Antibody to the α4 Integrin Decreases Infarct Size in Transient Focal Cerebral Ischemia in Rats

Kyra Becker, MD; Darin Kindrick, BS; Jane Relton, PhD; John Harlan, MD; Robert Winn, PhD

Background and Purpose—Inflammation, a process that involves neutrophils, lymphocytes, and monocytes, contributes to cerebral ischemic injury. Blockade of neutrophil adhesion to endothelium improves outcome after experimental stroke. In this study we sought to assess the contribution of lymphocytes and monocytes to ischemic brain injury.

Methods—Male Lewis rats underwent 3 hours of middle cerebral artery occlusion followed by 45 hours of reperfusion. Two hours after the onset of ischemia, one group of animals received an intraperitoneal injection of antibodies to the α4 integrin (n=16); another group was injected with an isotype control antibody (n=11). Neurological examination, body temperature, and body weight were assessed at different time points after stroke. Animals were killed 48 hours after the onset of ischemia for determination of infarct volume and leukocyte counts.

Results—There were no significant differences in body temperature or weight at any time. Neurological scores (deficits) were significantly less in animals treated with anti-α4 antibodies at 24 (2.0±1.2 versus 3.0±0.4; P=0.006) and 48 (2.0±1.2 versus 3.0±0.8; P=0.011) hours after ischemia. Peripheral blood leukocyte counts were significantly higher in anti-α4-treated animals (6.8±2.2×10⁷ versus 2.9±1.9×10⁷; P=0.001) and revealed a lymphocyte/monocyte predominance (86.0±16.2% versus 71.0±15.6%; P=0.008). Infarct volume was significantly less in animals treated with antibodies to α4 (120.1±51.21 versus 173.7±42.29 mm³; P=0.012).

Conclusions—These data support a role for lymphocytes and monocytes in cerebral ischemic injury and show that blockade of α4, even when instituted after the onset of ischemia, can improve neurological outcome and decrease infarct volume. (Stroke. 2001;32:206-211.)

Key Words: cell adhesion molecules • inflammation • integrins • lymphocytes • monocytes • stroke • rats

Inflammation is a stereotyped reaction of living tissue to injury and is the sum result of multiple biological processes, including capillary dilatation and leukocyte migration. In most tissues, the inflammatory response leads to edema, erythema, and an increase in temperature. In the brain, inflammation may be particularly detrimental because it can increase intracranial pressure due to increased blood flow and edema. Inflammation also causes fever, and fever early in the course of stroke is associated with poor outcome. Because anti-inflammatory agents improve outcome in experimental models of stroke, it appears that the inflammatory process plays a role in propagating ischemic brain injury. To date, attempts at controlling the postischemic inflammatory response in the brain have focused on the contribution of neutrophils to that response. Lymphocytes and monocytes, however, infiltrate the brain within 24 hours of stroke onset and secrete cytokines that worsen brain injury (ie, interleukin-1 and tumor necrosis factor). Lymphocytes and natural killer cells can also be cytotoxic. Little attention has been given to the therapeutic possibilities of manipulating the lymphocytic or monocytic response to reduce brain injury.

See Editorial Comment, page 211

Leukocyte trafficking to areas of inflammation depends on the complementary expression of adhesion molecules on circulating cells and the endothelium. Leukocytes express adhesion molecules known as integrins. Integrins are heterodimers composed of various combinations of α and β chains. Almost all leukocytes express CD11a/CD18 (leukocyte function–associated antigen-1 [LFA-1]) and CD11b/CD18 (Mac-1), which are integrins that contain a common β2 chain (CD18) and are thus known as β2 integrins. These integrins allow the cells to bind to endothelial intercellular adhesion molecule-1 (ICAM-1) and intercellular adhesion molecule-2 (ICAM-2) and migrate through the vessel. In addition to the β2 integrins, lymphocytes and monocytes express α4β1 (CD49d/CD29) and αβ7 (CD49d/CD103). Because these integrins contain a common α chain, they are referred to as αi integrins. CD49d/CD29, or αiβ1, is also known as very late activation antigen-4 (VLA-4), and CD49d/CD103, or αβ7, is also known as lymphocyte-Peyer’s patch adhesion molecule-1 (LPAM-1). Lymphocytes bind to the endothelium through the interaction of αi integrins with...
either vascular cell adhesion molecule-1 (VCAM-1) or mucosal adressin cell adhesion molecule-1 (MAdCAM-1). See Sharar et al\textsuperscript{13} for a review of the adhesion cascade.

