Inhibition of Polymorphonuclear Leukocyte Adherence Suppresses No-Reflow After Focal Cerebral Ischemia in Baboons

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Background and Purpose: While polymorphonuclear leukocytes may contribute to the “no-reflow” phenomenon after focal cardiac and skeletal muscle ischemia/reperfusion, their contribution to acute focal cerebral ischemia is unresolved. We have examined the role of polymorphonuclear leukocytes in microvascular perfusion defects after focal cerebral ischemia/reperfusion in a baboon model of reversible middle cerebral artery occlusion with the anti-CD18 monoclonal antibody IB4, which inhibits neutrophil adherence to endothelium.

Methods: Microvascular patency in the basal ganglia after 3-hour middle cerebral artery occlusion and 1-hour reperfusion (by india ink tracer perfusion) was quantified by computerized video imaging. Animals were randomized to receive intravenous IB4 infusion 15 minutes before reperfusion (n=7) or to receive no treatment (n=6). Binding of IB4 to baboon leukocytes was maximal within 5 minutes of infusion.

Results: In the untreated group, a significant reduction in patency was observed in microvessels <30 μm diameter; mean percent reflow was 51% in the capillary diameter class (4.0–7.5 μm) and 39% in the precapillary arteriole and postcapillary venule diameter class (7.5–30 μm). Infusion of IB4 before middle cerebral artery reperfusion increased reflow in microvessels of all size classes, most significantly in those 7.5–30 μm (p=0.049) and 30–50 μm (p=0.034) in diameter.

Conclusions: These results suggest that CD18-mediated polymorphonuclear leukocyte–endothelium adherence contributes to no-reflow predominantly in noncapillary microvessels and at least partially to that in capillaries. (Stroke 1992;23:712–718)

Key Words • antibodies, monoclonal • cerebral ischemia • microcirculation • neutrophils • baboons

Incomplete return of blood flow in the microvasculature after periods of transient global or hemispheric cerebral ischemia, the “no-reflow” phenomenon, has been documented in several experimental systems. Although a number of mechanisms for the no-reflow phenomenon have been proposed, including endothelial cell swelling,11 endothelial luminal membrane tags,2 and perivascular edema,3 recent studies of cardiac and skeletal muscle ischemia/reperfusion injury have suggested that polymorphonuclear (PMN) leukocytes may contribute to perfusion abnormalities in the microvasculature.12-14 In two previous studies using a closed nonhuman primate model of transient middle cerebral artery (MCA) occlusion and reperfusion, we have documented microvascular occlusions in the lenticulostriate arterial territory15 and microvascular obstruction containing PMN leukocytes within 60 minutes of MCA reperfusion.16 Their presence suggests a potential role of PMN leukocytes in the formation of microvascular perfusion defects or no-reflow early after MCA territory ischemia and reperfusion.

The rheological properties of the PMN leukocyte (large size and low deformability) and receptor-mediated adhesiveness to the endothelium after PMN leukocyte activation provide potential mechanisms for microvascular obstruction. PMN leukocyte adherence to postcapillary venular endothelium is mediated by a specific membrane-associated glycoprotein receptor, designated the CD11b/CD18 (Mac-1, Mol) complex.17 The well-defined murine monoclonal antibodies (MoAbs) 60.3 and 1^4, which are directed against the CD18 complex, have been shown to inhibit stimulated granulocyte aggregation, endothelial adherence, and granulocyte-mediated endothelial injury.9,18–22 To further examine the role of PMN leukocytes in microvascular no-reflow after focal cerebral ischemia and reperfusion in the MCA territory, we have used a systemic infusion of purified MoAb IB4 to block CD18-mediated PMN leukocyte–endothelial adherence before reperfusion in an awake baboon stroke model.

Materials and Methods

Thirteen adolescent male baboons (Papio anubis/cynocephalus) weighing 8.5–14.0 kg were used for the
present studies. All animals were conditioned and observed to be disease-free during a mandated quarantine period before entry into this study. All procedures were approved by the institutional Animal Research Committee and were performed in accordance with standards published by the National Research Council (the Guide for the Care and Use of Laboratory Animals), the National Institutes of Health policy on Humane Care and Use of Laboratory Animals, and the US Department of Agriculture Animal Welfare Act.

