Matrix Metalloproteinases and TIMPs Are Associated With Blood-Brain Barrier Opening After Reperfusion in Rat Brain

G.A. Rosenberg, MD; E.Y. Estrada, BS; J.E. Dencoff, BS

Background and Purpose—Reperfusion disrupts cerebral capillaries, causing cerebral edema and hemorrhage. Middle cerebral artery occlusion (MCAO) induces the matrix-degrading metalloproteinases, but their role in capillary injury after reperfusion is unknown. Matrix metalloproteinases (MMPs) and tissue inhibitors to metalloproteinases (TIMPs) modulate capillary permeability. Therefore, we measured blood-brain barrier (BBB) permeability, brain water and electrolytes, MMPs, and TIMPs at multiple times after reperfusion.

Methods—Adult rats underwent MCAO for 2 hours by the suture method. Brain uptake of 14C-sucrose was measured from 3 hours to 14 days after reperfusion. Levels of MMPs and TIMPs were measured by zymography and reverse zymography, respectively, in contiguous tissues. Other rats had water and electrolytes measured at 3, 24, or 48 hours after reperfusion. Treatment with a synthetic MMP inhibitor, BB-1101, on BBB permeability and cerebral edema was studied.

Results—Brain sucrose uptake increased after 3 and 48 hours of reperfusion, with maximal opening at 48 hours and return to normal by 14 days. There was a correlation between the levels of gelatinase A at 3 hours and the sucrose uptake ($P<0.05$). Gelatinase A (MMP-2) was maximally increased at 5 days, and TIMP-2 was highest at 5 days. Gelatinase B and TIMP-1 were maximally elevated at 48 hours. The inhibitor of gelatinase B, TIMP-1, was also increased at 48 hours. Treatment with BB-1101 reduced BBB opening at 3 hours and brain edema at 24 hours, but neither was affected at 48 hours.

Conclusions—The initial opening at 3 hours correlated with gelatinase A levels and was blocked by a synthetic MMP inhibitor. The delayed opening, which was associated with elevated levels of gelatinase B, failed to respond to the MMP inhibitor, suggesting different mechanisms of injury for the biphasic BBB injury. (Stroke. 1998;29:2189-2195.)

Key Words: blood-brain barrier • brain edema • cerebral ischemia • reperfusion injury • matrix metalloproteinases • type IV collagenses

Reperfusion of ischemic tissue with thrombolytic agents shortly after the infarct reduces ischemic damage. However, a delay in treatment increases the risk of hemorrhagic transformation and cerebral edema. Several mechanisms have been proposed to explain the tissue damage associated with reperfusion of ischemic tissue. Reintroduction of oxygenated blood into the damaged region enhances the production of free radicals, recruits neutrophils and macrophages, and releases proteases. Reperfusion causes a biphasic opening of the blood-brain barrier (BBB). Free radicals, cytokines, and proteases may mediate the attack on the capillary. Matrix metalloproteinases (MMPs) attack the basal lamina around cerebral capillaries, which contains type IV collagen, fibronectin, laminin, and heparan sulfate. Direct intracerebral injection of one of the MMPs, gelatinase A (72-kDa type IV collagenase or MMP-2), caused an increase in capillary permeability and hemorrhage. In rats with permanent middle cerebral artery occlusion (MCAO), a large increase in the MMP gelatinase B (92-kDa type IV collagenase or MMP-9) occurred 12 to 24 hours after the injury, when capillary permeability was elevated. Thus, the MMPs may be involved in the increased capillary permeability seen in reperfusion injury.

Proteolysis depends on the balance between the proteases and their inhibitors. Tissue inhibitors to metalloproteinases (TIMPs), which interfere with the activation and action of the MMPs, affect the extent and duration of proteolytic damage. Recently, quantitative measurement of TIMPs was described with the use of a reverse zymogram. Thus, combining measurements of MMPs by zymography with measurements of the TIMPs with reverse zymography provides insights into the proteolytic potential for damaging the extracellular ma-
MMPs and TIMPs in Reperfusion Injury

