurate.11 Values are mean±SEM. *p≤0.05 as compared with preischemic values. Blood flow was not different between groups at any time in any region. n=10, placebo; n=8, MoAb 60.3.

right caudate; 68±9% of preischemic values at 30 minutes of occlusion) during left MCA and bilateral common carotid artery occlusion (Table 1). This difference in response between groups at 30 minutes of occlusion cannot be attributed to an effect of MoAb 60.3 because drug was not administered until 40 minutes of ischemia.

During ischemia, SEP amplitude over the left somatosensory cortex was suppressed to 0–8% of preischemic values in both groups. During reperfusion, return of SEP amplitude was minimal (placebo, 8±7%; MoAb 60.3, 6±4%), and there were no differences between groups. Evoked potentials remained isoelectric throughout reperfusion in seven of 10 placebo-treated cats and six of eight MoAb 60.3–treated cats. All cats had normal latency of the wave measured over the second cervical vertebra. Changes in SEP amplitude recorded from the right somatosensory cortex were not different between groups throughout the protocol. However, in both groups SEP amplitude decreased from preischemic values by 180 minutes of reperfusion (MoAb 60.3, 66±20% of preischemic values; placebo, 74±19%).

There were no differences between groups in the volume of injured left hemisphere (MoAb 60.3, 37±5%; placebo, 38±7% of hemisphere volume) or injured caudate nucleus (MoAb 60.3, 60±7%; placebo, 55±8% of left caudate volume). Furthermore, in each of the twelve coronal sections, there was no difference in the area of cortical injury between groups. There was a negative correlation between left hemisphere injury volume and percent recovery of SEP amplitude recorded over the left somatosensory cortex (MoAb 60.3, r=−0.70, p≤0.002; placebo, r=−0.72, p≤0.001). No animal in either group demonstrated injury in the right hemisphere or right caudate nucleus.

Discussion

Cats exposed to 90 minutes of MCA and bilateral common carotid artery ligation followed by 180 minutes of reperfusion were found to have poor recovery of SEP amplitude and large volumes of neuronal injury in the left hemisphere and caudate nucleus. There were no differences between groups in any physiological variable measured or in the increase in leukocyte number in response to ischemia. Administration of MoAb 60.3 did not alter the distribution of blood flow reduction to regions ipsilateral to MCA occlusion, but administration was associated with fewer regions with reduced blood flow to regions contralateral to MCA occlusion. Both groups were found to have postischemic hyperemia to the ipsilateral caudate nucleus, but delayed hypoperfusion was not demonstrated in any region in either group. There was also no difference between groups for recovery of SEP amplitude or volume of cerebral injury measured in the left hemisphere or caudate nucleus. Our data, therefore, do not support the hypothesis that inhibition of neutrophil adherence with MoAb 60.3 results in decreased size of the injury or improved electrophysiological function after severe transient focal cerebral ischemia in cats.

Although it is possible that the lack of effect of MoAb 60.3 administration during transient focal cerebral ischemia is related to the dose or timing of administration, we believe that this is an unlikely explanation. The dosage of MoAb 60.3 was based on previous studies in the spinal cord5,6 and other organ systems7,8 that demonstrated therapeutic efficacy for antibody directed at the leukocyte glycoprotein complex CD18 either before or following ischemia and reperfusion. In cats, intra-arterial administration of 2...
mg/kg MoAb 60.3 before the onset of intestinal ischemia attenuated the increased microvascular permeability normally induced with ischemia and reperfusion.7 In rabbits, a similar dose of MoAb 60.3 was associated with elevation of antibody levels necessary to attenuate leukocyte migration for >24 hours.14 In addition, saturation of CD18 binding sites was 99.5% within 5 minutes of intravenous injection and remained >70% saturated at 24 hours after injection.14

It is possible that CD18-mediated leukocyte adhesion may not occur in the central nervous system. We think that this is an unlikely explanation for our data because leukocytes accumulate in brain regions with low blood flow produced by air embolism.1,2 leukocyte depletion before transient forebrain ischemia results in amelioration of postischemic hypoperfusion,3 and treatment with antibody directed at the CD18 glycoprotein complex before1 or at 30 minutes of reperfusion from transient spinal cord ischemia6 results in improved neurological outcome. Antibody to CD18 glycoprotein complex also inhibits recruitment of leukocytes across the blood–brain barrier in response to acute inflammatory stimuli of bacterial origin.21