The degree to which lymphocytes and monocytes contribute to the postischemic inflammatory process is unknown, and the relative importance of the $\beta_2$ and $\alpha_4$ integrin pathways to lymphocyte and monocyte activation and trafficking into the brain is unclear. Because neutrophils infiltrate the brain soon after the onset of ischemia, neuroprotective strategies that target neutrophils must be instituted early after stroke onset if they are to be effective. Since lymphocyte and monocyte infiltration into the brain is delayed relative to neutrophil infiltration, antilymphocyte and antimonocyte strategies might provide a longer time window for instituting neuroprotection. Figure 1 is based on several histological studies\textsuperscript{11,12,22–24} and depicts the time course of leukocyte infiltration into the brain after stroke and potential strategies for interfering with that infiltration. There is an early and transient influx of polymorphonuclear cells (PMNs), followed by a more sustained increase in mononuclear cells (monos) and T lymphocytes (T cells).\textsuperscript{11,12,22–24} All leukocytes express $\beta_2$ integrins (CD11/CD18), which mediate binding to the endothelium through interaction with ICAM-1, which is upregulated after stroke. Lymphocytes and other mononuclear cells also express the $\alpha_4$ integrin VLA-4, which adheres the cell to the endothelium through its interaction with VCAM-1. VCAM-1 expression in the brain is induced after ischemia.

![Image](85x551 to 243x718)

Figure 1. Time course of leukocyte infiltration into the brain after stroke and potential strategies for interfering with that infiltration. There is an early and transient influx of polymorphonuclear cells (PMNs), followed by a more sustained increase in mononuclear cells (monos) and T lymphocytes (T cells).\textsuperscript{11,12,22–24} All leukocytes express $\beta_2$ integrins (CD11/CD18), which mediate binding to the endothelium through interaction with ICAM-1, which is upregulated after stroke. Lymphocytes and other mononuclear cells also express the $\alpha_4$ integrin VLA-4, which adheres the cell to the endothelium through its interaction with VCAM-1. VCAM-1 expression in the brain is induced after ischemia.

Materials and Methods

Animals

All protocols were approved by the University of Washington Animal Care and Use Committee. Male Lewis rats (weight, 300 to 400 g) were anesthetized with halothane 2% and underwent middle cerebral artery occlusion by insertion of a filament into the internal carotid artery that was advanced approximately 17 to 20 mm.\textsuperscript{25} Body temperature was continuously monitored during surgery and maintained at 37°C to 38°C with a thermostatically controlled warming blanket, but animals were allowed to spontaneously thermoregulate thereafter. Three hours after middle cerebral artery occlusion, under brief anesthesia, reperfusion was established by withdrawing the suture. Neurological examinations were performed at 2, 3, 4, 5, 6, 24, and 48 hours with the use of the modified Bederson scale (Table).\textsuperscript{26} Body temperature was measured at baseline and at 2, 3, 4, 5, 6, 24, and 48 hours. Animals were weighed at baseline and at 24 and 48 hours after the onset of ischemia. Systemic blood was collected into heparinized syringes by cardiac puncture at the time of death.

Drug Administration

Two hours after middle cerebral artery occlusion (1 hour before reperfusion), animals were injected intraperitoneally with 2.5 mg/kg of either TA-2 antibodies (mouse anti-rat $\alpha_4$ IgG1; Seikagaku) (anti-$\alpha_4$; n = 16) or 1E6 antibodies (mouse anti-human LFA-3 IgG1; Biogen) (control; n = 11). TA-2 recognizes rat VLA-4, blocks the adhesion of rat lymphocytes to activated endothelium, and prevents the migration of rat lymphocytes to inflamed tissue.\textsuperscript{27,28} The 1E6 antibody serves as an isotype control.