Reverse Zymography
Reverse zymography was performed as recently described. Polyacrylamide minigels (15%) are prepared: 5.0 mL Protogel (30% ultrapure, National Diagnostics); 2.5 mL of 1.5 mol/L Tris-HCl, pH 8.8; 1.67 mL porcine gelatin at 15 mg/mL; 0.83 mL distilled water; 0.2 mL of 10% SDS; 6.4 μL of purified gelatinase A (0.252 μg/μL; a gift from Dr. W.G. Stetler-Stevenson at the National Cancer Institute); 50 μL of 10% ammonium persulfate; and 5 μL of N,N,N′,N′-tetra-methylthlenediamine (TEMED). Gels were allowed to polymerize for 1.5 to 2 hours before the stacking gel was added (5.6 mL distilled water; 4.15 mL 0.5 mol/L Tris, pH 6.8; 1.66 mL Protogel 30%; 1.25 mL (NE SDS; 10 μL TEMED; and 200 μL 10% APS). The stacker was allowed to polymerize for 1 hour before samples were loaded.

Tissue samples were thawed and were mixed 1:1 with nonreducing SDS loading buffer (New England Biolabs) before loading on gels. Prepared samples were not boiled or exposed to reducing agents. Prestained rainbow-colored molecular weight markers (Amersham Life Science) and HT1080 fibrosarcoma media, which contains TIMP-1 and TIMP-2, were run in every gel to determine molecular weights of TIMPs. After loading, gels were electrophoresed at 150 V for 2.5 hours. Following electrophoresis, all gels were agitated (2× 30 minutes) in 2.5% Triton X-100 to remove the SDS. Gels were rinsed 3 times in distilled water, then incubated for 16 hours at 37°C in 50 mL of 50 mmol/L Tris-HCl (pH 7.6) containing 0.2 mol/L NaCl, 5 mmol/L CaCl2, 0.02% Brij-35, and 0.02% Azide. Finally, all gels were stained for 1 hour in Coomassie G-250. Gels were then destained for approximately 1 hour in 10% acetic acid.

Zymogram and reverse zymograms were scanned with an Hewlett Packard ScanJet 1hc, and images were analyzed using an image analysis software program (NIH Image), running on a Macintosh PowerPC. Image standardization was accomplished using an optical density step tablet, and the MMP and TIMP activities were measured using the electrophoretic gel lane calculation option. The initial series of time points (3 to 24 hours) for TIMP measurements were run with a gelatinase kindly supplied by Dr. Dylan Edwards at the University of Calgary. The second series of experiments from 48 hours to 14 days were run with a recombinant gelatinase A from Dr. William Stetler-Stevenson. Data was expressed as relative lysis zone (pixels) divided by protein content in the tissue.

Brain Water and Electrolytes
Fifty-two rats had brain water and electrolyte content measured after 2 hours of MCAO followed by 3, 24, or 48 hours of reperfusion with or without treatment with MMP inhibitor. Brain water was measured in tissue with the wet/dry method. Tissue was frozen, and sections were taken from the ischemic and nonischemic hemispheres. The tissue was weighed wet and dried in a 100°C oven for 48 hours and reweighed. After drying, it was extracted for electrolyte measurement with a flame photometer (Corning Corp). Rats with temporary MCAO (tMCAO) for 2 hours were treated with a metalloproteinase inhibitor, BB-1101 (British Biotechnology), after either 3, 24, or 48 hours of reperfusion. The BB-1101 (30 μg/kg) was given intra- parenchymally at the onset of the ischemia and repeated after 2 hours of reperfusion in the 24-hour and 48-hour studies. For the BBB studies, treated rats underwent 2 hours of tMCAO followed by 3, 24, or 48 hours of reperfusion, and they were compared with untreated rats from the BBB series. For the edema and electrolyte studies, treated rats underwent 2-hour tMCAO followed by 3, 24, or 48 hours of reperfusion, and they were compared with untreated rats. The effect of the agent on physiological parameters was assessed in 5 rats. Blood glucose, mean arterial blood pressure, and temperature were measured before injection of BB-1101, which was given 10 minutes after the start of the experiment and at 4 and 24 hours later.