Preparation of the nonhuman primate model of right MCA occlusion and reperfusion and surgical implantation of the MCA occlusion device (Mentor Corporation, Goleta, Calif.) have been previously described in detail.15–16 Anesthesia was undertaken with halothane by intubation (3–5% induction) and maintained with the same agent (1.5–2.0%). Full recovery was routinely achieved 1–2 hours after completion of surgery. Subsequently, all subjects were allowed a 7-day, procedure-free interval before entry into the experimental protocol and displayed normal neurological function (score of 100) during the interval. At study onset, the subjects had an average hematocrit of 36.9±3.7, platelet counts of 510.5±93.0×10³/µl, and experiment baseline total white cell counts of 12.7±3.0×10³/µl (absolute PMN leukocytes, 5.7±2.8×10³/µl).

Animals were randomized into control (n=6) and IB4-treated (n=7) groups. Occlusion of the right MCA was accomplished by inflation of the extrinsic MCA balloon to 100 µl in the awake subject. Following a 3-hour period of MCA occlusion, reperfusion of the MCA territory for 1 hour was achieved by balloon deflation. The treatment group received an intravenous bolus of 2–4 mg/kg of the murine MoAb IB4 15 minutes before the balloon deflation (reperfusion) (Figure 1). The IB4 dose was based on preliminary in vitro studies that demonstrated 1) 57% inhibition of adherence of unstimulated baboon PMN leukocytes to human umbilical vein endothelium by 15 µg/ml MoAb 60.3 and 75% inhibition of adherence of phorbol myristate acetate (PMA)- and formyl-methionyl-leucyl-phenylalanine (FMLP)-stimulated PMN leukocytes, and 2) 80% inhibition of baboon PMN leukocyte adherence at 12 hours by a 2-mg/kg infusion of IB4 (data not shown).

The experiments were terminated by pressure-perfusion fixation with india ink tracer (scheme 2) 60 minutes after MCA balloon deflation. Perfusion-fixation consisted of an initial perfusion flush phase to wash out all blood elements under antithrombotic and isotonic conditions and a subsequent isosmotic fixation/tracer–perfusion phase. At the time of sacrifice, under pentothal Na+ (15 mg/kg infusion) anesthesia and assisted ventilation, the thorax was opened and the descending aorta and inferior vena cava clamped. The left ventricle was rapidly cannulated and perfused by a chilled flush solution containing 25 g/l bovine serum albumin (Sigma, St. Louis, Mo.), 2,000 units/l heparin, and 6.7 µM Na+ nitroprusside (Fisher Scientific, Fair Lawn, N.J.) in Plasmalyte (Baxter Healthcare, Deerfield, Ill.) adjusted to 340 mosmol/l with NaCl and pH 7.4 at a pressure of 160–180 mm Hg for 3 minutes. This was immediately followed by fixation with chilled carbon suspension-fixative solution consisting of India ink (Pelican Fount India, Pelican AG, Hannover, FRG) (1:1, vol/vol) in Plasmalyte/paraformaldehyde (2% final concentration)/glutaraldehyde (0.15% final concentration) for 17 minutes. High mean arterial perfusion pressures were chosen to maximize vascular patency.

The exposed brain was immersed in alcohol-formaldehyde-acid (AFA) solution (87% ethanol, 10% formaldehyde, 3% glacial acetic acid, vol/vol) for 1 week. The brain was subsequently removed, sectioned coronally at 1-cm intervals, and immersed for another week in AFA solution to achieve complete intravascular gelation of the carbon tracer. Tissue blocks (1.0 cm×1.0 cm×0.2 cm) from stereanatomically identical sites of the left and right basal ganglia and from the temporal lobe in the left (normal) side were embedded in glycol methacrylate (Polysciences, Inc., Warrington, Pa.), sectioned to 10-µm thickness, stained with basic fuchsin/methylene blue, and examined by light microscopy for the presence of India ink–filled (patent) microvascular structures.

