Adhesion of leukocytes to the endothelium can occur in a few hours after the onset of ischemia, and the actions of leukocytes have been suggested to aggravate reperfusion injury. Adhesion is a prerequisite for the harmful leukocyte actions. Rapid mediation of leukocyte adhesion and aggravation of reperfusion injury can occur through production of platelet-activating factor (PAF). The authors hypothesized that prevention of leukocyte adhesion during ischemia reperfusion would have beneficial effects and that these effects might be enhanced by a PAF antagonist. To test this hypothesis, rabbits were anesthetized with pentobarbital and subjected to severe spinal cord ischemia (25 minutes) followed by 30 minutes of reperfusion, at which time either vehicle, antibody against the CD11/CD18 (anti-CD) leukocyte adhesion molecule (1 mg/kg), or the anti-CD and PAF antagonist, WEB 2086 (3 mg/kg), was administered intravenously and the animals were monitored for 6.5 hours. Using a score from 0 to 5, recovery of motor function was improved at 5.5 hours by the CD antibody (2.0 ± 0.2 in the six animals in the vehicle group, p < 0.05). No further improvement was induced by WEB 2086 in the six anti-CD treated animals (1.6 ± 0.7). Spinal cord blood flow (laser Doppler flowmetry) at 6 hours was at the preischemic level in the control animals (−7% ± 20%), but clearly increased in the anti-CD group (+73% ± 29%, p < 0.5). The severity of blood-brain barrier damage in the spinal cord gray matter was decreased by the treatments. Extravasation of intravenously injected Evans blue albumin (EBA), measured by detection of EBA fluorescence, was reduced by approximately 50% in both treated groups (p < 0.05). The number of morphologically normal motor neurons in the lumbar anterior horns of the infarcted spinal cord showed protection in the seven animals in the anti-CD treated group at 6.5 hours: 12.7 ± 1.7 versus 5.3 ± 1.6 (vehicle), p < 0.05 without an additional effect by PAF antagonist 12.2 ± 2.6 (anti-CD + WEB 2086). Our results suggest that ultraracute treatment of reperfusion injury based on special inhibition of leukocyte effects may be beneficial. Platelet-activating factor antagonism failed to enhance this therapeutic effect, which may suggest dependency on a common mechanism.

KEY WORDS • adhesion molecules • leukocytes • platelet-activating factor • stroke • cerebral circulation • rabbit

Antagonism of neutrophil adherence in the deteriorating stroke model in rabbits

PERTTU J. LINDSBERG, M.D., PH.D., ANNA-LEENA SIRÉN, M.D., PH.D., GIORA Z. FEUERSTEIN, M.D., AND JOHN M. HALLENBECK, M.D.

Department of Neurology, F. Edward Hébert School of Medicine, Uniformed Services University of the Health Sciences, Bethesda; Stroke Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland; and Department of Neurology, University of Helsinki, Helsinki, Finland

Adhesion of leukocytes occurs acutely in areas of cerebrovascular injury in humans and experimental animals. Leukocytes have been suggested to contribute and aggravate ischemia-reperfusion injury, for which the adherence of leukocytes to the endothelium is an essential step. Leukocyte accumulation can occur in a few hours after the onset of ischemia and coincide with the development of postischemic hypoperfusion and progressive neuronal death. Leukocytes adhere to the endothelium by means of specific membrane-bound glycoprotein molecules such as the β2-integrin receptor complex, CD11/CD18. Synthesized rapidly by endothelial cells, platelet-activating factor (PAF) enhances leukocyte adhesion to the endothelium. Platelet-activating factor can also signal neutrophil chemotaxis and activation in addition to an array of direct noxious actions such as vasoconstriction, platelet aggregation, and release of other inflammatory mediators. The pathophysiological significance of these biological events in vivo remains unproven in ischemic brain injury.

Recent experimental studies have indicated that pharmacologically induced decrease in the number of circulating leukocytes or inhibition of their biological activity improved microcirculation, function, and survival of neuronal tissue after ischemia. Chemical inhibition of phagocytic and secretory function of mononuclear phagocytes improved long-term motor recovery even when treatment was delayed up to 6 hours following reperfusion after spinal cord ischemia in rabbits. The effect of more specific approaches targeted at inhibition of leukocyte adhesion is controversial. Clark, et al., pretreated rabbits with the anti-CD18 adhesion molecule, R3.3,
observed improvement of neurological function in ischemia–reperfusion of the spinal cord, but no effect in cerebral ischemia. Takeshima, et al., treated cats with CD11/CD18 antibody, MoAb 60.3, during cerebral ischemia followed by reperfusion and found no effect on the extent of injury, neuronal function, or blood flow.