Quantification of Leukocytes

Leukocytes were counted under a fluorescence microscope after dilution of whole blood with 0.9% NaCl (1:10), incubation in 4% paraformaldehyde (1:1), permeabilization of cell membranes with 0.2% Triton-X 100 (Sigma Chemical Co), and nuclear staining with 4,6-diamidino-2-phenylindole dihydroporphyrin chloride (10 μg/mL) (Sigma Chemical Co). The differential of these cells (lymphocytes/monocytes versus neutrophils) was determined by standard light microscopy after staining with Diff-Quick (Dade Behring AG).

Infarct Size

Animals were killed 48 hours after the onset of ischemia. Brains were removed, frozen in isopentane, and stored at −70°C. Sections (10 μm) were stained with cresyl violet (Cresyl Echt Violett, Chroma-Gesellschaft) and analyzed at 8 predetermined levels (bregma +2.2, +1.2, 0.2, −0.8, −1.8, −2.8, −3.8, −4.8). The stained sections were scanned and digitized; infarct size was determined with the MetaMorph Imaging System V4.1.1 (Universal Imaging Corp) by an investigator blinded to treatment status. Infarct size was corrected for edema.\textsuperscript{29}

Statistical Analysis

All data are expressed as median±SD unless otherwise noted. Comparisons were made by the Mann-Whitney U test. Statistical significance was set at $P<0.05$.

Results

There were no significant differences in the body temperatures of the 2 experimental groups at any time. The maximum recorded temperature in each group occurred 6 hours after the onset of ischemia and reached 38.7±0.29°C in the anti-$\alpha_4$–treated animals and 38.7±0.68°C in the control animals. Temperature at the time of death was 37.5±0.52°C in anti-$\alpha_4$–treated animals and 37.0±0.67°C in control animals. By 24 hours, the neurological score of anti-$\alpha_4$–treated animals was significant lower than that of control animals (2.0±1.2 versus 3.0±0.4; $P=0.006$; the difference persisted to 48 hours (2.0±1.2 versus 3.0±0.8; $P=0.011$) (Figure 2). There

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**Neurological Grading Scale**

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No deficits</td>
</tr>
<tr>
<td>1</td>
<td>Flexed forepaw</td>
</tr>
<tr>
<td>2</td>
<td>Inability to resist lateral push</td>
</tr>
<tr>
<td>3</td>
<td>Circling</td>
</tr>
<tr>
<td>4</td>
<td>Agitated circling</td>
</tr>
<tr>
<td>5</td>
<td>Stupor</td>
</tr>
</tbody>
</table>

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and 48 hours. Animals were weighed at baseline and at 24 and 48 hours after the onset of ischemia. Systemic blood was collected into heparinized syringes by cardiac puncture at the time of death.
were no significant differences in body weight between the 2 groups at any time during the course of the experiment.

At 48 hours after stroke, animals that received anti-α4 antibodies had significantly higher peripheral blood leukocyte counts than animals that received the isotype control antibodies (6.8±2.2×10^6 versus 2.9±1.9×10^6; P=0.001); there was a lymphocyte/monocyte predominance (86.0±16.2% versus 71.0±15.6%; P=0.008).

Infarct volume, after correction for edema, was significantly less in animals treated with antibodies to α4 compared with animals treated with isotype control antibodies (120.1±51.21 versus 173.7±42.29 mm^3; P=0.012; Figure 3). Two control animal brains were destroyed during processing (therefore, n=9).

Discussion

We have shown that blockade of the α4 integrin after the onset of transient focal cerebral ischemia induces a peripheral leukocytosis, improves neurological outcome, and decreases infarct volume in a rat model of stroke. These data support a role for lymphocytes and/or monocytes in the inflammatory response to cerebral ischemic injury and as potential therapeutic targets for limiting that injury.