Sections were analyzed with the aid of a computerized video imaging system consisting of an image system unit connected in-line with a Hamamatsu C2400–07 Newvicon NTSC video camera (Hamamatsu Photonics, Hamamatsu, Japan) staged vertically on the light microscope (VIDAS, Kontron and Carl Zeiss, Munich, FRG). Ninety nonoverlapping 526.1 µm×491.4 µm images at ×200 optical magnification (25 mm²) were processed in each section, and the minimum transverse diameter of India ink–filled microvessels was computed. The sections were taken at 30-µm intervals from one another from stereanatomically identical sites of both ischemic (right) and control (left) basal ganglia. An identical number of fields (4–7 sections) from each of the paired basal ganglia were analyzed until 2,000 vessels were counted in the left control basal ganglia. Reproducibility and reliability data have been reported previously.16

Adequacy criteria for complete analysis of microvascular patency were developed in the course of this and a previous study16: 1) when the hemispheric surface (gray matter) of the parietal or temporal cortex was inhomogeneously stained black by visual inspection, the India ink perfusion was judged inappropriate, and the animal was excluded (n=1); 2) partial packing of carbon elements in the vascular lumen and segmented carbon

FIGURE 1. Graph of serial change in IB4 binding to polymorphonuclear (PMN) leukocytes. Closed circles, PMN leukocyte CD18 occupancy by IB4; open circles, IB4 binding sites available. Each point represents n=7.
columns in the microvessels suggested incomplete india ink perfusion or failure of gelation and caused miscounting of the microvessels during video image processing. When such events were observed, the preparation was inappropriate for video imaging analysis, and the animal subject was excluded (n=1). With these exclusions, the ratio of patent capillaries and all microvessels in the left basal ganglia to the left temporal cortex (layers I–VI) was 0.54±0.27 and 0.59±0.27 (n=11; 0.25 cm² regions of interest; data not shown), respectively. This compares favorably with previous observations in which the distribution and morphology of patent microvessels in the left basal ganglia were judged excellent. Comparable ratios from animals excluded by criterion 1 fall >2 standard deviations (SDs) from the mean, and by criterion 2 fall within the mean±2 SD for patent capillaries and all microvessels.

The extent of reflow is expressed as “percent reflow,” the ratio of the number of microvessels containing carbon (i.e., patent) in the ischemic to control basal ganglia expressed per 100 vessels. Microvessels include those vessels of <100 μm minimum diameter. Data are displayed as a continuum of vessel diameters and as discrete vessel size classes. For the purpose of this study, microvessels were divided into four diameter classes: 1) vessels 4.0–7.5 μm in diameter constitute capillaries; 2) vessels 7.5–30 μm in diameter include precapillary arterioles (metarterioles) and postcapillary venules; 3) vessels 30–50 μm in diameter correspond to small arterioles and connecting venules; and 4) vessels >50 μm in diameter correspond to those histologically characterized as muscular arterioles and venules.

For IB4–PMN leukocyte binding determinations, peripheral blood samples obtained by venipuncture were drawn into Na² heparin (100 units/ml) at fixed intervals before IB4 infusion and at 5, 10, 15, 30, and 60 minutes after IB4 infusion. The whole blood specimens stored at 4°C were immediately analyzed for CD18 receptor occupancy and receptor availability by flow cytometric analysis. All procedures were performed at 4°C. A 0.2-ml aliquot of blood was centrifuged at 2,000g; the pellet was washed twice with 1 ml cold phosphate-buffered saline (PBS), pH 7.4, and reconstituted with 0.2 ml PBS at 4°C. IB4 bound to the surface of the cells was detected with FITC-conjugated goat anti-mouse secondary antibody (GAM; Sigma). Fifty microliters of a 1:80 dilution of GAM was added to 50 μl diluted reconstituted blood and incubated on ice for 30 minutes. The samples were washed twice with cold PBS before analysis. As an indication of the proportion of surface CD18 molecules that were occupied by IB4, FITC-labeled IB4 antibody was added to the blood samples at saturating concentration (2.5 μg/ml). Samples were washed once with cold PBS before flow cytometric analysis. All samples were analyzed on a Becton Dickinson FACSscan flow cytometer (San Jose, Calif.) after lysis of red blood cells with 10 μg/ml saponin. The PMN leukocyte population was selected on the basis of characteristic forward versus orthogonal light scattering. Results are expressed as median green fluorescence channel number for GAM-stained samples or number of fluorescent IB4 molecules bound to the PMN leukocytes. Fluorescent calibration particles (Flow Cytometry Standards Corp., Research Triangle Park, N.C.) were used to calibrate the FACSscan to allow calculation of numbers of antibody molecules bound.

