Reduction of Central Nervous System Ischemic Injury in Rabbits Using Leukocyte Adhesion Antibody Treatment

Wayne M. Clark, MD; Ken P. Madden, MD, PhD; Robert Rothlein, PhD; and Justin A. Zivin, MD, PhD

Activated leukocytes appear to be directly involved in ischemic central nervous system injury. A surface glycoprotein (CD18) on the leukocyte is required for endothelial adherence and subsequent function and can be blocked with leukocyte adhesion antibody treatment. We used two animal models to determine the efficacy of anti-CD18 antibody treatment in preserving neurologic function after central nervous system ischemia. We gave a dose of 1 mg/kg anti-CD18 to treatment rabbits 30 minutes before inducing irreversible ischemia in the brain with intraarterial microspheres or in the spinal cord using reversible aortic occlusion. Treatment with anti-CD18 produced a significant reduction in neurologic deficits in the reversible spinal cord model, but not in the irreversible microsphere model. This protective effect supports the active role of leukocytes in central nervous system reperfusion ischemic injury and offers potential for future therapy. (Stroke 1991;22:877–883)

The appearance of leukocytes in injured ischemic tissue traditionally has been believed to represent a pathophysiologic response to existing injury. Recent evidence indicates they also may be important in the pathogenesis and extension of ischemic injury.1–4 Two proposed mechanisms of leukocyte potentiation of ischemia are 1) microvascular occlusion from direct mechanical obstruction and cytotoxic effects on the endothelium, and 2) central nervous system (CNS) tissue infiltration and neuronal cytotoxic injury.5–7 The leukocyte-mediated tissue damage may be irreversible even if blood flow is restored. Leukocyte adhesion is an early step in both of these mechanisms.

Recent studies have identified a specific leukocyte membrane glycoprotein complex (CD18) that is critically important for adherence. Monoclonal antibodies (anti-CD18) to these glycoproteins have been shown to inhibit adherence-dependent leukocyte functions in vitro.8,9 In vivo studies using anti-CD18 have shown a reduction of ischemic injury and decreased leukocyte tissue infiltration in heart, lung, intestinal, and systemic shock models.10–14 These data indicate that leukocyte endothelial adhesion is probably a critical early event in the process leading to neutrophil-mediated tissue reperfusion injury. To date, there have been no published studies using anti-CD18 to reduce CNS ischemic injury. It is not known how the blood–brain barrier influences leukocyte adhesion and migration or if it would alter anti-CD18 effects. Because neutrophil infiltration is present early in CNS ischemic injury,15–17 it is anticipated that leukocytes also will have a critical role in CNS ischemic injury. To test this hypothesis, we evaluated the therapeutic efficacy of anti-CD18 in two models of selective CNS ischemia in rabbits. These models were chosen to compare the treatment efficacy of anti-CD18 in a reperfusion model versus an irreversible microemboli model.

Materials and Methods

We used male New Zealand White rabbits weighing 2–3 kg. We selected the rabbit because of the high CD18 leukocyte density and because the majority of previous anti-CD18 studies have used this species. All animal procedures were approved by our Internal Animal Care Use Committee. The rabbit spinal cord ischemia model has been described previously in detail.18
We anesthetized rabbits with halothane and placed a snare ligature occluding device around the abdominal aorta just below the left renal artery, leaving the end of the occluder accessible through the skin. The rabbit was allowed to recover for a minimum of 2 hours. To induce ischemia, we tightened and clamped the occluder, making all rabbits completely paraplegic within 2 minutes. The animals showed no evidence of discomfort, and the procedure appeared to be painless, based on a lack of previously measured changes in heart rate, blood pressure, or circulating catecholamine levels. At the end of a variable predetermined occlusion period, the device was unclamped, removed, and the skin closed with surgical staples.

Animals were exposed to varying durations of ischemia. Those with shorter occlusion times tended to regain function, whereas those with longer occlusion durations remained permanently paraplegic. Each animal was scored as paraplegic (no hind limb movement) (0) or functional (normal/paretic) (1). We then observed the animals for 3 additional days for evidence of changes in neurologic function, performing manual expression daily and maintaining adequate hydration as necessary.

To induce irreversible CNS ischemia, we have devised previously a rabbit multiple cerebral embolism model using microspheres.19 The rabbit was anesthetized with halothane and, through a lateral neck incision, a 3-cm long 20-gauge catheter was implanted into the common carotid artery after the external carotid was ligated. The end of the catheter was left externally exposed and plugged with an injection cap. Microspheres (50 μm) were mixed (1:100 iodine-125 labeled to unlabeled spheres), suspended in acetone, and dried. For each rabbit, a variable weight of dried spheres was added to 100 μl 0.05% Tween in normal saline. The amount of radioactivity present was determined and the specific activity calculated. After the animals fully recovered from anesthesia, the microsphere suspension was carefully injected into the catheter. The animals showed no evidence of pain with the microsphere injection. Animals that received a small amount of microspheres remained essentially normal; those that received a large amount showed persistent behavioral abnormalities or death at 18 hours. An examiner blinded to group and amount of spheres observed the rabbits at 18 hours and scored them as grossly abnormal (hemiplegia or obtundation)/dead (0) or essentially normal (mild circling or nystagmus)/normal (1). The animals then were killed, the brains quickly removed and sectioned, and the radioactivity present measured in a gamma counter. From the specific activity of the injected mixture of microspheres and the amount of radioactivity recovered from each brain, we calculated the total weight of microspheres delivered to each brain. For each group, a range of microsphere amounts was used to generate an ischemia dose–response curve.