We have previously demonstrated the formation and pathological effect of PAF in deteriorating stroke in rabbits at 2 hours after reperfusion. Platelet-activating factor antagonist treatment during ischemia inhibited tissue edema and post-ischemic hypoperfusion and reduced neutrophil accumulation in a model of focal brain damage. In the present study, we examined the hypothesis that therapeutically administered specific antibody directed against the β subunit of the integrin LFA-1, a leukocyte adhesion molecule, protects neuronal tissue and function and that these effects might be enhanced by a PAF antagonist through inhibition of leukocyte-independent PAF effects.

Materials and Methods

Surgical Preparation and Laser Doppler Flowmetry

We used the well-described rabbit spinal cord model of deteriorating stroke. Male New Zealand albino rabbits weighing 1.8 and 3.8 kg were housed at 22°C in a 12-hour light/12-hour dark cycle with food and water ad libitum. The rabbits were sedated with ketamine hydrochloride (50 mg/kg intramuscularly) and anesthetized with 40 mg/kg of sodium pentobarbital given intravenously through the right ear vein. Each animal underwent tracheostomy and received a muscle relaxant (gallamine triethiodide; 3 mg/kg intravenously) and was respirated artificially† (stroke volume 17 cc/kg and rate 30–35 minute) with oxygen given directly into the endotracheal tube. Anesthesia was maintained with supplementary doses of pentobarbital. The physiological state was observed and maintained within the following ranges as described earlier: pH, 7.3 to 7.48; PCO₂, 25 to 37 mm Hg; and PO₂, 100 to 125 mm Hg. Body temperature was measured with a rectal thermometer and maintained within normal range with a water-jacketed heating pad. A Swan Ganz No. 4 French catheter was advanced via the right femoral artery to the abdominal aorta to reach a level just above the left renal artery. The correct internalized catheter length was estimated in preliminary studies and established post mortem in each case. Heparin (500 IU) was infused to prevent clotting of the catheter. The mean arterial pressure (MAP) proximal to the occlusion was monitored with an on-line laser Doppler flowmetry. If this level of ischemia was not reached during a test occlusion of approximately 30 seconds, the Swan Ganz catheter was advanced slightly to ensure occlusion of all radicular arteries supplying the lumbosacral spinal cord.

During the course of these experiments, the laminotomy site was in some instances placed at L-4 to ensure that the level of ischemia was homogeneous throughout the lumbosacral spinal cord sections. After 25 minutes of occlusion the balloon was deflated and the catheter was flushed with 1 to 2 ml of 100 IU/ml heparin–saline solution. Blood gases and rectal temperature were recorded before ischemia and 15 minutes after reperfusion, and corrected if necessary. Thirty minutes after reperfusion, the drug infusions were given intravenously through the ear vein (see drug treatments below). Thereafter, anesthesia was no longer maintained. The Swan Ganz catheter was removed while the left femoral catheter was heparinized, closed, and left in place in the femoral skin pouch. The laminotomy site was closed with staples. Blood oxygenation was ensured during spontaneous breathing with supplemental oxygen, if necessary. Blood gases were monitored at a maximum of 45 minutes apart and at 2 hours of reperfusion; sampling for complete blood counts and rectal temperature measurement were repeated.

The neurological score (see below) was recorded 5.5 hours after reperfusion, at which time the rabbits had regained consciousness. The rabbits were reassedated with pentobarbital, the blood gases were monitored and corrected as needed. Samples of complete blood counts were obtained. The rabbits were again stabilized and prepared for laser Doppler flowmetry recording as described above. A volume of 1 ml/kg of 2% Evans blue dye and 1% bovine albumin solution (EBA) was injected intravenously and allowed to circulate for 30 minutes, after which (at 6.5 hours) the animals were killed with a pentobarbital overdose (2 ml of 65 mg/ml). The spinal cord was rapidly dissected. A lumbosacral cord tissue block underneath L-5 lamina was immersion fixed for at least 48 hours in 10% phosphate-buffered formalin for histopathological examination and a neighboring caudal section was immediately frozen in liquid nitrogen–cooled isopentane to be analyzed for blood-brain barrier (BBB) integrity.

The experiments reported herein were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Resources, National Research Council, DHHS, Publication No. 85-23 of the National Institutes of Health and were approved by the Uniformed Services University laboratory animal review board.

