P-Selectin and Intercellular Adhesion Molecule-1 Expression After Focal Brain Ischemia and Reperfusion

Yasushi Okada, MD; Brian R. Copeland, MD; Etsuro Mori, MD; Ming-Ming Tung, BS; Winston S. Thomas, MD; Gregory J. del Zoppo, MD

Background and Purpose Polymorphonuclear leukocytes have been implicated in the development of the "no-reflow" phenomenon after focal cerebral ischemia and reperfusion. To further understand the role of granulocytes in microvascular occlusions, the responses of the granulocyte-endothelial cell adhesion molecules P-selectin and intercellular adhesion molecule-1 during middle cerebral artery ischemia and reperfusion were characterized.

Methods Twelve adolescent male baboons were used for 2-hour middle cerebral artery occlusion (n=3) or for 3-hour occlusion with 1-hour (n=3), 4-hour (n=3), and 24-hour (n=3) reperfusion, and three separate unoperated primates served as controls. A quantitative immunohistochemical study of the microvascular distribution of P-selectin and intercellular adhesion molecule-1 was performed using 10-μm frozen sections from basal ganglia analyzed with computerized light microscopy video imaging.

Results Significant (P<.05) persistent upregulation of P-selectin (beginning during ischemia) and transient upregulation of intercellular adhesion molecule-1 (at 1 and 4 hours of reperfusion) were observed on endothelium of selected postcapillary microvessels of the ischemic lenticulostriate artery territory. Platelet accumulation also occurred in this territory and was responsible for a significant proportion of the nonendothelial P-selectin signal at 24 hours after reperfusion.

Conclusions Focal cerebral ischemia/reperfusion stimulates endothelial P-selectin and intercellular adhesion molecule-1 expression in brain microvessels in the ischemic zone, which may contribute to enhanced leukocyte adherence and persistent activation. (Stroke. 1994;25:202-211.)

Key Words • cell adhesion molecules • endothelium • leukocytes • microcirculation • reperfusion

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ments the previous finding of PMN leukocytes in this territory.1,2

Materials and Methods

Twelve adolescent male baboons (Papio anubis/cynocephalus) weighing 8.7 to 11.4 kg were used for the MCA occlusion/reperfusion studies, and three separate primates weighing 9.2 to 25.0 kg served as controls. Before entry into this study, all animals lacked evidence of disease during a mandated standard quarantine period. The protocols throughout this study 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 on Humane Care and Use of Laboratory Animals, and the US Department of Agriculture Animal Welfare Act. In compliance with these standards, every effort was made to ensure that the subjects were free of pain or discomfort. The principal investigator, veterinarian, and prime handling staff were present for all procedures.

Preparation of the non-human primate model of right MCA occlusion and reperfusion by surgical implantation of the MCA occlusion device (PS Medical, Goleta, Calif) has been described in detail elsewhere.1,2 Halothane anesthesia administered as 3% to 5% induction followed by 1.5% to 2.0% maintenance was routine during the surgical procedures. Following surgical recovery, all animals were allowed a 7-day interval before entry into the experimental protocol. All animals entered into the study were clinically free of infection or apparent inflammation and had normal neurological function (score, 100).21

In this study, 3 animals underwent right MCA occlusion for 2 hours; 9 animals (3 each) underwent MCA occlusion for 3 hours and subsequent reperfusion for 1 hour, 4 hours, or 24 hours; and 3 animals did not undergo the surgical implantation procedure and served as a control group. The experimental paradigm previously reported was followed here exactly.1-3 Each experiment was terminated by pressure perfusion with isosmotic perfusion flush solution during reperfusion.3 Pressure perfusion at the end of 2 hours of MCA occlusion only required simultaneous deflation of the balloon. Before termination, each animal underwent craniotomy under pentothal sodium (15 mg/kg infusion) anesthesia and mechanical ventilation.5 A left occipital quadrilateral cranial window was extended caudally to the foramen magnum and rostrally by segmental removal. Hemorrhage was minimal (10 to 20 mL). Perfusion of the superior thoracic and cranial arteries was extended caudally to the foramen magnum and was immersed in ice. The brain was immediately excised en bloc from the cranium and was immersed in ice. It was subdivided into 1-cm coronal sections and was stored at -70°C until sectioning.