The anti-CD18 monoclonal antibody used in all of these experiments was a monoclonal mouse IgG1 immunoglobulin specific for the β unit of CD18 (a spontaneous class switch variant of R 3.3), which was a gift from Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, Conn.). This antibody binds to all leukocyte classes. It has been used previously in multiple experimental studies in other organs (dose, 1 mg/kg) and inhibits leukocyte adhesion for up to 18 hours.20 Thirty minutes before ischemia was induced, 1 mg/kg anti-CD18 in saline (5.5 mg/ml) was given as an intravenous bolus. Control animals received a similar injection of saline. We chose a 30-minute pretreatment period based on the cardiac studies

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<th>Ischemic duration (min)</th>
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For each duration of ischemia, the number of animals exhibiting each neurologic grade is shown.

Table 1. Efficacy of Leukocyte Adhesion Antibody Treatment in Rabbit Spinal Cord Ischemia Model
and because it allows time for maximal leukocyte binding.

For both the spinal cord ischemia model and the multiple cerebral embolism model, we constructed quantal dose–response curves from the neurologic function ratings at 18 hours. This analysis involves iterative fitting of a logistic function. The derivation of this type of analysis has been described in detail, and its utility as a pharmacologic screen has been demonstrated. For the spinal cord ischemia model, at each point on the abscissa, the percentage of paraplegic animals in the group is calculated and plotted as the ordinate. The total increases from 0% at the shortest duration of occlusion to 100% at the longest duration. The resulting sigmoid curve represents the effect of a range of durations on neurologic outcome. The point at which 50% of the animals are paraplegic (termed ET₅₀ for effective time) is calculated and is the average length of ischemia that produces impairment in 50% of the animals. An analogous calculation is made for the multiple cerebral embolism model. In this model, at each point on the abscissa, the percentage of abnormal animals is calculated and plotted as the ordinate. The total increases from 0% at the lowest weight of microspheres to 100% at the highest. The resulting sigmoid curve represents the effect of a range of weights on neurologic outcome. The point at which 50% of the animals are abnormal (termed ES₅₀ for effective stroke dose) is calculated and is the average length of ischemia that produces impairment in 50% of the animals. An agent effective in reducing neurologic damage will shift the curve (and ET₅₀ or ES₅₀) to the right; that is, the animals will, on average, tolerate a longer period of ischemia or greater amount of spheres. To determine significance, we performed t tests and considered a value of p<0.05 to be significant.

### Results

Table 1 shows the results of anti-CD18 treatment in the spinal cord ischemia model. The average length of ischemia that produced impairment (ET₅₀) in the treatment group (n=16) was 32.35±2.88 minutes (mean±SEM), and the ET₅₀ in the control group (n=16) was 22.88±1.90 minutes. This difference was significant at the p=0.02 level (t=2.45). Thus, pretreatment with anti-CD18 produced a significant reduction in ischemic injury in this model. Figure 1 shows the quantal dose–response curves for these groups. We observed the animals in our study for a

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**Table 2. Efficacy of Leukocyte Adhesion Antibody Treatment in Rabbit Multiple Cerebral Emboli Model**

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<th>Microsphere weight (mg)</th>
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<th>Abnormal or dead (n)</th>
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For each quantity of microspheres in the brain, number of animals exhibiting each neurologic grade is shown.
total of 4 days. During this time, none of the animals changed from their 18-hour category (normal/paretic [1] or plegic [0]). However, our binary scoring system may have missed a partial delayed deterioration.

Table 2 presents the results of anti-CD18 treatment in the multiple cerebral embolism model. The average weight of microspheres that produced impairment (ES₅₀) in the treatment group (n=19) was 0.52±0.16 mg, and the ES₅₀ in the control group (n=14) was 0.44±0.12 mg. This difference was not significant (t=0.40).

We performed histopathologic examination on randomly selected animals (spinal cord ischemia model only; n=6) (see Figure 2). A reduction in hypercellularity occurred in all of the anti-CD18-treated animals studied relative to untreated animals with similar ischemic durations.