* New Zealand albino rabbits obtained from Hazelton Laboratories, Gaithersburg, Maryland.
† Rodent ventilator, Model 613, obtained from Harvard Apparatus, Natick, Massachusetts.
‡ MK IV Physiograph obtained from Narco, Houston, Texas.
§ Stereotactic spinal cord unit, Model 1500, manufactured by David Kopf Instruments, Tujunga, California.
¶ Fiber optic laser Doppler flowmetry probe, Model P436, obtained from TSI, Inc., St. Paul, Minnesota; electrode carriers, Model 1500, obtained from David Kopf Instruments, Tujunga, California.
* Laser Doppler flowmeter, Model BPM 403a, obtained from TSI, Inc., St. Paul, Minnesota.
Leukocyte adherence in deteriorating stroke

**Pharmacological Protocol**

Solutions for pharmacological treatments were prepared a maximum 1 hour before infusion. Two separate intravenous infusions were administered over a 30-second period, 2 minutes apart at 30 minutes after reperfusion. The potent and selective PAF antagonist, WEB 2086, in a dose of 3 mg/kg was suspended in normal saline (3 mg/ml) at 20° to 30°C and dissolved with the aid of an ultrasonic bath. Selection of this dose was based on previous reports in the literature and our preliminary experiments with rabbits, which demonstrated total inhibition at 2 hours and more than 80% inhibition of PAF-induced hypotension at 5 hours after treatment (Fig. 1). A dosage of 1 mg/kg of the monoclonal antibody for CD11/CD18 (anti-CD) leukocyte adhesion molecule (anti-LFA-1 (β subunit); clone R15.7) was administered in 1 to 3 ml of normal saline. This dose was based on previous experimental data, which showed that 1.25 mg/kg of anti-CD18 (β subunit; R3.3) was effective in inhibiting neutrophil migration in rabbits for up to 20 hours. The following treatment groups were assigned (seven rabbits in each): A, Vehicle I + Vehicle II; B, Vehicle I + anti-CD; and C, WEB 2086 + anti-CD solution. The investigator performing all experiments was unaware of which treatment was actually given.

**Data Processing and Statistical Methods**

Data in figures and tables are expressed as mean ± standard error for the given number of animals. The p values and statistical significance are indicated as computed by analysis of variance (ANOVA) followed by the least-squares difference method. The data of the hemodynamic state and the evolution of the circulating cells were analyzed with ANOVA for the repeated measures. The nonparametric motor score data were analyzed by the Mann-Whitney U test. To examine the relationship between the motor score, neuronal counts, and neutrophil counts, correlation analysis was performed based on Pearson’s product moment coefficient followed by significance estimation according to t distribution. A p value of less than 0.05 was considered significant.

**Results**

The physiological variables were monitored frequently (Table 1) and corrected if necessary after each measurement. The rectal temperature was measured at a level that approximated the abdominal aorta occlusion. The temperature remained within the normal range (Table 2). The baseline levels of circulating leukocytes, platelets, and erythrocytes were within the normal ranges. No differences between the groups were observed in the levels of circulating erythrocytes, hematocrit, or hemoglobin. The platelet counts in the three groups 5.5 hours after reperfusion were Group A, 251 ± 26; Group B, 192 ± 36; and Group C, 192 ± 30 × 10^3, nonsignificant. The two CD11/CD18 antibody (anti-CD) treated groups (B and C) exhibited significant peripheral leukocytosis during reperfusion (at 5.5 hours they were: A, 5.7 ± 0.7; B, 12.6 ± 1.3, p < 0.001; C, 10.0 ± 0.8 × 10^3, p < 0.001). Although the number of lymphocytes was moderately increased in Group B (A, 2.7 ± 0.3; B, 4.5 ± 0.7, p < 0.05; C, 3.6 ± 0.2 × 10^3, nonsignificant), the neutrophil counts were significantly elevated in the two anti-CD-treated groups as early as 2 hours after reperfusion (Fig. 2). Mean arterial pressure showed the expected pattern during ischemia-reperfusion (Table 3). The MAP dis-

† Scanning laser fluorescence image analyzer manufactured by Meridian Instruments, Inc., Okemos, Michigan.

‡ WEB 2086 and monoclonal antibody for the CD 18 (anti-CD) leukocyte adhesion molecule (anti-LFA-1 (β subunit); clone R15.7) generously provided by Boehringer Ingelheim Pharmaceuticals, Ridgefield, Connecticut.