Leukocyte trafficking in and out of the brain requires the expression of complement-mediated adhesion molecules on the surface of leukocytes and endothelial cells. The molecules most important for leukocyte trafficking in general and lymphocyte trafficking in particular include L-selectin, the β2 integrins, ICAM-1, VCAM-1, and VLA-4. Almost all leukocytes express L-selectin, which tethers the cell to the vessel wall. The cells become firmly adherent to the endothelium through the interaction of the β2 integrins with ICAM-1 and ICAM-2. ICAM-1 is expressed constitutively on brain microvessels, and its expression is upregulated during ischemia. Lymphocytes, monocytes, and eosinophils also express α4 integrins, providing another pathway for adhesion as well as a means for activation. In certain situations, however, neutrophils may also express VLA-4. α4β1 (VLA-4) tethers lymphocytes to the endothelium through binding to VCAM-1, while αβγ (LPAM-1) tethers cells to MAdCAM-1. VCAM-1 is not expressed in normal brain tissue, but its expression is induced within the central nervous system microvasculature by ischemia. VCAM-1 can also be expressed on activated astrocytes and cytokine-stimulated neural cells. Since VCAM-1, but not MAdCAM-1, can be induced in brain, it is the interaction of VLA-4 with VCAM-1 that appears to be important for the trafficking of lymphocytes into the central nervous system. The VLA-4/VCAM-1 interaction may also be necessary for production of matrix metalloproteinases, which allow for the transmigration of cells through tissue. Finally, interaction of VCAM-1 with VLA-4 provides costimulatory signals for T-cell activation, and, when activated, lymphocytes can become cytotoxic and secrete cytokines that worsen brain injury.

Soluble ICAM-1 and shed L-selectin are persistently elevated in patients with stroke risk factors. After stroke, however, further increases in the plasma concentrations of these adhesion molecules are not reliably documented. Stroke patients express increased levels of LFA-1 and Mac-1 on their leukocytes, and blocking the interactions of these β2 integrins with ICAM-1 improves outcome in experimental models of stroke. Neutralization of L-selectin, on the other hand, is not of convincing benefit in animal models of cerebral ischemia, which may be related to the fact that L-selectin is not required for leukocyte trafficking into the inflamed brain. The therapeutic potential of blocking VCAM-1–mediated cell adhesion has not yet been explored, but soluble VCAM-1 (sVCAM-1) levels are elevated by 4 hours after the onset of stroke and remain elevated for at least 5 days. In patients with autoimmune disease, sVCAM-1 levels are a reliable surrogate for disease activity and, presumptively, organ-specific expression of VCAM-1. The relationship between sVCAM-1 and brain VCAM-1 is not
known, but sVCAM-1 levels might serve as a surrogate for the degree of lymphocyte infiltration into brain.

Given the promise of the experimental data, there have been 2 clinical trials of antiadhesion therapy for treatment of acute stroke. In the first, a monoclonal antibody directed against ICAM-1 (enlimomab) was administered to patients within 6 hours of stroke onset; its use was associated with increased morbidity and mortality.57 The poor outcome has been attributed to the fact that the antibody was of murine origin and, as a foreign protein, may have incited an inflammatory response that contributed to the injury process.58 In fact, subsequent studies suggest that the enlimomab antibody activates neutrophils in a complement-dependent fashion.59 In the present study, rats received an antibody of murine and therefore foreign origin. While it is possible that the heterologous protein 1E6 initiated an immune response and exacerbated injury, the temperature profiles and infarct volume of 1E6-treated animals are similar to those seen in other control animals in our laboratory. Another clinical study using a humanized monoclonal antibody to CD18 (LeukArrest) was recently halted at interim analysis when it was claimed to show no effect, either beneficial or detrimental.60

While lymphocytes express L-selectin, CD18, and VLA-4, there appear to be differences in the importance of these adhesion molecules for lymphocyte trafficking and activation. In models of experimental allergic encephalomyelitis (EAE), inhibition of the VLA-4/VCAM-1 interaction appears to be more effective than inhibition of the CD18/ICAM-1 interaction61–63 and L-selectin–mediated adhesion64 in preventing or limiting disease. In fact, monoclonal antibodies to VLA-4 can even reverse the clinical signs of EAE when given after disease onset.65 Thus, on the basis of models of EAE and what is known about expression of adhesion molecules after stroke, modulation of the VCAM-1/VLA-4 interaction may be a more reasonable approach for limiting the postischemic inflammatory response than blockade of other adhesion molecules. Studies suggest that the role of antiadhesion therapy in treating EAE may have little to do with leukocyte trafficking and more to do with cell activation initiation of the immune response.63

