Experimental Acute Thrombotic Stroke in Baboons

GREGORY J. DEL ZOPPO, M.D., BRIAN R. COPELAND, M.D., LAURENCE A. HARKER, M.D., THOMAS A. WALTZ, M.D., JACK ZYROFF, M.D., STEPHEN R. HANSON, PH.D., AND ELENA BATTENBERG, B.A.

SUMMARY To study the effects of antithrombotic therapy in experimental stroke, we have characterized a baboon model of acute cerebrovascular thrombosis. In this model an inflatable silastic balloon cuff has been implanted by transorbital approach around the right middle cerebral artery (MCA), proximal to the take-off of the lenticulostriate arteries (LSA). Inflation of the balloon for 3 hours in six animals produced a stereotypic sustained stroke syndrome characterized by contralateral hemiparesis. An infarction volume of $3.2 \pm 1.5 \text{cm}^3$ in the ipsilateral cortex at 14 days was demonstrated by computerized tomographic (CT) scanning at 10 days following stroke induction and $3.9 \pm 1.9 \text{cm}^3$ in the right corpus striatum. The safety and efficacy of antithrombotic therapies in acute stroke should reproduce the human vascular anatomy and thrombotic processes. Additionally, the in situ thrombotic stroke may be useful to assess the safety and efficacy of thrombolytic therapies.

Although an animal model of in situ cerebrovascular thrombosis should be valuable to assess the safety and efficacy of antithrombotic therapies in acute stroke, none of the available models is satisfactory. In the animal models described, thromboembolic stroke and reversible cerebral ischemia have been produced in animals phylogenetically distant from humans using methods that have not produced potentially reversible in situ thrombosis.

To be useful in the study of antithrombotic therapies, an experimental animal model of thrombotic stroke should reproduce the human vascular anatomy and thrombotic processes. Additionally, the in situ occluding thrombus should be induced without compromising vascular continuity so that measures to establish reperfusion can be assessed. In light of these considerations, the baboon is a suitable animal since both the hemostatic apparatus and the cerebral vascular anatomy of the baboon have been well-characterized and closely resemble the human systems. Furthermore, the baboon is readily adapted to standard clinical, surgical, and radiographic procedures.

In this context, we have studied a modification of the baboon model of cerebral ischemia that utilizes transorbital implantation of an inflatable silastic cuff around one middle cerebral artery (MCA). In this model, prolonged MCA occlusion produces a defined region of cerebral infarction in the area supplied by the lenticulostriate branches of the MCA (i.e. caudate nucleus, putamen, and internal capsule) and a consequent contralateral hemiparesis and facial paresis. Since the MCA remains patent after deflation of the cuff, we reasoned that thrombus forms in the lenticulostriate arteries and their perforating branches which subserve the region of cerebral infarction. In this report we present data confirming this postulate.

Methods

Animals Studied

Twenty-two adolescent male baboons (Papio cynocephalus/anubis), approximately 12–14 kg in weight,
have been employed for the functional, radiologic, neuropathological, and isotopic studies. All animals were dewormed and observed to be disease-free for at least six weeks prior to study. Baseline circulating platelet concentrations in the study animals averaged $382,000 \pm 129,000$ platelets/\(\mu l\) ($\pm 1$ SD), hematocrits were $35 \pm 3\%$, and the mean white cell count was $8,300 \pm 4,100/\mu l$. The animals were documented to have normal neurologic function prior to their involvement in the experimental procedure.

All procedures were approved by the institutional Animal Care and Use Committee and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

**Procedure for Device Implantation**

A miniature inflatable silastic balloon cuff assembly (Mentor Corporation, Goleta, CA), similar to that described by Spetzler, et al,\textsuperscript{16} was placed around the right MCA using a transorbital approach. The device consisted of an eccentric inflatable balloon (capacity 0.05 ml) with stainless steel artery hooks for stabilization about the right MCA, and a silastic connector tube and plug. The latter portion of the device was placed subcutaneously for remote post-operative inflation of the balloon. The device was loaded with diatrizoate meglumine (MD-76\textsuperscript{®}; Mallinkrodt, St. Louis, MO) prior to implantation to facilitate subsequent radiographic documentation of balloon inflation in vivo.

Anesthesia was achieved and maintained with pentothal sodium (10 mg/kg induction bolus and 10 mg/kg/hr). The procedure involved a right transorbital approach, with enucleation, removal of the medial sphenoid wing and portions of the adjacent middle fossa, exposure of the internal carotid artery and MCA, and placement of the occluding device about the MCA proximal to the take-off of the lenticulostriate arteries. Fixation of the device and catheter portion of the device was completed with papaverine-impregnated Gelfoam and radiolucent methylmethacrylate in the enucleated orbit. The connector tube and plug were placed subcutaneously near the apex of the head in a tunnel under the scalp.

Postoperative recovery typically occurred within 2–6 hours without neurologic deficit; the animals were fully active within 12–24 hours. Neurologic function was monitored for an additional 5 days prior to any experimental procedure.