Statistical Analysis
BBB data was tested for statistical significance with an ANOVA, using the Bonferroni correction for multiple t tests (Prizm, Graph-
Figure 1. Uptake of 14C-sucrose into brain in rats with temporary occlusion of the MCA by the suture method. The artery was occluded with an intraluminal thread for 2 hours and then withdrawn to reperfuse the brain. Adult rats were injected with 14C-sucrose at the end of the reperfusion period and killed 10 minutes later. Samples from brain and blood were collected, and brain sucrose uptake was calculated as a percentage of sucrose in brain to that in blood (Sucrose Space %). The sucrose spaces in the ischemic sides were compared by ANOVA with Bonferroni corrections. A significant increase in the uptake of sucrose occurred at 3 and 48 hours compared with all other times. *Statistically significant increases with $P<0.05$.

Results

BBB Permeability and Brain Water and Electrolytes

Uptake of sucrose into the brain was significantly increased after 3 hours of reperfusion and was maximal at 48 hours (Figure 1). By 3 hours of reperfusion, the brain water was increased, reaching maximal levels by 24 and 48 hours, with sodium and potassium paralleling the changes in water content (Table 1). Significant increases in water content were found at 3, 24, and 48 hours. Sodium was significantly increased, reaching maximal levels by 24 and 48 hours, with potassium was increased on the nonischemic side at 48 hours. Potassium was significantly increased at 3, 24, and 48 hours compared with sham-operated controls at 24 and 48 hours, whereas the sodium was increased in the ischemic side compared with sham-operated controls at 24 and 48 hours. Sodium was significantly increased, reaching maximal levels by 24 and 48 hours, with potassium was increased on the nonischemic side at 48 hours. Potassium was significantly decreased at 24 hours and increased on the nonischemic side at 48 hours.

MMPs and TIMPs

Representative zymograms and reverse zymograms from reperfused tissues are shown in Figure 2. Elevated levels of gelatinase B at 92-kDa are seen in the reperfused tissue at 48 hours (Figure 2A). Normally, MMP-9 is undetectable in rat brain. All tissue samples showed bands at 72 kDa from MMP-2, which is constitutively expressed. Reverse zymograms showed dark bands at 28 kDa and 21 kDa from TIMP-1 and TIMP-2, respectively (Figure 2B).

To allow comparison over multiple time points from samples run on different gels, the amount of gelatinase B in the ischemic side was normalized by dividing it by the nonischemic side. Optical density measurements showed a significant increase in gelatinase B ratios by 48 hours (Figure 3A). The increase in gelatinase B began by 15 hours but was not significant at that time. After 48 hours, there was a drastic reduction in the levels of gelatinase B. Gelatinase A ratios were maximal at 5 days (Figure 3B).

Although the mean levels of gelatinases A and B were not increased at 3 hours, plotting the individual values for gelatinase A against sucrose space revealed a strong correlation at 3 hours ($P<0.0003$) and lower significance for

TABLE 1. Water and Electrolytes in Cerebral Tissue After 2-Hour MCAO With Various Times of Repерfusion

<table>
<thead>
<tr>
<th>Occlusion/Reperfusion</th>
<th>Water, %</th>
<th>Sodium, mEq/L</th>
<th>Potassium, mEq/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ischemic</td>
<td>Nonischemic</td>
<td>Ischemic</td>
</tr>
<tr>
<td>2/3 (n=9)</td>
<td>81.16±0.33†</td>
<td>78.80±0.21</td>
<td>308±23.0</td>
</tr>
<tr>
<td>2/24 (n=9)</td>
<td>83.07±0.48†</td>
<td>79.34±0.27</td>
<td>459±40†</td>
</tr>
<tr>
<td>2/48 (n=6)</td>
<td>83.42±0.53†</td>
<td>79.04±0.24</td>
<td>591±44†</td>
</tr>
<tr>
<td>Sham* (n=6)</td>
<td>76.62±0.09</td>
<td>78.38±0.06</td>
<td>216±4</td>
</tr>
</tbody>
</table>

*Sham-operated rats had the suture inserted and removed. Brains were removed and studied 24 hours later.

Statistical significance was determined by ANOVA with Bonferroni correction for multiple t tests. Significance levels were †$P<0.001$; ‡$P<0.01$; and §$P<0.05$. 