OCT compound (Miles, Inc, Elkhart, Ind) in Plasmalyte (Baxter Healthcare, Deerfield, Ill) adjusted to 340 mOsm/L with NaCl, pH 7.4, to wash structures was achieved by infusion of chilled (4°C) perfusion fluid at 180 to 210 mm Hg for 4 minutes (700 to 800 mL/min flow) with endogenous peroxidase activities, then with streptavidin horse-radish peroxidase complex (Vector Laboratories) for 30 minutes. Antibody-bound peroxidase was detected with the chromogen substrate 3-amino-9-ethyl carbazole (AEC Kit, Biomeda Corporation, Foster City, Calif), freshly prepared at 0.02% in 20 mmol/L sodium acetate buffer and 0.03% hydrogen peroxide, and incubated for 10 minutes. Sections were washed in tap water, counterstained with Mayer's hematoxylin (Biomeda Corporation) for 1 minute, and blued in saturated sodium bicarbonate solution. All immunostained specimens were then mounted with clear mounting medium (Biomeda Corporation). The following immunohistochemical controls were routinely performed on each tissue type: (1) deletion of the primary antibody, (2) deletion of the secondary antibody, and (3) TIB115, a murine MoAb against the irrelevant SV40 large T viral antibody.

To permeabilize the endothelium29 to assess the intracellular content of endothelial P-selectin, adjacent sections were fixed with acetone and incubated with the anti-P-selectin antibody for 10 minutes.

The absolute number and minimum transverse diameters of manually identified peroxidase-stained microvessels from the basal ganglia of post–ischemic/reperfused and nonischemic territories were determined with the computerized video-imaging system previously described.9 Minimum transverse diameters of peroxidase-stained vascular structures from non-overlapping images (×400) in a 1000-field matrix (66.4 mm²) were computed from each section with a linear measurement program. Off-line analysis of the microvascular distribution was performed with resident statistical programs.

To determine the exact frequency of P-selectin– and ICAM-1-positive vessels, those more than 100 µm in diameter (microvessels) stained by the MoAb CLB-HEC-75 was regarded as the total number of microvessels.3,23 P-selectin and ICAM-1 expression is presented as the fraction of peroxidase-stained vessels, P-selectin/CD31 and ICAM-1/CD31,
FIG 1. Top left, P-selectin-containing endothelium with intensity level 1 stain (arrows) in the post-ischemia/reperfusion (I/R) zone at 1 hour of reperfusion. Top right, P-selectin-containing endothelium with intensity level 2 stain in the post-I/R zone at 24 hours of reperfusion. Bottom left, LlP4 (anti-glycoprotein IIb/IIIa)-positive microvessels in the post-I/R zone at 24 hours of reperfusion, from section adjacent to that of top right panel. Magnification bar=50 μm.
respectively. Since P-selectin expression is seen on both stimulated endothelium and platelets, the contribution of platelet (α-granule)-related P-selectin to the overall increase was measured indirectly with the specific anti-platelet GPIIb/IIIa antibody, LJ-P4. The difference in total P-selectin- and GPIIb/IIIa-positive vessels was regarded as the number of microvessels with endothelium-associated P-selectin expression. The intensity of P-selectin and ICAM-1 peroxidase stain associated with the microvascular endothelia in each section was assessed visually by three observers not aware of the specimen source, timing, or antibody, who graded the stain according to a 3-point scale: 0, no stain; 1, light stain; and 2, strongly stained (Fig 1). Included in the group with intensity level 1 stain were a small number of microvessels with punctate stain in the vascular lumen adjacent to the endothelium, often at acute bends. Concordance of the intensity judgments with this scale was 97%. The presence of ischemic injury, indicated by the relative degree of neuron damage, was assessed by the method of Etke et al. The center of the ischemic zone was defined as the area with type IV neuronal damage and nonischemic tissue by the presence of type I cells only. Among the post–ischemia/reperfusion specimens, 33.6±11.0% of the area analyzed had type IV neuron damage. This area corresponded to variable degrees of visible contiguous tissue disturbance (center of ischemic zone). Tissue peripheral to this injury had some type II and III neuron damage.