**Experimental Stroke Protocols**

Four experiment formats were employed for the studies reported here: 1) long-term (14 day) experiments to correlate neurological functional outcome, with cerebral infarction size determined by CT (cerebral) scan and neuropathology following the 3 hour MCA occlusion (n = 6); 2) short-term (4 hour) experiments to examine the lenticulostriate (LSA) dependent microvascular bed following the 3 hour MCA occlusion (n = 10); 3) short-term (4 hour) experiments to evaluate the effect of combination antiplatelet/anticoagulant agents on LSA microcirculatory occlusions (n = 4); and 4) short-term experiments to evaluate the possibility that platelets might accumulate in the ischemic region after previous ischemic injury (n = 2).

In long term (14 day) experiments, six animals with MCA occlusion devices served as untreated controls according to the protocol set out in table 1. In the long-term experiments, following the 3 hour MCA occlusion, lateral skull films and selective right carotid angiography were performed at 3–3.5 hours, after which a 1 hour intracarotid infusion of saline at 30 ml/hr was begun. Serial CT cerebral scans were performed within 1 hour, at 24 hours, and at 10 days post-infusion; serial neurological examinations were performed as noted above; and the experiments were terminated at 14 days by pressure perfusion-fixation. The results of these experiments are reported in table 5.

In one set of short term (4 hour) experiments, ten animals with MCA occlusion devices served as un-

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Time (hours)</th>
<th>Agents</th>
<th>Ne</th>
<th>Angio</th>
<th>Lat SF</th>
<th>CT scan</th>
<th>Neuropath</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Right MCA occlusion (inflation of device)</td>
<td>0</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>2. Release of occlusion (deflation of device)</td>
<td>3</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>3. Placement of catheter in right carotid artery</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>4. Initiate IC saline infusion</td>
<td>3.5</td>
<td>IC saline</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>5. Terminate IC saline infusion</td>
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<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>6. Assessment of intracerebral hemorrhage</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. Documentation of cerebral infarction/hemorrhage</td>
<td>24</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
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<td></td>
<td>48</td>
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<td></td>
<td>72</td>
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<td></td>
<td>96</td>
<td>X</td>
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<td></td>
<td>240</td>
<td>X</td>
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<tr>
<td></td>
<td>336</td>
<td>X</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**TABLE 1 Experimental Protocol of Long Term Experiments (14 day)**

Lat SF = lateral skull films; Angio = selective right carotid angiography; IC = intracarotid infusion; CT scan = serial CT cerebral scans; Ne = serial neurological examinations.
TABLE 2  Protocol of Short Term Experiments (4 hour) — Control Animals

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Time (hours)</th>
<th>Agents</th>
<th>Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Preparation of $^{111}$In autologous platelets</td>
<td>-4</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>2. Infusion of $^{111}$In autologous platelets (2 mCi)</td>
<td>-1</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>3. Infusion of Evans blue dye (10 ml/kg)</td>
<td>-0.5</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>4. Right MCA occlusion (inflation of device)</td>
<td>0</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>5. Release of occlusion (deflation of device)</td>
<td>3</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>6. Perfusion-fixation</td>
<td>4</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>7. Whole brain imaging ($\gamma$ camera)</td>
<td>5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations same as table 1.

treated controls according to the protocol set out in table 2. In this set of experiments, lateral skull films, angiography, and IC saline infusions were not performed. Six animals received an intravenous infusion of Evans blue dye (10 ml/kg) only prior to MCA occlusion and served to identify thrombotic occlusions in the LSA territory by fluorescence and light microscopy, and by electron microscopy. Four animals received $^{111}$In-labeled autologous platelets and Evans blue dye prior to MCA occlusion and the results are reported here in detail in table 6. The experiments were terminated by pressure-perfusion fixation 1 hour after release of the occlusion and imaging of the whole brain was performed in the group receiving $^{111}$In-labeled platelets.

In a second set of short-term (4 hour) experiments, four animals received the antiplatelet/anticoagulant regimen ticlopidine/heparin in addition to the $^{111}$In-labeled autologous platelets and Evans blue dye according to the protocol set out in table 3. Radiographic studies were not performed in this group, and the experiments were terminated by pressure-perfusion fixation at 4 hours. All fixed whole brain specimens were imaged for 12 hours by $\gamma$ scintillation camera. The results are presented in table 6.

Finally, two untreated animals received $^{111}$In-labeled autologous platelets directly following the Evans blue infusion and 3 hour MCA occlusion and reperfusion, to serve as imaging controls. Following perfusion-fixation 1 hour after the infusion of $^{111}$In-labeled platelets, the fixed whole brain specimens were imaged for 12 hours by $\gamma$ scintillation camera.

For each experiment, the animal was fitted with a stabilization collar and placed into an acute restraining chair of the Davis type (Primate Products, Woodside, CA). While awake and alert, and under local anesthesia, the distal terminus of the occluder device was exposed by sterile surgical cut-down on the scalp. The right MCA balloon was inflated by infusion of 0.05 ml diatrizoate meglumine (MD-76®) via a calibrated syringe attached to the exposed terminus of the device. The balloon device was maintained inflated for 3 hours. Deflation of the occluding balloon was achieved by removal of the radiocontrast material, the end of the device was replaced, and the skin incision closed.