Figure 2. Representative zymogram and reverse zymograms. The samples are from the 48-hour to 14-day series. A, Gelatin-substrate zymogram with molecular weights as shown on the side determined from HT1080 run in a lane that is not shown. Lane 1 is from nonischemic (NI) tissue from the caudate after 2-hour MCAO and 48-hour reperfusion. Lane 2 is the same except the tissue is from the ischemic side (I). Lanes 3 through 6 are 120 hours and 336 hours of reperfusion as labeled. B, Reverse zymogram from brain tissue at the same time points as in A. Molecular weights are derived from TIMP standards and HT1080. The higher band at 28 kDa is from TIMP-1 and possibly a glycosylated form of TIMP-3, while the lower 21-kDa band contains TIMP-2. The massive increase in TIMP-1 is seen at 48 hours in the reverse zymogram, corresponding to the increase in gelatinase B at the same time in the zymogram.
gelatinase B ($P<0.04$) (Figure 4). No correlation was found at 24 hours for either of the enzymes (data not shown). The marked increase in gelatinase B at 48 hours coincided with the maximal increase in sucrose uptake.

Ratios of TIMP-1 in the 2 sides showed a statistically significant elevation at 48 hours that was absent at 5 and 14 days (Figure 3C). Ratios of TIMP-2 were more erratic, with maximal levels seen at 5 days, reaching significance compared with the 3-, 6-, and 24-hour values. Maximal ratios were seen at 5 days when the ratios for gelatinase A were also increased (Figure 3D).

**Effect of Treatment With a Metalloproteinase Inhibitor**

The synthetic metalloproteinase inhibitor BB-1101 did not affect blood glucose level, mean arterial blood pressure, or temperature for the 24 hours during which these parameters were measured (Table 2). BB-1101 significantly decreased uptake of sucrose at 3 hours in the ischemic hemisphere and at 48 hours in the nonischemic hemisphere (Figure 5A). It also lowered the sucrose space at 48 hours in the ischemic side, but the results did not achieve statistical significance.

Brain water was significantly reduced by the BB-1101 at 24 hours (Figure 5B). No effect of the agent was seen at 48 hours, however. Administration of the drug after either a 1- or 2-hour delay failed to affect the cerebral edema at 24 or 48 hours, suggesting that the agent was acting to alter an early event (data not shown).

**Discussion**

Reperfusion for 3 and 48 hours after 2-hour occlusion of the MCA opened the BBB, with the maximal opening at 48 hours. The initial opening corresponded with increased levels of gelatinase A, whereas the second one occurred when gelatinase B was markedly increased. Maximal levels of gelatinase A were seen at 5 days after reperfusion, during the time when the repair process had begun. An inhibitor to metalloproteinases blocked the initial opening of the BBB.

**TABLE 2. Effect of BB-1101* on Blood Glucose, Temperature, and Mean Arterial Blood Pressure at 0, 4, and 24 Hours After Injection**

<table>
<thead>
<tr>
<th></th>
<th>0 Hours</th>
<th>4 Hours</th>
<th>24 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose, mg/dL</td>
<td>160±16†</td>
<td>200±15</td>
<td>170±5</td>
</tr>
<tr>
<td>Temperature, °C</td>
<td>37.7±0.12</td>
<td>37.6±0.24</td>
<td>38.0±0.0</td>
</tr>
<tr>
<td>Mean arterial blood pressure, mm Hg</td>
<td>79.0±1.0</td>
<td>81.0±3.7</td>
<td>87.5±4.8</td>
</tr>
</tbody>
</table>

*Dose of BB-1101 was 30 mg/kg IP at 10 minutes after start of the experiment.

†Five nonfasted rats used for each measurement.
...and also the edema at 24 hours, suggesting that they were related to a metalloproteinase. Tissue inhibitor to metalloproteinase-1 was markedly increased at 48 hours, and TIMP-2 was maximally increased at 5 days. These results show that MMPs and TIMPs are dramatically affected by reperfusion but contribute in a complex manner to reperfusion injury.