Neurological function was assessed according to a well-described quantitative (100-point) scale weighted toward unilateral motor function loss.

All values are expressed as literal values (e.g., fluorescence intensity) or as the mean±SD. Data were analyzed using either paired or unpaired Student’s t test (one tailed and two tailed), and statistical significance was set at p<0.05 or 2p<0.05 where appropriate. Because microvascular diameter is a continuous variable, vascular data were classified in biologically relevant categories of size for statistical analysis (see above).

Results

Binding of the MoAb IB4 to baboon PMN leukocytes plateaued within 5 minutes of IB4 infusion and remained unchanged throughout the duration of the experiments (Figure 1). A reciprocal mean 83.3% reduction of free PMN leukocyte CD18 binding sites was observed from 4.1±2.0x10⁴ free sites before IB4 infusion to 6.8±2.4x10⁴ free sites after IB4 infusion. IB4 does not bind to primate platelets (data not shown).

Two animals in the IB4-treated group were excluded from patency analysis because of inhomogeneous (n=1) and incomplete India ink perfusion (n=1), so that six animals were included in the control untreated group and five animals in the IB4-treated group. Baseline hematocrit, platelet counts, total white blood cell counts, and neurological scores after occlusion of the MCA were not significantly different between the groups (Tables 1 and 2). An increase in the mean total white blood cell count and absolute PMN leukocyte count from baseline was noted in the initial 60 minutes of MCA occlusion in both cohorts. The IB4 infusion did not alter the total white blood cell counts or fraction of PMN leukocytes of the treated subjects (Table 1).

In the control group, within 60 minutes after ischemia/reperfusion, a statistically significant reduction in the number of patent microvessels was observed in microvessels of <30 μm minimum diameter (Figure 2). The reduction occurred in the capillary range (4.0–7.5 μm minimum diameter, p=0.042), but was most significant in the range 7.5–30 μm (p=0.009). This range is consistent with the precapillary arteriole–postcapillary venule diameter class (Figure 3). The percent reflow in microvessels >50 μm in diameter varied greatly with individual animals because of the small number of vessels counted in this size class.

Infusion of IB4 immediately before MCA reperfusion resulted in a significant increase in the microvascular percent reflow (Figures 2 and 4). While an increase in reflow was apparent in all vessel classes <100 μm, the difference in percent reflow between the IB4-treated and control groups was statistically significant in the classes of 7.5–30 μm diameter (postcapillary venule/precapillary arteriole, p=0.049) and of 30–50 μm diameter (connecting venule/small arteriole, p=0.034).

To determine whether these findings could be explained by microvascular dilatation, the proportion of the total number of vessels in the ischemic and the contralateral basal ganglia was calculated (Figure 5). No significant shift in the distribution of microvessels undergoing ischemia/reperfusion compared with the nons ischemic terri-
Discussion

The experiments presented here are the first direct evidence that modulation of PMN leukocyte adherence may alter microvascular reflow after focal cerebral ischemia and reperfusion. Since the suggestion by Ames and coworkers1 that the postischemia no-reflow phenomenon, regarded as occlusion of carbon tracer perfusion, was confined to the microvasculature in global cerebral ischemia and reperfusion in the MCA territory.16 The adhesive interaction between activated PMN leukocytes and microvascular endothelium mediated by the leukocyte membrane CD11b/CD18 complex occurs in postcapillary venules and may be abrogated by MoAb directed against the complex.19,21 That IB4 could prevent the sudden rise in vascular resistance known to accompany ischemia/reperfusion in a canine skeletal muscle preparation when given before reperfusion26 is consistent with the experiments reported here.

Improvement of local cerebral blood flow (CBF) after ischemia/reperfusion has accompanied partial depletion of PMN leukocytes by anti-neutrophil serum in a rabbit model of hypotension/embolic stroke,27 whereas pres ischemic depletion of PMN leukocytes by intraperitoneal injection of an anti-neutrophil serum in a rat hypotension/carotid occlusion model increased local cortical CBF significantly but basal ganglionic CBF marginally.28 This experience is also consistent with our results. However, polyclonal sera may cause cellular and vascular effects through alternative mechanisms (e.g., platelet depletion, vasomotor reactivity through complement activation) which may also contribute to alterations in CBF. The failure to enhance regional CBF in