Discussion
In this study, we found that leukocyte adhesion antibody treatment reduced CNS ischemic injury. We saw a significant protective effect in a model of large-vessel reperfusion (spinal cord ischemia model), but not in a model of irreversible microvascular occlusion (multiple cerebral embolism model). This difference in therapeutic efficacy may provide a clue to the mechanisms of leukocyte-mediated injury.
Leukocytes are relatively large cells with high viscoelastic properties and require considerable deformation on their passage through capillaries. When chemotactic substances activate them during ischemia, their cytoplasmic stiffness increases and they develop adhesion properties to the capillary endothelium. Under conditions of reduced perfusion pressures, they may be unable to traverse the capillary, leading to obstruction of the microcirculation. Data from white blood cell perfusion studies on isolated muscle and kidney preparations show that leukocytes alone may obstruct the microcirculation under low-flow states, increasing the resistance by 30%. This leukocyte capillary plugging also may be the major mechanism of the “no-reflow phenomenon.” This phenomenon, first described in the CNS by Ames et al., is defined as the incomplete restoration of normal blood flow after a period of ischemia. Areas of parenchyma that might be viable when blood flow returns are not adequately reperfused and ultimately die.

This microvascular obstruction also can be potentiated by damage to the endothelium. Leukocyte granule contents, reactive oxygen metabolites, and membrane phospholipases have been found to injure endothelium. By constricting capillary lumen and increasing leukocyte adhesion, these endothelial effects can potentiate low-flow states. Because reduced perfusion pressures may be seen both in the ischemic penumbra (static low flow) and during the initial phase of reperfusion (early reflow), treatment with leukocyte adhesion antibody could reduce injury by improving blood flow in both of these conditions. We chose the two ischemic models in this study to evaluate which of these possible leukocyte obstruction mechanisms (reduced flow to the penumbra or no reflow during reperfusion) is of prime importance.

Because the rabbit has poor spinal cord collaterals, the spinal cord ischemia model produces relatively little ischemic penumbra during the occlusion time (only at rostral edge). When the occlusion is released, there is reperfusion to a large ischemic area (maximal reflow with relatively little ischemic penumbra). In the multiple cerebral embolism model, the rabbit has excellent cerebral collaterals; therefore, this model produces multiple small areas of irreversible ischemia with surrounding areas of low blood flow (maximal penumbra with minimal reflow). Our finding that treatment with anti-CD18 reduced ischemic injury in the spinal cord ischemia model, but not in the multiple cerebral embolism model, suggests that decreased reperfusion may be the primary obstructive mechanism of acute leukocyte-mediated ischemic injury. This study used a pretreatment strategy and did not assess the efficacy of anti-CD18 in delayed “resuscitative” treatment. Further studies evaluating the efficacy of treatment initiated during the reperfusion phase are planned.

Although possible, it is unlikely that the differences in treatment efficacy found in these two models are due to differences in brain and spinal cord...
leukocyte properties because both areas of the CNS have similar “blood-brain barriers.” Vascular endothelium, meninges, and glia, and both have previously documented leukocyte infiltration.\textsuperscript{15-17} For the middle cerebral embolism model results, the possibility that we did not detect a real treatment effect because of inadequate sample size must be considered (Type II error). Based on the assumption that the observed 18% difference in ET\textsubscript{50} values between the treatment and control groups is real, if we had chosen to design the experiment to have a power of 90% and set the \( \alpha \) error level at the usual 5%, we would have needed 227 animals in each group to find a statistically significant difference.\textsuperscript{28} Although embolism models do have greater inherent variability, we previously have found the multiple cerebral embolism model to be a sensitive test of therapeutic efficacy. Using sample sizes comparable to this study, we have found significant treatment effects with various neuroprotective agents.\textsuperscript{19,22} Thus, it is unlikely that increasing our sample size within reasonable limits would have altered our conclusions.

An additional proposed mechanism of leukocyte potentiation of ischemia is direct CNS tissue infiltration and neuronal cytotoxic injury. This mechanism is felt to be involved in the delayed (days) deterioration seen after ischemic reperfusion (delayed reperfusion injury) that has been observed previously in the spinal cord ischemia model.\textsuperscript{18,20} Because the dose of anti-CD18 used in this study is felt to block adhesion for 12–18 hours, some inhibition of early leukocyte infiltration is possible, which may have contributed to the treatment effect. This is supported by our histopathologic study. However, a multiple-dose regime would be required to inhibit the period of maximal infiltration, 24–48 hours.\textsuperscript{30} Inhibition of leukocyte infiltration and delayed reperfusion injury recently has been produced in a variation of the spinal cord ischemia model using the nonspecific neutrophil inhibitor colchicine.\textsuperscript{16} Repeated treatments with anti-CD18 may be equally efficacious, with less potential for side effects.

We conclude that treatment with leukocyte adhesion antibody reduces CNS ischemic injury in a reperfusion model, but not in an irreversible occlusion model. These findings support the role of leukocytes as active participants in CNS injury, presumably through potentiating reperfusion injury. Leukocyte adhesion antibody may offer potential for future CNS ischemic therapy. Finding an agent that reduces CNS reperfusion injury would have significant clinical benefit, especially given the potential role of stroke thrombolytic therapy, with resulting reperfusion.

References


**KEY WORDS** • cell adhesion • cerebral ischemia • leukocytes • rabbits
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