---

**Fig. 1.** Graph illustrating platelet-activating factor (PAF)-induced systemic hypotension used to test the in vivo potency of PAF antagonist treatment. These original recordings demonstrate the inhibition by 3 mg/kg intravenous WEB 2086 of the systemic hypotension produced by bolus injections of PAF (0.5 nmol/kg). The solid line illustrates a typical hypotensive response in a rabbit that did not receive the PAF antagonist. Inhibition was complete at 2 hours (dotted line) and 84% effective at 5 hours (dashed line) after the WEB 2086 treatment as revealed by area analysis of the mean arterial pressure curves. Solid circles indicate starting points of the recordings.

**Tissue Evaluation**

Fifteen-micrometer serial sections were cut for histopathological examination and stained with hematoxylin and eosin (H & E). The number of large anterior horn neurons with histological characteristics of normal and abnormal structure (intracytoplasmic vacuolization, shrinkage and hyperdensity of the nuclei (pyknosis), perineuronal large halo, eosinophilic or dark, shrunken cytoplasm with indistinct neuronal processes (Table 1) and corrected if necessary after each measure-

**Neurological Score**

The severity of hindlimb paralysis was observed separately in each hind limb at 5.5 hours after reperfusion. A six-point scale was used; 0, complete paraplegia; 1, paraplegia with minimal functional movement; 2, functional movement present, cannot hop; 3, hopping, ataxia and paresis present; 4, hopping, minor impairment; and 5, normal. The average performance of the hindlimbs was accepted as the motor score in each rabbit. Muscle spasticity was also tested in the hindlimbs. Pinprick sensation was examined to identify rabbits with complete cutaneous sensory loss in the hindquarters and feet.
tal to abdominal aorta occlusion was well below the autoregulatory capacity of the rabbit spinal cord. There was a tendency toward hypotension at the end of the experiments, but no significant differences were observed between groups at any time point. The depth of spinal cord ischemia was similar in all groups: A, 78% ± 5%; B, 80% ± 3%; and C, 76% ± 4% (percent decreases (sign −) from baseline). Reperfusion was successful in all rabbits; the averaged continuous blood flow recordings during the first 15 minutes after reperfusion ranged from −7% to +373% of baseline with no significant differences between the groups. At 5.5 hours, spinal cord blood flow was approximately at baseline in the vehicle group (−7% ± 20%), but the drug-treated groups showed variably hyperemic (sign +) states (B, +73% ± 29%; p = 0.05; C, +24% ± 23%, nonsignificant). Due to technical difficulties or incomplete recovery from anesthesia, not all rabbits could be evaluated for the various end-points, and the number of animals in each data set is indicated separately. Hindlimb motor function did not recover in the vehicle group by 5.5 hours after reperfusion, but significant improvement of motor function took place in the anti-CD–treated group in the six-point motor score scale (Fig. 3). Platelet-activating factor antagonist treatment did not enhance this effect. In the groups of six rabbits, four showed hindlimb muscle spasticity in the vehicle group and two in both of the treated groups. Complete cutaneous sensory loss in the hindquarters and feet was recorded in three rabbits in the vehicle group and in one and two rabbits in the anti-CD–treated and anti-CD with WEB 2086–treated groups, respectively.

Quantitative analysis of total EBA fluorescence in the gray matter of the fresh-frozen spinal cord sections revealed significantly lower fluorescence intensities in both drug-treated groups (Fig. 4), which was interpreted to indicate decreased diffusion of EBA into the gray matter and decreased BBB permeability. Light microscopic examination of the stained spinal cord sections demonstrated histopathological signs such as cytoplasmic vacuolization, nuclear pyknosis, perineuronal halo formation, and shrunken cytoplasm with indistinct neuronal processes. These changes were particularly evident in rabbits showing no hindlimb motor function (Fig. 5). The number of normal-appearing anterior horn large neurons in spinal cord cross-sections was significantly increased compared to the vehicle-treated group in both drug-treated groups (Fig. 6). Correlation analysis was performed to clarify whether the presumed nominal effect of the treatments on the circulatory behavior of neutrophils was indeed related to the histopathological and motor outcomes. Interestingly, the number of circulating neutrophils at 5.5 hours correlated directly with motor score and the total number of anterior horn large neurons (Fig. 7) and indirectly with the amount of Evans blue fluorescence in the infarcted spinal cord gray matter (r = 0.52, p = 0.036, not illustrated). As expected, the motor score seemed to depend on the severity of the morphological changes observed in the anterior horn neurons but also to some degree on the amount of Evans blue fluorescence in the gray matter (r = −0.54, p = 0.027, not illustrated). Significant correlations were not found between blood flow and the above variables.