Since immunocytochemistry was not performed in this study, it is unclear how binding of the TA-2 antibody to the α₄ integrin produced neuroprotection. Administration of the TA-2 antibody is known to induce lymphocytosis due to alteration in cell distribution and/or trafficking.28 In the present study, the numbers of nonmonocytic, nonlymphocytic cells in TA-2– and 1E6-treated animals are roughly equal (9.52×10⁷ versus 8.41×10⁷), consistent with the premise that monocytes and lymphocytes are selectively involved. Presumptively, the change in lymphocyte distribution and trafficking also reflects decreased leukocyte extravasation into the brain. In addition to the changes in leukocyte trafficking, the TA-2 antibody may also alter the activity of α₄-bearing cells, either through inhibition or activation of those cells. Finally, this experiment did not directly address which cellular phenotype is responsible for the inflammatory brain injury in stroke; it merely shows that cells bearing the α₄ integrin are involved.

There are quantitative and qualitative differences in leukocyte antigen expression,66 and the relative importance of each in adhesion and activation is unclear. Whether the appropriate adhesion molecules are being targeted in acute stroke remains to be seen. The optimal time for institution of anti leukocyte therapies depends on the cell being targeted and the intervention being used. It is reasonable to assume, as depicted in Figure 1, that antineutrophil-based therapies will need to be delivered earlier than anti lymphocyte- or antimonoocyte-based therapies. Since the interaction of VCAM-1 with VLA-4 leads to lymphocyte and potentially neutrophil35 activation, inhibition of this interaction may be crucial in preventing postischemic inflammation in the brain.

Conclusions

Inflammation exacerbates cerebral ischemic injury. Under normal circumstances, the trafficking of lymphocytes into the brain and the activation of those lymphocytes are limited because of the relative absence of adhesion molecules (ie, VCAM-1) in the brain. After ischemia, however, adhesion molecule expression within the central nervous system allows for leukocyte influx. Interventions that interfere with lymphocyte trafficking into brain and activation of those lymphocytes may therefore be viable therapeutic options for limiting brain injury after stroke. Because the influx of lymphocytes and monocytes is delayed relative to that of neutrophils, neuroprotective strategies that interfere with lymphocyte- and monocyte-mediated injury may provide a longer time window for therapy than those that interfere with neutrophil-mediated injury. We have shown that administration of an antibody to α₄, an antibody known to block lymphocyte binding to endothelium and to prevent infiltration of lymphocytes into inflamed tissue, can decrease infarct volume and improve neurological outcome even when given after the onset of ischemia.

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References


In this article, companion to the article by Relton et al.,1 Becker and colleagues2 have shown that administration of TA-2, an antibody against the α4 integrin, reduces infarct size and improves neurological outcome when given 2 hours after a stroke. The α4 integrin is thought to be found predominantly in lymphocytes and monocytes, rather than neutrophils. The contribution of these former leukocyte populations to ischemic brain injury is not well known, but the results of this study suggest that anti-lymphocyte/monocyte strategies are effective. Given that monocytes migrate into the brain after neutrophils, such strategies may offer a longer temporal therapeutic window than antineutrophil strategies. However, the authors are careful to state that the distribution and cellular localization of this integrin has not yet been convincingly shown. The peripheral counts in the treated animals did show a lymphocyte and monocyte predominance, and might suggest that this could result from the TA-2 treatment’s preventing migration into the brain. However, the study by Relton et al showed that TA-2 treatment was associated with overall brain leukocyte reduction and not specific subpopulations. Nevertheless, the findings of these two articles suggest the possibility of a novel new anti-inflammatory target for stroke treatment.

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References
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