Table 1. Serial Hematologic Studies in IB4-Treated and Control Groups

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Baseline</th>
<th>60 minutes</th>
<th>120 minutes</th>
<th>180 minutes*</th>
<th>Reperfusion 60 minutes*</th>
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<tr>
<td>WBC (x10^9/μl)</td>
<td>Control 6</td>
<td>11.0±2.1†</td>
<td>21.8±6.2</td>
<td>29.1±6.9</td>
<td>27.4±6.4</td>
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<tr>
<td></td>
<td>IB4 7</td>
<td>13.9±2.9†</td>
<td>25.6±3.8</td>
<td>28.1±7.8</td>
<td>26.5±3.4</td>
<td>24.7±3.9</td>
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<td>PMN leukocyte (x10^3/μl)</td>
<td>Control 6</td>
<td>4.4±1.7</td>
<td>15.9±6.3</td>
<td>21.7±6.1</td>
<td>21.2±5.2</td>
<td>19.8±2.8</td>
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<tr>
<td></td>
<td>IB4 7</td>
<td>6.2±3.4</td>
<td>18.6±3.6</td>
<td>21.9±7.3</td>
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<tr>
<td>Platelet (x10^3/μl)</td>
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<td>492±27</td>
<td>540±81</td>
<td>552±72</td>
<td>562±70</td>
<td>581±102</td>
</tr>
<tr>
<td></td>
<td>IB4 7</td>
<td>511±128</td>
<td>570±78</td>
<td>558±145</td>
<td>531±126</td>
<td>513±101</td>
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<tr>
<td>Hematocrit (volume %)</td>
<td>Control 6</td>
<td>37.6±4.7</td>
<td>38.5±4.9</td>
<td>37.9±4.6</td>
<td>38.4±4.6</td>
<td>34.7±3.8</td>
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<tr>
<td></td>
<td>IB4 7</td>
<td>35.9±2.9</td>
<td>36.7±2.5</td>
<td>37.3±3.1</td>
<td>36.1±2.9</td>
<td>33.8±4.2</td>
</tr>
</tbody>
</table>

Values are mean±SD. MCA, middle cerebral artery; WBC, white blood cell count; PMN, polymorphonuclear.
†p=0.069; all other comparisons 2p>0.20.

Table 2. Serial Neurological Score in IB4-Treated and Control Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Baseline</th>
<th>60 minutes</th>
<th>120 minutes</th>
<th>180 minutes*</th>
<th>Reperfusion 60 minutes*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>100</td>
<td>60.7±24.2</td>
<td>52.7±15.3</td>
<td>57.0±18.5</td>
<td>57.0±18.5</td>
</tr>
<tr>
<td>IB4-treated</td>
<td>5</td>
<td>100</td>
<td>65.2±24.1</td>
<td>59.2±22.9</td>
<td>57.4±24.0</td>
<td>59.2±22.9</td>
</tr>
</tbody>
</table>

2p = 0.764 0.585 0.976 0.864

Values are mean±SD. MCA, middle cerebral artery.
a cohort receiving intravenous anti-neutrophil serum may be explained by microvascular trapping of residual leukocytes (group S28). Also, antineoplastic agents used to induce leukopenia affect bone marrow production of all cell lines (myelocytic and megakaryocytic), so that a role for platelets in the genesis of ischemia/reperfusion injury as suggested previously15,16,29 cannot be excluded by those experiments.

An important concern is the activation state of the neutrophil under various experimental conditions of ischemia. The increase in PMN leukocyte number noted here represents a response to stress common to several animal species.30,31 IB4 is known to inhibit the endothelial binding of activated PMN leukocytes in humans and other species.17 The absence of an increase in free CD18 receptor sites during MCA occlusion implies negligible up-regulation of this receptor and minimal overt systemic PMN leukocyte activation during focal ischemia in these experiments (Figure 1).