**Discussion**

The present study supports the earlier observation that leukocyte adhesion based on the CD11/CD18 molecule family may have a pathophysiological role in central nervous system ischemia-reperfusion injury. We now demonstrate that this concept offers a basis for effec-

---

**Table 1**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group*</th>
<th>Baseline</th>
<th>15 Min</th>
<th>2 Hrs</th>
<th>5.5 Hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>A</td>
<td>7.38 ± 01</td>
<td>7.36 ± 01</td>
<td>7.40 ± 02</td>
<td>7.34 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>7.39 ± 02</td>
<td>7.36 ± 01</td>
<td>7.39 ± 02</td>
<td>7.38 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>7.40 ± 02</td>
<td>7.36 ± 02</td>
<td>7.38 ± 03</td>
<td>7.31 ± 0.2</td>
</tr>
<tr>
<td>CO₂ mm Hg</td>
<td>A</td>
<td>33.3 ± 1.3</td>
<td>31.8 ± 1.6</td>
<td>29.7 ± 0.6</td>
<td>29.6 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>31.6 ± 1.8</td>
<td>30.2 ± 2.2</td>
<td>33.3 ± 2.3</td>
<td>29.8 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>32.1 ± 1.7</td>
<td>32.0 ± 1.3</td>
<td>30.0 ± 1.5</td>
<td>30.7 ± 1.7</td>
</tr>
<tr>
<td>O₂ mm Hg</td>
<td>A</td>
<td>111.9 ± 4.6</td>
<td>118.6 ± 10.8</td>
<td>117.5 ± 6.4</td>
<td>109.5 ± 10.6</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>119.1 ± 4.8</td>
<td>135.4 ± 6.2</td>
<td>109.2 ± 4.8</td>
<td>121.2 ± 8.0</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>116.5 ± 8.4</td>
<td>123.9 ± 5.7</td>
<td>110.3 ± 5.0</td>
<td>119.4 ± 9.6</td>
</tr>
</tbody>
</table>

* Treatment groups were assigned as follows: A = vehicle, B = anti-CD, C = anti-CD and WEB 2086.

---

**Table 2**

<table>
<thead>
<tr>
<th>Group*</th>
<th>Baseline</th>
<th>Ischemia</th>
<th>15 Min</th>
<th>2 Hrs</th>
<th>5.5 Hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>39.8 ± 0.2</td>
<td>39.5 ± 0.3</td>
<td>39.9 ± 0.4</td>
<td>39.1 ± 0.6</td>
<td>39.0 ± 0.3</td>
</tr>
<tr>
<td>B</td>
<td>39.4 ± 0.3</td>
<td>39.3 ± 0.3</td>
<td>39.6 ± 0.3</td>
<td>39.1 ± 0.3</td>
<td>38.7 ± 0.4</td>
</tr>
<tr>
<td>C</td>
<td>39.5 ± 0.3</td>
<td>39.1 ± 0.3</td>
<td>39.7 ± 0.3</td>
<td>39.5 ± 0.3</td>
<td>38.8 ± 0.3</td>
</tr>
</tbody>
</table>

* Treatment groups were assigned as follows: A = vehicle, B = anti-CD, C = anti-CD and WEB 2086.
Leukocyte adherence in deteriorating stroke

![Graph](image1.png)

**Fig. 2.** Graphs illustrating the levels of circulating neutrophils (upper) and lymphocytes (lower) that were measured repeatedly during the experimental protocol. Blood samples were taken before ischemia and at 15 minutes, 2 hours, and 5.5 hours after reperfusion, as indicated. Squares represent the vehicle group, circles the anti-CD group, and triangles the anti-CD with WEB 2086 group. Repeated-measures analysis of variance suggested a significant treatment effect and the asterisks indicate statistical significance in comparison to vehicle group. * and ** indicate p < 0.05 and p < 0.01, respectively.

![Graph](image2.png)

**Fig. 3.** Graph showing the hindlimb motor score at 5.5 hours after reperfusion. A six-point scale was used: 0 = complete paraplegia; 1 = paraplegia with minimal functional movement; 2 = functional movement present, cannot hop; 3 = hopping, ataxia, and paresis present; 4 = hopping, minor impairment; 5 = normal. The asterisk indicates the statistical significance of the difference between anti-CD and vehicle group (p < 0.05). The therapeutic effect was not enhanced by WEB 2086. There were six rabbits in each group.

Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>Occlusion</th>
<th>15 Min</th>
<th>5.5 Hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>99.9 ± 1.8</td>
<td>32.5 ± 1.6†</td>
<td>100.7 ± 3.0</td>
<td>85.0 ± 2.6‡</td>
</tr>
<tr>
<td>B</td>
<td>96.0 ± 2.0</td>
<td>34.1 ± 2.2†</td>
<td>94.2 ± 4.1</td>
<td>85.7 ± 4.6§</td>
</tr>
<tr>
<td>C</td>
<td>95.4 ± 3.1</td>
<td>35.4 ± 1.8†</td>
<td>92.4 ± 4.6</td>
<td>89.0 ± 5.4</td>
</tr>
</tbody>
</table>

* Treatment groups were assigned as follows: A = Vehicle, B = CD11a/18-antibody, C = CD11a/18-antibody and WEB 2086.
† Analysis of variance for repeated measures suggested highly significant decrease of MAP distal to the occlusion (p < 0.001) in all groups and § significant systemic hypotension at 5.5 hours in Groups A and B (p < 0.05). There was no significance between group differences.

revealed a therapeutic window for preventing the delayed deterioration 1 to 2 days after injury by inhibiting the chronic inflammatory response. The present data also demonstrated the existence of an acute therapeutic window for altering the course of very severe ischemic injury. In ischemic stroke, three therapeutic strategies may be warranted: clot lysis to enhance reperfusion, control of acute reperfusion injury, and prevention of delayed deterioration.

Past experience with this stroke model has established that, following complete loss, hindlimb motor function can be regained by four hours after reperfusion before secondary deterioration occurs by 24 hours. The present series of experiments does not exclude the possibility that the beneficial effects observed at the acute phase of therapy given at a single time point could be lost during later evolution of the multiple pathophysiological processes. However, acute recovery of motor function seems necessary for preservation of some degree of motor function in the late phase, because spontaneous delayed motor recovery is rarely observed. A recent report indicated that...
breakdown of the BBB in the spinal cord gray matter is an acute, transient phenomenon that occurs at 30 minutes after reperfusion and coemerges again with delayed deterioration of motor function at later time points (8 to 24 hours). Based on these observations, the present treatment protocol was designed to improve motor recovery at the acute stage with a treatment presumably inhibiting early BBB damage. Extravasation of EBA in the spinal cord gray matter was significantly lower (Fig. 4) in both drug-treated groups compared to the vehicle-treated group, and this correlated with motor score. Therefore, early opening of the BBB may be primed by actions of leukocytes and might be an important aspect of subsequent functional deterioration.

The pathophysiological significance of the level of postischemic blood flow in ischemia-reperfusion models remains a matter of controversy. The evolution of circulatory states of the infarcted spinal cord after reperfusion in the present model has been relatively well described with laser Doppler flowmetry and iodoantipyrine autoradiography. After a transient reperfusion hyperemia of approximately 30 minutes, blood flow returns to baseline but starts to decline 1 to 2 hours after reperfusion. The postischemic hyperemia at 2 hours after reperfusion, its severity and duration, depend on the duration of ischemia. At 2 hours, the spinal cord microcirculation showed dysregulation of CO2 reactivity during hypercapnia and dose-dependent ischemia-induced tissue edema. Therapeutic administration of a PAF antagonist (BN 50739, 10 mg/kg intraperitoneally) during 25 minutes of ischemia prevented the hyperperfusion and reduced tissue edema at 2 hours after reperfusion. At the time when some motor function could be regained, typically at 4 to 6 hours postischemia, hyperperfusion still persisted at approximately −30% of baseline in the ventral and intermediate gray matter. During the period of hyperperfusion, extravasation of EBA was milder than at 0.5 hours or at 12 to 24 hours. However, a delayed phase of hyperemia (12 to 24 hours) developed in these areas, which seemed to correlate with a poor prognosis of motor function in that study. It was suggested that the delayed hyperemia in the presence of BBB damage enhanced edema formation, which was 7.7% greater in the gray matter of nonhopping rabbits (motor scores 0, 1, 2) at 24 hours than in the rabbits with a better motor score. It appears from the above investigations that, in the presence of BBB damage, the perfusion state influences the passive extravasation of proteins and fluid according to Starling’s law. However, the hyperperfusion at 1 to 4 hours may represent a pathological correlate of BBB damage in that both can be inhibited by treatments aimed at preservation of microcirculation. This is supported by the present data demonstrating decreased gray matter Evans blue fluorescence (Fig. 4) and somewhat higher blood flow values in the treated groups. In contrast to the study of the natural course of this