Matrix-degrading proteases are important in many normal and pathological processes, including motility of developing cells, spread of cancer cells, inflammatory responses, and tissue repair after injury. The balance between the proteases and the inhibitors determines whether there is proteolytic breakdown of extracellular matrix or inhibition of proteolysis with buildup of extracellular matrix. Normally, gelatinase A is present in brain tissue in a latent form, which is activated by a membrane-bound protease, membrane-type metalloproteinase (MT-MMP). Attachment of TIMP-2 to MT-MMP is required for the activation MMP-2. Because the activation reaction proceeds on the membrane surface, proteolysis is spatially constrained. Gelatinase A is constitutively expressed, making it available for early tissue injury. Along with the serine protease, plasminogen activator, MMP-2 is also a critical factor in the controlled proteolysis during blood vessel regrowth. On the other hand, gelatinase B is a proinflammatory protease that is released during the neuroinflammatory response by the stimulation of the cytokines and immediate early genes. The gelatinase B promoter region contains both AP-1 and nuclear factor-kB sites, which respond to a wide variety of proinflammatory stimuli. Released in a proform, gelatinase B requires an activation step, which has not been reported for the in vivo situation, but may involve stromelysin, other proteases, or free radicals.

Several brain cells produce gelatinases under resting and stimulated conditions. Astrocytes normally produce latent gelatinase A. Microglial cells produce gelatinase B after stimulation by proinflammatory agents. Cerebral capillaries express gelatinase A and also produce gelatinase B, elastase, and cathepsins, which may contribute to the disruption of the tissue in the secondary inflammatory response.

Intracerebral injection of activated gelatinase A opened the BBB, and TIMP-2 blocked the gelatinase-induced opening. Tumor necrosis factor-α, which is formed in ischemic tissue, increased gelatinase B at 24 hours when the BBB was maximally opened, and an inhibitor to metalloproteinases, Batimistat, reduced the capillary injury. Cerebral capillaries are surrounded by a basal lamina composed of type IV collagen, fibronectin, and laminin, which are attacked by the proteases, including gelatinases. Ischemia/reperfusion injury in monkeys causes loss of laminin in the basal lamina, which adds evidence to the concept that proteolytic disruption of the basal lamina contributes to the BBB injury.

The initial rise in capillary permeability may have been due to hyperemia that accompanies the early stages of reperfusion. Treatment with the hydroxamate-type synthetic inhibitor to MMPs reduced this initial increase in capillary permeability at 3 hours and modified the permeability at 48 hours on the nonischemic side. There was a correlation between the levels of gelatinases at 3 hours and the increases in the BBB that suggests that they are linked. One possible explanation for the early effect is that the inhibitor blocked the activation of the endogenous MMP-2 by MT-MMP. BB-1101 is a potent inhibitor of MT-MMP (K. Miller, personal communication, 1998). We have observed by immunohistochemistry that the MMP-2 is normally found around the cerebral vessels in astrocytic processes that abut on the capillary wall and that MT-MMP is also present (S. Mun-Bryce, J. Wallace, unpublished data, 1998). Another possibility is that the inhibitor blocked the conversion of tumor necrosis factor-α from a latent to an active form, preventing it from damaging the capillary. The delayed opening of the BBB seen at 48 hours was associated with a large increase in gelatinase B, which may have been endogenous from astrocytes and microglia or exogenous from the invading neutrophils and macrophages. However, the MMP inhibitor failed to interfere with the BBB damage or edema at that time.

A marked increase in TIMP-1 was seen around the time of maximal rise in the gelatinase B. At 5 days, the BBB was closed, and the levels of both MMP-9 and TIMP-1 had fallen drastically. TIMP-2 was maximal at the time of maximal increases in the levels of MMP-2. TIMP-1 inhibits the action of MMP-9, and TIMP-2 inhibits MMP-2; both have been implicated in other functions, but the role of TIMPs in brain is uncertain.

Brain water content rose significantly from the onset of reperfusion, while the changes in capillary permeability fluctuated. A composite graph showing the changes in multiple parameters is shown in Figure 6. At 24 hours when the...
brain water was high, the sucrose space had returned to normal, suggesting that the cytotoxic component of the brain edema predominated. Sodium content was high and potassium low at that time, as expected for cytotoxic edema. However, by 48 hours the water content remained high with elevated levels of sodium and potassium. Furthermore, the sucrose space was maximal. Thus, vasogenic edema seemed to also contribute to the water changes at the later time.