**Neurologic Function**

Neurologic function was objectively assessed at 10 minutes, 1 hour, 2 hours, and 3 hours following MCA occlusion by means of a quantitative neurological scale (table 4) developed by Spetzler and associates. Because the ischemic lesions produce a unilateral loss of motor function, the 100-point scale was weighted toward motor loss, with less important contributions for other changes. Assessment was performed independently by two blinded observers with experience in evaluating primate neurologic functions. All animals

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Time (hours)</th>
<th>Agents</th>
<th>Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Ticlopidine (50 mg/kg/d)</td>
<td>Ticlopidine</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>2. Preparation of $^{111}$In autologous platelets</td>
<td>-4</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>3. Infusion of $^{111}$In autologous platelets (2 mCi)</td>
<td>-1</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>4. Infusion of Evans blue dye (10 ml/kg)</td>
<td>-0.5</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>5. Infusion of heparin (1000 U/kg)</td>
<td>-0.4</td>
<td>Heparin</td>
<td>X</td>
</tr>
<tr>
<td>6. Right MCA occlusion (inflation of device)</td>
<td>0</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>7. Release of occlusion (deflation of device)</td>
<td>3</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>8. Perfusion-fixation</td>
<td>4</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>9. Whole brain imaging ($\gamma$ camera)</td>
<td>5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations same as table 1.
employed in these experiments developed a stereotypic stroke syndrome (see "Results").

Radiographic Procedures

Standard lateral skull radiographs were routinely performed to document device placement and function in long-term experiments. In six animals, unilateral (right) carotid angiography was also performed via the transfemoral route using an arteriotomy of the right femoral artery for introduction of a 4.1 French angiographic catheter (Harwood-Nash Spec 2, Cook, Inc., Bloomington, IN). The catheter was advanced into the ipsilateral carotid artery to the carotid bifurcation. Each angiographic study employed a one second injection of 3 ml diatrizoate meglumine (MD-60®). The animals were sedated with ketamine HCl (8 mg/kg) for the procedure.

Serial computerized tomographic (CT) scans of the cerebral hemispheres provided documentation and evaluation of experimental cerebral infarction or hemorrhage into the region of ischemia. All scans were performed on a modified EMI 1005 CT Scanner with 60-second scan time. In long-term experiments, serial CT cerebral scans were routinely obtained baseline (postoperative) and within 1 hour, at 24 hours, and at 10 days post-MCA occlusion. The scanning equipment employed was shown to resolve a volume of hemorrhage (high attenuation) of less than 0.5 ml (data not shown). The volume of cerebral infarction was estimated by summation of the individual areas of low attenuation multiplied by the slice thickness from the CT scan obtained at 10 days (table 5). The volume approximation obtained in this fashion was compared with that obtained by neuropathology. In general, the method of infarction volume approximation from CT scan employed by Pullicino and Kendall1718 was consistently less than that used in this study (data not shown).

Gross Neuropathology

Gross whole brain specimens were prepared in situ by pressure perfusion-fixation for 30 minutes with 10–15 liters of 10% phosphate buffered formalin. The pressure-perfusion apparatus consisted of a high pressure N₂ cylinder in series with a manometer, fixative reservoir, and an in-line flow meter in the fixative delivery tubing.

Under ketamine analgesia and deep barbiturate anesthesia both internal jugular veins were cannulated for subsequent exit drainage. While maintaining mechanical ventilation, a median sternotomy was performed. Following division of the pleura, the perfusion delivery tube was inserted by 10-gauge needle into the left ventricle, and the aorta was clamped distal to the takeoff of the left common carotid artery. To maintain

<table>
<thead>
<tr>
<th>TABLE 4 Quantitative Neurological Scoring for Stroke in Baboons</th>
</tr>
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<tbody>
<tr>
<td>I. Motor Function (70-10)</td>
</tr>
<tr>
<td>Normal strength normal function</td>
</tr>
<tr>
<td>favors opposite extremity</td>
</tr>
<tr>
<td>Hemiparesis mild</td>
</tr>
<tr>
<td>severe</td>
</tr>
</tbody>
</table>

II. Behavior (20-0)

Normal aggression (swings on cage bars) | 20 |
Aware of surroundings moves in response to examiner | 15 |
not active | 5 |
Coma | 1 |
Death | 0 |

III. Ocular and Cranial Nerve Function (10-2)

Facial movement normal | 5 |
paretic | 1 |
Visual field normal | 5 |
paretic | 1 |

Score

<table>
<thead>
<tr>
<th>TABLE 5 Effects of 3 Hour Reversible Occlusion of Right Middle Cerebral Artery in Baboons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Functional score</td>
</tr>
<tr>
<td>Baseline 3 hr 24 hr 14 d</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>100 32</td>
</tr>
<tr>
<td>100 27</td>
</tr>
<tr>
<td>100 31</td>
</tr>
<tr>
<td>100 46</td>
</tr>
<tr>
<td>100 35</td>
</tr>
<tr>
<td>100 27</td>
</tr>
</tbody>
</table>

100 33±7 36±11 36±11 0.7±0.3 3.2±1.5 3.9±1.9

*Volume of cerebral infarction.
CT derived volumes represent the summation of (average area of low attenuation) x (slice thickness). Volumes prior to balloon inflation (baseline) represent presence of balloon device preceding stroke induction.
Neuropathology derived volumes represent the summation of (area of infarction) x (slice thickness).
See Methods for details.
†Perivascular extravasation of erythrocytes.
normal distension of the cerebral vasculature, the fixative flow in the perfusion system was adjusted to achieve 90–100 torr intraarterial perfusion pressure within the cerebral circulation. Flow rates of approximately 500 ml/min were required to maintain (carotid) arterial pressure within these limits when jugular outflow was unchecked. Upon completion of 30 minutes perfusion-fixation, the brain was removed and immersed in 10% phosphate buffered formalin at 4°C until brain cutting.