**Fig. 4.** Graph showing quantitative analysis of the total Evans blue fluorescence in the gray matter of fresh-frozen spinal cord sections dissected at 6.5 hours after reperfusion. Fluorescence emissions were excited by scanning argon laser beam, gathered by computerized microscopy followed by image analysis, and expressed in arbitrary fluorescence units. Identical scanning parameters were used in the examination of all sections. The asterisks indicate statistical significance (analysis of variance) in comparison to vehicle group (p < 0.05). There were six rabbits in each group.

**Fig. 5.** Photomicrographs showing histopathology of spinal cord sections. The light microscopic appearance of the sections demonstrates histopathological changes such as cytoplasmic vacuolization, nuclear pyknosis, perineuronal halo formation, and shrunken cytoplasm with indistinct neuronal processes (arrowheads). These changes were particularly distinct in rabbits showing no hindlimb motor function. Upper: Histopathology in a representative paraplegic vehicle-treated animal. Lower: Histological appearance of a corresponding field of the spinal cord section in an anti-CD–treated rabbit, which had regained its hopping ability. The arrowheads indicate examples of normal appearing anterior horn large neurons, which were counted as illustrated in Fig. 6. H & E, original magnification × 200.
Leukocyte adherence in deteriorating stroke

Experimental stroke, hyperemia in the drug-treated rabbits with reduced BBB damage was not associated with worsened motor performance. These data also support the assumption that leukocytes can acutely perturb microcirculation in stroke.

Although the circulating neutrophil counts remained stable in the vehicle group, they were significantly elevated in both treatment groups at 1.5 hours after drug infusions and increased further by 5 hours (Fig. 2). Complete blood count differentials showed that neutrophils increased up to threefold by anti-CD treatment and lymphocytes only up to twofold from baseline. It is well known that the rare “leukocyte adhesion deficiency syndrome” caused by lack of synthesis of the β subunit of the CD11/CD18 complex is associated with persistent peripheral leukocytosis even in the absence of infectious episodes. The leukocytosis is believed to reflect the inability of neutrophils to marginate and adhere loosely to the endothelium. Individuals who suffered from the severest form of the disease (total lack of granulocyte CD11/CD18 surface antigens) exhibited white blood cell counts of 26 × 10^9/mm^3 at a minimum, whereas patients whose leukocytes expressed 2% to 7% of normal level of these antigens had white blood cell counts of 15 × 10^9/mm^3 at a minimum. Because the antibody that we used binds to the same β subunit, we consider the liberation of leukocytes into the circulation in our drug-treated rabbits to be a probable indicator of the efficacy of the adhesion antagonism. This interpretation fits with the fact that the treatment-induced changes in the circulating behavior of neutrophils correlated significantly with motor recovery and with neuronal survival (Fig. 7) and indirectly with the degree of BBB damage.

Clark and colleagues found the CD18 antibody (R3.3) effective in the ischemia-reperfusion model of the spinal cord, but ineffective in the model of multiple irreversible cerebral emboli. They concluded that inhibition of leukocyte adhesion is therapeutic only in association with reperfusion injury. The experiments by Takeshima, et al., do not seem to support this view. A monoclonal antibody against the CD18 molecule (MoAb 60.3; 2 mg/kg) was ineffective when given to cats prior to reperfusion injury induced by 90 minutes of ischemia of the middle cerebral artery territory and followed by 180 minutes of reperfusion. Although this is a very acute protocol for treatment effects to be demonstrated, the antibody failed to improve the recovery of evoked potential response and to decrease the tissue area not staining with triphenyltetrazolium chloride. Blood flow was not significantly reduced in any of the most perturbed cortical areas at 180 minutes of reperfusion, but it was on a sharp decline. It remains to be demonstrated, whether secondary deterioration can be prevented in similar models at a slightly later time point.

![Graph 6](image6.png)

**Fig. 6.** The number of normal appearing anterior horn large neurons was counted in each histological cross-section (solid bars). The open bars indicate the total number of anterior horn large neurons with or without pathological morphological changes. Each bar represents the average of neuronal counting in four sections in the caudal lumbar sacral spinal cord. The asterisks represent statistical significance (analysis of variance) in comparison to vehicle group. * and ** indicate p < 0.05 and p < 0.01, respectively. There were seven rabbits in each group.