In our experiments, MoAb 60.3 was administered during the time of ischemia, which may have limited access to leukocytes activated in the ischemic core. However, because administration of antibody to CD-18, even at 30 minutes of reperfusion from spinal cord ischemia, is associated with improved recovery of blood flow and neurological function,5 we believe that administration of an antibody during ischemia (before reperfusion) should have therapeutic benefit. However, neutrophil adherence to endothelium can be induced with thrombin, leukotriene C4, interleukin-1, and tumor necrosis factor by a time-dependent mechanism involving endothelium independent of neutrophil CD11/CD18 complex.24,25 Thus, we cannot totally exclude neutrophil involvement through a non-CD11/CD18 mechanism or neutrophil involvement at a longer time of reperfusion.

The role of circulating leukocytes in mediating cerebral injury following transient ischemia is in question. In rats, although delayed hypoperfusion following incomplete global cerebral ischemia is attenuated by administration of anti-neutrophil serum before ischemia,3 it was not attenuated when administered shortly after reperfusion despite a rapid decrease in circulating leukocytes.3 Dogs treated with anti-neutrophil serum before transient complete ischemia were actually found to have a decreased survival during the first 24 hours of reperfusion, and there was no improvement in neurological outcome in survivors.4 Other therapeutic modalities (e.g., indomethacin) that attenuate postischemic hypoperfusion are also not associated with a reduction in leukocyte accumulation in the brain during reperfusion.2

More recently, mononuclear phagocytes have been hypothesized to play an important role in mediating postischemic injury in the spinal cord.26 For example, when chloroquine and colchicine are administered up to 6 hours after transient spinal cord ischemia to decrease activity of mononuclear phagocytes,27 there is decreased accumulation of mononuclear phagocytes found within damaged gray matter and improved recovery of neurological function.28 Although MoAb 60.3 would be expected to inhibit monocyte function,28 the antibody may not cross the blood–brain barrier and, therefore, may not inhibit microglia function.

During MCA and bilateral carotid occlusion, the degree and extent of reduction of regional CBF was similar to previous reports from our laboratory using the same model of focal ischemia but with a different anesthetic.19,29 However, the percent reduction in SEP amplitude during ischemia was greater in this study with halothane-anesthetized cats as compared with our previous study with fentanyl/N2O-anesthetized cats.29 The lack of therapeutic benefit for MoAb 60.3 in this study may be because the current insult is too severe.

Areas of brain destined for injury as indicated by TTC had blood flow values of <10 ml/min/100 g during ischemia. Consistent with our previous observations39 is the finding that at 180 minutes of reperfusion, blood flow to areas that did not subsequently

<table>
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<th>Brain region</th>
<th>Preischemia</th>
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<th>Ischemia (min) 90</th>
<th>Reperfusion (min) 10</th>
<th>Reperfusion (min) 60</th>
<th>Reperfusion (min) 180</th>
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<tr>
<td>Caudate</td>
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<td>92±10*</td>
<td>96±13*</td>
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<td>86±30</td>
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Values are mean±SEM. Flows are expressed as ml/min/100 g. *p<0.05 compared to preischemic value. There were no differences between groups at any time in any region. MoAb 60.3, monoclonal antibody 60.3.
stain with TTC was well above values normally considered to be the ischemic threshold. Because these regions of brain lack metabolism (as indicated by no TTC staining and an isoelectric SEP) but have continued blood flow, interpretation of other experimental protocols based solely on blood flow may be misleading.

In conclusion, we found that treatment with MoAb 60.3 during transient focal cerebral ischemia produced by combined left MCA and bilateral common carotid artery occlusion in cats does not ameliorate brain injury as measured by TTC staining or SEP amplitude. These data would also not support a role for antibody to leukocyte glycoprotein complex CD18 to prevent reperfusion injury after thrombotic therapy.

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Key Words • cerebral blood flow • evoked potentials • leukocytes • cats
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