Good quality serial coronal sections of 2 mm (± 0.1 mm) thickness of the perfused-fixed brains were obtained by rostral to caudal slicing with a rotary slicing device (Intedge; Roseland, NJ).

At 10–14 days after MCA occlusion, cerebral infarction was evident as well defined regions of tissue softening, necrosis, and cystic formation involving all or portions of the putamen, globus pallidus, and the intervening anterior limb of the internal capsule. In some cases portions of the adjacent temporal cortex demonstrated changes consistent with recent infarction.

Standard photographic prints (from Kodak Ektachrome® EN 35 mm transparencies) were made of full thickness (2 mm) coronal sections of the perfused-fixed brains. Infarction mapping of individual sections was accomplished by planimetry of the respective prints with a Lasico L30 manual polar planimeter (Los Angeles Scientific Instrument Co., Inc., Los Angeles, CA). The infarction volume of each section was computed as surface area by planimetry × thickness adjusted for magnification. The total infarction volume was determined by summing individual slice infarction volumes.

In sixteen animals Evans blue dye (10 ml/kg) was injected 10 minutes preceding the inflation of the balloon occluder device. Following 3 hours of MCA occlusion, the device was deflated and pressure-perfusion fixation was performed thereafter. Residual blue stain in serial brain sections represented both the retention of Evans blue dye in those cerebral vessels not accessible to perfused fixative because of occlusion to flow, and its presence in ischemic tissues.

**Histologic Neuropathology**

Tissue sections 10 μ thick from ischemic and contralateral control areas were prepared by paraffin embedding for light microscopic (hematoxylin-eosin and fluorescence) evaluation. In six animals given Evans blue dye prior to inflation of the balloon device, sections from the ischemic and contralateral control corpora striata were examined by fluorescence microscopy for the presence of Evans blue stain within the lenticulostriate artery branches. Sections were viewed under a Zeiss Universal microscope equipped with an epi-illumination system and a standard Rhodamine filter pack. (Evans blue dye fluoresces bright red upon incidence of light under the designated filter pack.)

Specimens (approximately 2 × 3 × 3 mm) from ischemic (3 hour) and control corpora striata from the six animals sacrificed acutely were additionally fixed by immediate immersion in 5% glutaraldehyde (Polysciences, Warrington, PA) in 0.1 M phosphate buffer, pH 7.4, for 24 hours. Blocks were briefly rinsed in phosphate buffer, post-fixed in 2% osmium tetroxide (Polysciences) in 0.1 M phosphate buffer for 1 hour, dehydrated in a graded series of alcohols, and embedded in TAAB resin (Taab Lab, Reading, U.K.). Ultra thin sections, counterstained with uranyl acetate and lead citrate, were examined and photographed under a Zeiss EM10 transmission electron microscope.

On eight brains (table 6) a morphometric estimate of the presence of thrombotic occlusions in the microcirculation of the corpora striata ipsilateral to an MCA occlusion and in contralateral anatomically paired control corpora striata was performed. All brains were fixed by pressure-perfusion with 10% formalin immediately following the 3 hour period of MCA occlusion. Ten random 10 μ thick sections (hematoxylin-eosin) from each of four paraffin-embedded blocks (8 × 8 × 2 mm) obtained from the Evans blue-stained corpora striata and from anatomically paired regions of control corpora striata were examined for eosinophilic complete vascular occlusions by light microscopy. A score was determined for each corpus striatum (maximum = 40 arbitrary units), representing the summation of individual scores from each 10 μ section (maximum = 1 unit = presence of complete thrombotic occlusion; minimum = 0 units = absence of occlusions in any

<table>
<thead>
<tr>
<th>Animals</th>
<th>Thrombotic mass (ml)*</th>
<th>Vascular occlusion score (arbitrary units)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right</td>
<td>Left</td>
</tr>
<tr>
<td>Untreated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.30</td>
<td>38</td>
</tr>
<tr>
<td>2</td>
<td>1.22</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>0.60</td>
<td>33</td>
</tr>
<tr>
<td>4</td>
<td>0.06</td>
<td>36</td>
</tr>
<tr>
<td>mean</td>
<td>0.55 ± 0.49</td>
<td>34.8 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>0.5 ± 0.6</td>
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<tr>
<td>Ticlopidine and heparin treated</td>
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<tr>
<td>1</td>
<td>0.05</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>-0.003</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>-0.02</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>0.01</td>
<td>7</td>
</tr>
<tr>
<td>mean</td>
<td>0.01 ± 0.03</td>
<td>10.8 ± 7.4</td>
</tr>
<tr>
<td>Significance: (treated vs untreated)</td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.01</td>
</tr>
</tbody>
</table>

*Whole blood equivalent activity of 111In-labelled platelets.
†Vascular Occlusion Score represents the sum of individual scores from 40 10 μ sections in which 1 unit = the presence of complete thrombotic occlusion and 0 units = the absence of occlusions in any vessel or the presence of individual erythrocytes in patent vessels. Sections were removed from the appropriate corpora striata: Right = Specimens from the Evans blue-stained territory ipsilateral to the MCA occlusion and Left = Specimens from the control territory at anatomically comparable regions.
vessel or presence of individual erythrocytes). The scores obtained in four untreated animals undergoing acute MCA occlusion were compared with the scores of four animals treated with the antithrombotic combination ticlopidine (50 mg/kg/d for 4 days; Syntex Corp, Palo Alto, CA) and heparin (1000 U/kg intravenously) immediately prior to MCA occlusion. All sections were blinded prior to scoring.