![Graph 7](image7.png)

**Fig. 7.** Graphs illustrating the correlation analysis between the number of circulating neutrophils at 5.5 hours, the hindlimb motor score, and the number of anterior horn large neurons. Data points of all animals are pooled together regardless of which treatment was received.
when postischemic hypoperfusion and, in fact, accumulation of neutrophils have been observed simultaneously.14

We have recently discussed our concepts of the mechanism of ischemia-reperfusion brain injury, in which locally released mediators (for example, PAF, cytokines) expressing proinflammatory and procoagulant effects at the blood–endothelium interface play a key role in early microvascular perturbations leading to deterioration of tissue blood circulation and secondary brain damage.20 Progress in research of the signaling and tethering molecules governing leukocyte adhesion to endothelial cells has revealed that events both in endothelium and leukocytes participate in the regulation of inflammatory response.30 Whereas adhesion molecules expressed on endothelial cells, such as P-selectin, ICAM-1, ICAM-2, and E-selectin (ELAM-1), serve an important role in localization of the granulocyte response, other newly synthesized signaling molecules, such as interleukin-8 and PAF, make the adhesion tighter by upregulation of the neutrophil-based integrin receptors (CD11/CD18) and induce transendothelial migration.30 Rapidly produced endothelial PAF may be involved in the first steps of this process by influencing neutrophil “rolling” and then tight adherence through coordinate interaction with tethering molecules L-selectin on the neutrophils and P-selectin on the endothelium.25,30 The significance of the various consequences of leukocyte adherence has not been systematically studied in brain injury. However, observation of increased adhesion of granulocytes to membrane and tissue matrix proteins in acute stroke patients13 emphasizes the importance of such investigations. Our second hypothesis, that PAF antagonism might further improve the outcome in ischemia-reperfusion injury, in which leukocyte adherence is already inhibited, is not supported by the present data. Testing such a hypothesis with the present protocol, however, is relevant because all deleterious PAF effects are not mediated by leukocytes, and PAF antagonism was timed at a period when PAF production and its tissue effects have been demonstrated in this model.24 Although PAF antagonists have been shown to possess significant therapeutic effects in the present and other experimental stroke models,20 we should raise the possibility that significant PAF production occurs shortly after reperfusion and the postischemic treatment is not effective. However, these data do not exclude a role for PAF in the initiation of an inflammatory tissue signal and other PAF effects, which may be mediated in part by the actions of leukocytes. Because anti-CD treatment prevented the leukocyte adhesion to the endothelium, it is possible that PAF synthesized by endothelium and acting in situ had no chance to activate leukocytes. Furthermore, a recent report suggested that CD18-dependent leukocyte effects mediated the proinflammatory PAF effects in the brain, but CD18-independent PAF effects were observed in the lung.3 The possible effect of postischemic PAF antagonism in this model should be examined separately, perhaps with the duration of ischemia lasting less than 25 minutes.

Today, stroke patients can be recruited in clinical studies of thrombolytic therapy within 90 minutes after the onset of symptoms.4 In the future, the beneficial effect of thrombolytic therapy can probably be enhanced with an adjuvant regimen to protect the tissue against reperfusion injury. The timing of the present pharmacological intervention seems compatible with acute stroke treatment that restores tissue perfusion and permits salvage of ischemic tissue. Control of leukocyte activity deserves further study as one component of tissue resuscitation in ischemic central nervous system injury.

Acknowledgments

We thank Drs. Robert Rohleim and Ronald Faa, Boehringer Ingelheim Pharmaceuticals Inc., Ridgefield, Connecticut, for providing the CD18 antibody and WEB 2086.

Disclaimer

The opinions and assertions contained herein are the authors’ and are not to be construed as official or as necessarily reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

References

Leukocyte adherence in deteriorating stroke


Manuscript received August 26, 1993. Accepted in final form May 9, 1994.

This work was supported in part by PHS Grant NS28225, Uniformed Services University of the Health Sciences protocol RD19261, and an award from the Finnish Academy.

Preliminary results of this investigation were previously presented and published as a proceedings paper in the XVth International Symposium on Cerebral Blood Flow and Metabolism: Brain-91.21

Address reprint requests to: Perttu J. Lindsberg, M.D., Ph.D., Department of Neurology, University of Helsinki, Haartmaninkatu 4, SF-00290 Helsinki, Finland.