In previous studies of cerebrovascular no-reflow using tracer perfusion techniques, the microvascular component of no-reflow was not quantified.13,5,10 In this study, no-reflow was quantified by comparison of microvascular patency in the ischemia/reperfusion zone with that of the contralateral nonischemic side. Homogeneity of tracer perfusion is a prerequisite. Adequacy of tracer perfusion was addressed by the ratio of patent microvessels in the basal ganglia to the temporal cortex of the normal side. The capillary ratio obtained in this study (0.54±0.27) compares favorably with that reported in rodents as determined by fluorescent tracer (caudate nucleus/auditory cortex=0.73).12 The relatively small difference may represent a variation among species. A pairwise comparison between ischemic and contralateral nonischemic basal ganglia would be expected to cancel any common alterations in perfusion conditions within either vascular territory. This assertion is valid when the basal ganglia have a comparable number and
distribution of microvessels. No difference in the normalized microvascular distribution of the ischemic or normal territories in both cohorts was observed.

Microvascular vasodilatation may explain an enhancement of reflow after IB4 infusion. Significant alteration of microvascular diameter does not occur in the myocardium after 1 hour of ischemia, although capillary dilatation is known to occur 24 hours after ischemia/reperfusion in the MCA territory. The absence of bias or shift to larger diameter in the normalized distribution of the ischemic territory in the present experiments argues strongly against a significant contribution of vascular enlargement. Recruitment of previously unperfused microvessels may also explain reflow enhancement, although the concept of recruitment is controversial. If it does exist, a disproportionate increase in capillary size class would be required. IB4 is not known to stimulate vasodilatation or vascular recruitment in other vascular beds. With the techniques used here, plasma- and erythrocyte-containing microvessels are patent, as demonstrated in the control tissues, unless obstruction occurs.

Although it was not possible to differentiate postcapillary venules from precapillary arterioles in this study, PMN leukocyte—endothelial adhesion on the venular side may have been responsible for the occlusion of noncapillary microvessels. In the simplest model of the lenticulostriatal microvasculature, persistent capillary obstruction could also result from precapillary arteriolar occlusion; however, release of PMN leukocytes from the arteriolar side might be expected to further obstruct the capillary field and decrease reflow. Partial reflow in capillaries and near-complete reflow in noncapillary microvessels after prererefusion IB4 treatment suggests structural complexity to this microvasculature, significant postcapillary vascular obstruction, or capillary flow restriction. The low deformability of PMN leukocytes suggests a significant hemodynamic resistance which, during a low-flow state with altered endothelial integrity, may produce functional or permanent capillary obstruction. Active receptor-mediated adhesion by endothelial adhesion molecules (e.g., ELAM-1 and GMP140) or other attachment proteins, platelet—PMN leukocyte complexes, or fibrin may contribute to microvascular obstructions during either ischemia or reperfusion in this model. Obstruction of the postcapillary venules by PMN leukocyte clusters may lead to retrograde occlusion and secondary thrombosis of some capillaries in the preparations studied here. Although these results imply that thrombosis alone is not the primary cause for the microvascular perfusion defect, recent observations in untreated animals indicate the presence of fibrin in association with PMN leukocyte—platelet aggregates. The possibility that thrombosis and PMN leukocyte occlusion may occur at different sites in the microvasculature cannot be discounted. This is further supported by the association of the potent procoagulant tissue factor in nonischemic basal ganglia with noncapillary microvessels. Prereperfusion anti-PMN leukocyte strategies have been shown to significantly reduce functional indicators of ischemia/reperfusion injury and to lower mortality in noncerebral ischemic experimental models. Data regarding functional outcome in cerebral ischemia/reperfusion after anti-PMN leukocyte strategies is quite limited, but encouraging. Preischemic use of vinblastine to induce leukopenia was accompanied by partial improvement in electroencephalographic and somatosensory-evoked potential changes in a rat model of reversible incomplete forebrain ischemia. A significant reduction in infarction volume and intracranial pressure (due to edema) in a rabbit carotid thromboembolism/hypotension model was observed after pretreatment with an anti-neutrophil serum.

The experimental paradigm used here is clinically relevant in that IB4 was initiated immediately before reperfusion after MCA occlusion. This setting accords with documented early reperfusion following the use of fibrinolytic agents in focal cerebral ischemia. However, in the present study, despite the increase in reflow, there was little change in the neurological score within 1 hour after reperfusion between control and IB4-treated groups. In these short-term studies, as suggested by clinical trials, the observation period was not sufficient to detect any meaningful clinical alteration.

To further elucidate the clinical effect of IB4, studies in which neuropathological and neurological outcomes are observed for longer periods are required.

Acknowledgment

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References

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