$^{111}$In-Platelet Imaging

In ten animals (including the eight animals used for occlusion scoring), autologous baboon platelets were labeled with $^{111}$In-oxine (Amersham Corp., Arlington Heights, IL) according to the following protocol. Whole blood (100 ml) was collected directly into plastic bags (TA-3, Fenwal Labs, Deerfield, IL) containing 20 ml acid-citrate-dextrose anticoagulant (NIH formula A). The blood was centrifuged in the bag at 300 x g for 10 minutes. The supernatant platelet-rich plasma (PRP) was transferred to a second bag and the pH adjusted to 6.5 by the addition of 0.15 M citric acid (0.1 ml/10 ml PRP). The red blood cell fraction was returned to the donor animal. The platelets were formed into a pellet by centrifugation of the PRP at 1300 g for 15 minutes. The supernatant platelet-poor plasma (PPP) was completely decanted and discarded. To remove residual plasma proteins, the bag containing the platelet pellet was carefully washed once by overlaying with 30 ml of Ringer’s citrate dextrose (RCD; pH 6.5) which was decanted and discarded. The pellet was then gently resuspended in 5.0 ml RCD, and incubated for 30 minutes with 2 mCi $^{111}$In-oxine (Amersham Corp.). Contaminating red cells were removed by a final slow centrifugation at 200 x g for 5 min. Labeling efficiency was greater than 95%.

The resultant labeled platelet suspension was passed over a Sepharose 2B column prepared with RCD to remove the non-platelet radioactivity. Ten ml of $^{111}$In-labeled platelet suspension (PRP) was then injected intravenously into the recipient animals. Following infusion of labeled platelets and Evans blue dye, introduction of acute stroke, and subsequent deflation of the balloon device, the brains were immediately pressure-perfusion fixed in situ with 10% buffered formalin as previously described. This procedure removed the blood-containing $^{111}$In-platelets from patent vessels; focal residual $^{111}$In-activity represented retention of platelets in occluded vessels that were not accessible to perfusion.

The pressure-perfusion fixed brains were imaged by gamma scintillation camera for residual sites of $^{111}$In-activity. Good spatial resolution was achieved by imaging of the low energy $y$ photon peak of $^{111}$In (172 keV) only with a high sensitivity $^{99m}$Tc collimator and a 5% energy window (see “Results”). Images of the perfused brains were acquired with a Picker DC 4/11 Dyna scintillation camera (Picker Corp. Northford, CT) and stored on and analyzed by a Medical Data System SIMUL computer (Medtronic, Ann Arbor, MI) interfaced with the camera. This system permitted simultaneous acquisition and analysis of data in 64 x 64 word mode. Immediately prior to sacrifice an autologous whole blood standard was drawn and imaged in order to express the localized residual $^{111}$In-platelet activity in the brain in terms of equivalent whole blood volume.

 Autoradiography

Autoradiographs were made of selected 2 mm coronal sections of fixed brains from two untreated animals which received $^{111}$In-labeled platelets and Evans blue dye prior to the 3 hour MCA occlusion. The sections were dried onto Whatman 3 MM paper under vacuum, and used to expose Kodak XR-S, X-Omat R film. Exposure time was 5 days at —70°C.

Statistics

The data are expressed as mean values ± 1 SD. Statistical comparisons were made using Student’s t-test (unpaired data) when the results were normally distributed or by the Wilcoxon Mann-Whitney rank sum test for the remaining results.

Results

Characterization of the Stroke Model in Baboons

Occlusion of the right MCA in awake baboons produced an acute stroke syndrome within 5–10 minutes characterized by a dense left hemiparesis, left facial paresis, and mydriasis of variable degree. A 3 hour period of MCA occlusion produced a permanent neurological deficit that generally did not change during the subsequent 7–14 days in six animals studied. Objective assessment of neurological function using a 100-point scale demonstrated a score of 36 ± 11 in six stroke animals followed for 14 days (table 5). None of the stroke animals died during the period of observation preceding their sacrifice.

Selective cerebral angiography of the right carotid system performed during balloon inflation demonstrated occlusion of the right MCA in the six animals studied above. After balloon deflation, patency of the right MCA was reestablished (fig. 1) in all animals. The subtending lenticulostriate arteries could not be visualized by angiography.

Serial CT cerebral scans demonstrated the development of a region of low attenuation consistent with that region of the brain supplied by the right lenticulostriate arteries i.e. the internal capsule, putamen, and caudate nucleus (fig. 2). The region of low attenuation had a volume averaging 3.2 ± 1.5 cm³ (table 5) at 10 days following stroke induction.