We found that the synthetic metalloproteinase inhibitor prevented the increase in brain water at 24 hours but not at 48 hours. The initial opening of the BBB was also limited by the inhibitor, suggesting that it was affecting the initial increase in capillary permeability. The inability of the inhibitor to act at 48 hours suggests that the second phase of injury was related to the neuroinflammatory response, which involves the infiltrating leukocytes and the endogenous microglial cells. Free radicals and other proteases, such as elastase, which are not inhibited by the MMP inhibitor, may be important because they are released by neutrophils and macrophages and are disruptive to the capillary. Another possible explanation of the failure of the agent to affect the edema at 48 hours is that the delayed BBB opening is beneficial by helping to remove fluid from the injury site. Such a mechanism has been proposed earlier to explain the finding of increased capillary permeability without cerebral edema after an ischemic injury. Our results suggested that the MMP inhibitor reduced the capillary permeability at 48 hours, while the edema was slightly increased, which is consistent with the notion that the opening of the BBB then may be beneficial, but further studies are needed to clarify this point.

Our results suggest that the balance between the MMPs and their inhibitors play a role in reperfusion injury. Inhibition of the metalloproteinases altered the early damage to the capillary and controlled the brain edema at 24 hours. However, the failure of the MMP inhibitors to affect the delayed injury suggest a multifactorial process. Because of the complex manner in which the increases and reductions in the proteases and inhibitors are woven together during the reperfusion process, attempts at altering the patterns with drugs will be complicated.

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**Figure 6.** Composite figure showing the relationship and time course of the brain edema, sucrose (Suc) space, and gelatinases. The data is from the earlier figures showing the individual results. Water content is increased by 3 hours and continues to steadily rise. Capillary permeability shows a biphasic response with peaks at 3 and 48 hours. Gelatinase B peaks at 48 hours, while gelatinase A is increased maximally at 5 days. Gel/Gel indicates gelatinases as ratio of ischemic (I) to nonischemic (II) side.

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**Acknowledgments**

These studies were supported by grants from the National Institutes of Health (RO1-NS21169-10) to Dr Rosenberg. The synthetic metalloproteinase inhibitor was a gift from Dr A. Gearing of British Biotechnology.

**References**

Breakdown of blood-brain barrier (BBB) after cerebral ischemia–reperfusion may involve a multitude of molecular events, including the engagement of such mediators as free radicals, cytokines, and others. In the preceding article by Rosenberg and colleagues, a time-dependent correlation of postischemic BBB breakdown and the expression of metalloproteinases (MMPs) was noted. An increase in sucrase space reflecting the extent of BBB breakdown was correlated with gelatinase A expression at 3 hours after ischemia. The enhanced proteolytic activity, suggesting a perturbed balance of proteases and their inhibitors, may contribute to the postischemic opening of the BBB. Rosenberg and associates also found an increase in tissue MMP inhibitor activity, especially at later time points. It is interesting to note that the early BBB opening, which was presumably related to early gelatinase A expression, was reduced by a synthetic MMP inhibitor, BB-1101. However, the second phase of postischemic BBB breakdown and the ultimate outcome was not affected by treatment with this protease inhibitor.

Dr Rosenberg’s laboratory was the first to apply zymograms and reverse zymograms to determine changes in the activities of MMPs and their inhibitors in the rat stroke model. This study, together with earlier works by the same group, provide interesting findings on the activation of a complex biochemical cascade that may contribute to ischemic brain edema.

It should also be noted that in the present study, the correlation of MMP activities and BBB breakdown, based on sucrase space and brain water content, was not as tight as expected. This may be partly caused by the sampling method applied to this study. Lack of BB-1101 effects on the ultimate outcome at 48 hours, especially on the brain water content, suggests the need to measure MMP activities following BB-1101 treatment to possibly maximize the intended therapeutic effects.

In summary, the Rosenberg group pioneered in the demonstration of an activated MMP pathway in the setting of cerebral ischemia with or without reperfusion. MMP expression may contribute to the postischemic BBB breakdown after ischemic stroke. However, additional studies are needed before the roles of MMPs in the pathogenesis of ischemic brain edema can be fully defined.

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*Stroke*. 1998;29:2189-2195
doi: 10.1161/01.STR.29.10.2189

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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