Animal sacrifice and pressure perfusion-fixation was performed on day 14. Neuropathology on those specimens confirmed the presence of cerebral infarction at the site of low attenuation (fig. 3; table 5). Hemorrhage was not evident by CT scan or by gross pathology in any animal studied in this group; however, perivascular collections of erythrocytes were noted (hematoxylin-eosin preparation) in the affected ischemic corpus striatum in a single animal. The infarction volume, determined by morphometry, of neuropatho-
logic specimens in four animals was $3.9 \pm 1.9 \text{ cm}^3$. Temporal cortical infarction was observed in three animals at 14 days. This component was combined with the corpora striatal defect to obtain the overall infarction volume shown in table 5.

**Acute Thrombotic Occlusion of the Lenticulostriate Artery Branches**

To assess the status of the lenticulostriate arterial territory immediately following the 3 hour MCA occlusion, 16 animals fitted with the silastic balloon device received an infusion of Evans blue dye (10 ml/kg) 30 minutes prior to elective occlusion. All animals tolerated the infusion without adverse effects. After the 3 hour period of MCA occlusion the balloon occluder device was deflated and the animals were then subjected to pressure-perfusion fixation with 10% buffered formalin (see Methods for detailed protocols). This procedure removed the Evans blue dye from all patent cerebral vessels. Discrete areas of Evans blue stain in the right internal capsule, putamen, and caudate nucleus were consistently present in coronal sections of the pressure perfusion-fixed specimens (fig. 4). No blue stain was observed in the contralateral control side of hemiparetic animals.

In eight animals, 10 μ thick sections (hematoxylin-eosin) from each of four paraffin-embedded blocks were obtained from each ischemic area (Evans blue-stained region) of the right corpus striatum and matched with sections from blocks prepared from anatomically comparable control regions of each brain. Both sets were compared for the presence of complete vessel occlusion by light microscopy. Vascular occlusion was scored as described in the "Methods." Occlusion of microcirculatory vessels by eosinophilic material resembling thrombus was consistently observed in sections from the Evans blue stained regions of the right corpora striata (fig. 5). A vascular occlusion score of $34.8 \pm 2.8$ was obtained from four animals undergoing 3 hour MCA occlusion (table 6). In four animals receiving the antithrombotic regimen ticlopidine and heparin prior to MCA occlusion, the occlusion score was $10.8 \pm 7.4$. This difference was statistically significant ($p < 0.01$).

Scores for untreated and treated contralateral control sections were $0.5 \pm 0.6$ and $0.75 \pm 0.9$, respectively.

Fluorescence microscopy was performed on specimens from six additional untreated animals. Fluorescent material (Evans blue stain and erythrocytes) was demonstrated to fill the lumen of microcirculatory vessels the size of precapillary arterioles in the right corpus striatum (fig. 7), but not those of the contralateral control side (fig. 8).

The presence of thrombotic material occluding perforating branches in the right lenticulostriate territory was confirmed by transmission electron microscopy (fig. 9). In contrast to the preservation of normal perivascular tissue on the control side (fig. 9A), the integrity of the surrounding cerebral tissue in the region of the occluded vessels was found to be disrupted (fig. 9B).

Extravascular hemorrhage was observed in sections from one untreated animal and one treated animal.
Figure 3. Gross neuropathology of stroked baboon. Coronal section (2 mm thick) of perfusion-fixed brain at 14 days following a 3 hour occlusion of the right MCA demonstrated regions of necrosis in the right corpus striatum.

Figure 4. Coronal section (2 mm thick) of perfused-fixed brain immediately following a 3 hour occlusion of the MCA. An area of Evans blue stain in the right corpus striatum is demonstrated, following MCA balloon deflation and subsequent perfusion-fixation.

Figure 5. Hematoxylin and eosin stained preparation of paraffin-embedded section (10 μm) from the right corpus striatum. Occlusion of a perforating arteriole with eosinophilic material is demonstrated in this section taken from a region containing Evans blue stain (see Methods). Endothelial nuclei may be seen. Magnification bar = 10 μm.

Figure 6. Gamma scintillation image of isolated whole brain (vertex view). The infusion of 111In-labeled autologous platelets (2 mCi) was followed by a 3 hour period of right MCA occlusion, balloon deflation, and immediate perfusion-fixation. The occiput is inferior; scan time was 2 hours.

Figure 7. Fluorescence microscopy of paraffin-embedded section (10 μm) from right caudate nucleus. Fluorescent material (Evans blue dye and erythrocytes) fill the lumen of a small blood vessel. This longitudinal section of the vessels was prepared from the Evans blue containing region (see Methods). Magnification bar = 5 μm.

Figure 8. Fluorescence microscopy of paraffin-embedded section (10 μm) of the contralateral (control) corpus striatum. In comparison to figure 7 patent perforating arteries in longitudinal section are demonstrated. Fluorescence of endothelial cell nuclei is evident. Magnification bar = 10 μm.
Localization of $^{111}$In-Labeled Autologous Platelets

Three types of experiments were performed in ten animals to assess the role of platelet-containing thrombus and the effect of antithrombotic therapy in the genesis of cerebral infarction in this model. In the first experiment, $^{111}$In-labeled autologous platelets (2 mCi each animal) and Evans blue dye were infused into four untreated animals one hour prior to occlusion of the right MCA. One hour following the 3 hour occlusion and deflation of the silastic balloon, the animals underwent pressure perfusion-fixation with 10% buffered formalin. Using y camera imaging of the perfused-fixed brain, significant $^{111}$In-platelet activity was found to be localized to the region of the right internal capsule, putamen, and caudate nucleus in all four animals (fig. 6). The focal $^{111}$In-platelet activity in this region of the brain averaged 0.55 ± 0.49 ml equivalent of whole blood (table 6). No activity above background was demonstrated in the comparable contralateral region of the fixed brain.

Autoradiographs of selected coronal sections demonstrated that the region of $^{111}$In activity was coincident with the area of Evans blue stain in the right corpus striatum (fig. 10). Standard light microscopy of H and E preparations of serial coronal sections through the region of Evans blue stain within the right corpora striata of the four animals, confirmed the presence of thrombus occluding perforating vessels (table 6).

In the second experiment, four animals were given potent antithrombotic therapy prior to the injection of autologous $^{111}$In-platelets (2 mCi each animal) and Evans blue dye and the 3 hour period of MCA occlusion. Antithrombotic therapy consisted of the platelet inhibitor ticlopidine (50 mg/kg/d for 4 days prior to study) and heparin (1000 U/kg) immediately prior to MCA occlusion. One hour after deflation of the MCA balloon the animals were subjected to pressure perfusion-fixation with formalin. The fixed brains were imaged for 12 hours. There was no detectable focal uptake in any of the animals given combination antithrombotic therapy, i.e., equivalent to less than 0.01 ± 0.03 ml of whole blood (table 6).

In a third experiment, $^{111}$In-labeled autologous platelets were infused into two additional animals immediately following the 3 hour period of right MCA occlusion and subsequent balloon deflation (data not shown). Both animals received Evans blue dye prior to MCA occlusion. This control experiment was designed to assess the possibility that platelets might react to preformed thrombus or endothelium injured by balloon occlusion or ischemia. In situ pressure perfusion-fixation of the brains was performed after the labeled platelets had circulated for 1 hour following balloon deflation. Continuous y scintillation imaging for 12 hours failed to discriminate regions of focal uptake in the region of either corpus striatum. No significant localization of activity in the right corpus striatum or right hemisphere was detected within the error of the technique, i.e., equivalent to less than 0.003 ml of whole blood.

Discussion

Although several nonhuman primate models of acute stroke have been employed to study cerebral infarction, there is little direct evidence to show that thrombosis of cerebral arteries distal to a mechanical
occlusion occurs in any of these models. We have demonstrated histologically and by in-platelet \gamma camera imaging in a primate model that thrombus forms in the territory of the lenticulostriate branches of the MCA during 3 hours of external occlusion produced by an inflatable silastic balloon cuff. Thrombotic material occluding the perforating arteries of the corpus striatum subserved by the right lenticulostriate arteries was also documented by fluorescence microscopy and confirmed by electron microscopy. In all cases the vascular occlusions were observed in animals that displayed acute neurological deficit(s) consistent with ischemia of the tissue supplied by the right lenticulostriate arteries. The extent of thrombotic occlusions was significantly decreased and in-platelet activity in specimens from the right corpus striatum was absent in animals given combination antithrombotic therapy prior to MCA occlusion.

The limitations and advantages of experimental models of focal cerebral ischemia have been described. The use of the baboon model of middle cerebral artery (MCA) territory cerebral ischemia in the present studies has significant advantages over other primate models: 1) A reproducible, persistent, and predictable nonfatal stroke syndrome results from a 3 hour inflation of the MCA silastic cuff situated proximal to the take-off of the lenticulostriate arteries. 2) A deep cortical (and temporal cortical) infarction results in the territory supplied by the lenticulostriate arteries. 3) The stroke syndrome can be induced and assessed in the alert animal at a time remote from surgical implantation. 4) The precise moment of MCA obstruction is elective and controlled. 5) The development of the region of cerebral infarction can be assessed noninvasively by CT cerebral scan or magnetic resonance imaging techniques. 6) It has been noted that acute neuropathological changes following vascular occlusion are those of a nonhemorrhagic cerebral infarction.

The angiographic demonstration that thrombosis does not occur in the MCA trunk itself following a 3 hour period of external compression is in accord with previous experience with this model. From prior carmine gelatine perfusion experiments, the extent of cerebral infarction following proximal MCA ligation or electrocoagulation-induced occlusion has been localized to the lenticulostriate and perforating vessels. In those experiments the patency of the lenticulostriate or perforating arteries was not examined. Immediate in situ pressure perfusion with 10% buffered formalin produces rapid fixation of brain tissue with high quality morphologic and histologic definition. In contradistinction to conventional methods of brain removal and ex vivo fixation, in situ pressure perfusion-fixation preserves vascular structures and intravascular pathology. Previous reports of primate models of acute stroke employing proximal middle cerebral artery occlusion or lenticulostriate interruption have used ex vivo fixation techniques and have failed to demonstrate thrombi in the occluded vessel or its branches. In some reports, perfusion techniques have included local carmine gelatine or radio-opaque silicone infusions, or systemic saline infusion with small volume formalin injections, followed by in situ formalin fixation for variable periods. In other models in which in situ perfusion fixation was reported, the effect of MCA occlusion on the dependent vascular bed was not examined. The failure to observe occlusive material in perforating vessels in the regions of a lenticulostriate artery-dependent cerebral infarction in various primate models when specimens were prepared remotely after stroke induction may be explained by active endogenous fibrinolysis of occlusive thrombi within these small vessels within hours of thrombus formation as recently demonstrated for acute thrombotic occlusion during myocardial infarction.

Previous morphologic studies employing in situ perfusion fixation have been inconclusive. While thrombotic occlusion of precapillary arterioles and capillary branches of the lenticulostriate arteries following transient ipsilateral MCA occlusion was not observed in one electron microscopic study, their presence was documented in another. Alterations in perivascular astrocytic processes were described by Garcia, et al. We observed similar changes adjacent to patent vessels in ischemic tissue with additional loss of tissue integrity characterized by structural disruption adjacent to occluded vessels. This observation is most probably due to a combination of ischemic injury and inadequate local tissue perfusion with fixative in the region subserved by the thrombosed blood vessels.

The retention of Evans blue dye in the caudate nucleus, internal capsule, and putamen following a 3 hour MCA occlusion, deflation of the balloon, and immediate perfusion-fixation is consistent with occlusion of the lenticulostriate arteries or their perforators and impairment of the blood-brain barrier. The coincidence of eosinophilic material and fluorescent material in the perforating vessels of the affected corpus striatum supports the conclusion that thrombus occludes these functional end-arterioles. Electron microscopic examination of representative vessels confirmed the presence of degranulated platelets, leukocytes, and erythrocytes in the occluding material.

The size of the region containing Evans blue dye and subsequent infarction was variable without close correlation to the severity of neurological deficit in a given animal. The variation in extent of Evans blue retention is in part related to variation in distribution of the lenticulostriate arteries supplying the corpus striatum. Although the principal blood supply to the posterior lateral portion of the corpus striatum in the baboon is derived from the lenticulostriate arteries, variability in the distribution of the lenticulostriate arteries and anatomic variants with regard to their origin in the baboon have been described. The actual site of placement of the silastic balloon device about the MCA in this model is, therefore, important in determining the functional outcome after its inflation.

There was no evidence of hemorrhage in the region of acute cerebral ischemia by CT scan or gross neuroanatomic examination in any animal undergoing perfu-
tion-fixation acutely or at 14 days (table 4). However, perivascular extravasation of erythrocytes was documented by light microscopy in one 14-day specimen. Microscopic hemorrhages was also observed in specimens from two to eight animals (one untreated, one treated with triplidine/heparin) receiving perfusion-fixation immediately following the 3-hour period of MCA occlusion and subsequent MCA balloon deflation. This is in contrast to the claim that this model is one of “nonhemorrhagic cerebral infarction.” In this regard this model resembles the neuropathological outcome of proximal MCA territory stroke in humans. 

Although the explanation for microscopic hemorrhage in the single animal from the group of animals studied at 14 days is not known, this finding may imply that attempts to establish reperfusion with thrombolytic agents in ischemic cerebral tissue in this model should be initiated early after the period of MCA occlusion before vascular necrosis develops.

The precise mechanism underlying the development of thrombosis in this model of acute stroke has not been established by our studies. However, we reasoned that since sustained external balloon compression of the MCA may produce focal (e.g., ischemic) endothelial disruption, the coagulation cascade may be activated locally through both extrinsic (via tissue thromboplastin and Factor VII) and intrinsic (via Factor XII) pathways. In association with consequent prolonged complete stasis, as produced experimentally in the end-arterioles of the affected corpus striatum, sufficient thrombin may be generated to form occluding thrombus. In the absence of complete stasis, as in the MCA, thrombin generation would be insufficient for thrombus formation.

The results obtained in the In-platelet imaging studies comparing untreated animals with animals given antithrombotic therapy are in accord with this interpretation. When In-platelets were injected preceding stroke induction in untreated animals, they remained only in the region of the LSA and their branches after perfusion removed blood from all patent vessels. Combination antithrombotic therapy significantly reduced both In-platelet deposition and thrombus formation as assessed isotopically and histologically.

The observation that localized In-platelets were not detected in the corpora striata when the labeled platelets were injected immediately following deflation of the MCA balloon indicates that any platelet interaction with preformed thrombus occluding the lenticulostriate vessels or balloon-induced exposed subendothelium injury of the MCA is not detectable by this technique. Furthermore, although it is has been proposed that cerebral infarcts release thromboplastin and platelet-mediated thromboxane A2 in the region of vascular obstruction, the absence of In-platelet deposition following the 3 hour period of MCA occlusion suggests that thrombus extension in the perforating arteries by this mechanism (or any other) does not develop early in this model following the acute occlusive event.

We conclude that erythrocyte rich, platelet-containing thrombus formation in the capillaries and precapillary arterioles of the lenticulostriate territory is dependent on stasis in this microcirculatory bed. It is possible that similar events occur following thromboembolism to and prolonged occlusion of the M1 segment of the MCA in humans.

In summary, we have shown that transient external compression of the MCA at its origin for a period of 3 hours produces occlusive thrombus in situ in perforating branches of the ipsilateral LSA with consequent infarction of the dependent corpus striatum and that this process is prevented by antithrombotic therapy. This model of acute thrombotic stroke may be useful to assess the safety and efficacy of thrombolytic and other antithrombotic strategies.

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References


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