### Results

The appearance of contralateral neurological deficits (hemiparesis and/or unresponsiveness to tactile and visual stimuli) occurred within 10 minutes after MCA occlusion in each subject. A significant and persistent reduction in neurological score to 44.2±13.9 at 1 hour was observed in both the MCA occlusion and MCA occlusion/reperfusion groups. Baseline data of cell blood count (total leukocyte count, 9.2±2.8×10⁶/μL; hematocrit, 35.5±3.6%; platelet count, 432±104×10⁶/μL) were not different in each cohort.

The ability of the anti-P-selectin polyclonal antibody and the anti–ICAM-1 MoAb to identify their respective vascular epitopes is indicated in Figs 1 and 2. The number of microvessels with the endothelial epitope CD31 in both basal ganglia in all subjects was the same as previously reported and did not differ significantly (Table 1). In the control subjects, the fraction of vessels with endothelial surface–expressed P-selectin or ICAM-1 was low (<0.6% and <1.5% of CD31, respectively) (Tables 2 and 3). In the controls, the small number of microvessels with peroxidase stain for each epitope had intensity level 1 only. The remainder of the vessels had no peroxidase stain. Both P-selectin and ICAM-1 were limited to noncapillary microvessels greater than 7.5 μm in diameter (Figs 3 and 4).

### Microvascular endothelial-associated P-selectin (fraction P-selectin/CD31) in sections of the non-ischemic/reperfused basal ganglia at each time point was not different from the basal ganglia of the control subjects (Table 2, Fig 5). In contrast, microvascular P-selectin expression in the post–ischemic/reperfused basal ganglia was significantly increased (P<.05), mainly in the precapillary and postcapillary vessel range (7.5 to 30.0

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**TABLE 1. Number of CD31-Positive Vessels**

<table>
<thead>
<tr>
<th>Duration</th>
<th>Non-I/R</th>
<th>Post-I/R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>889.2±35.3</td>
<td>898.3±40.3</td>
</tr>
<tr>
<td>MCA:O</td>
<td>817.7±95.2</td>
<td>903.5±76.2</td>
</tr>
<tr>
<td>MCA:O/R</td>
<td>973.0±175.4</td>
<td>980.7±171.7</td>
</tr>
<tr>
<td>1 h</td>
<td>960.8±146.8</td>
<td>1000.2±157.0</td>
</tr>
<tr>
<td>24 h</td>
<td>879.2±91.2</td>
<td>1000.0±231.0</td>
</tr>
</tbody>
</table>

Values are mean±SD per 1000 fields. Non-I/R indicates non-ischemia/reperfusion zone; post-I/R, post-ischemia/reperfusion zone; MCA:O, 2-hour middle cerebral artery occlusion; and MCA:O/R, 3-hour middle cerebral artery occlusion with indicated periods of reperfusion.
were detected in the non-ischemic/reperfused basal ganglia. At 2 hours of ischemia/reperfusion, particlarly at 4 and 24 hours, an apparent increase in mean fraction P-selectin/CD31 was noted (Fig 5).

Because of the specificity of the anti-P-selectin preparation for activated platelets, the potential contribution of platelets to the P-selectin signal in microvessels of both non-ischemic/reperfused and post-ischemic/reperfused zones was examined. No GPIIb/IIIa-positive vessels were detected in the non-ischemic/reperfused basal ganglia. At 2 hours of ischemia (—1 hour of reperfusion) and also at 1 hour of reperfusion, P-selectin was clearly associated with the endothelial lining. During reperfusion, particularly at 4 and 24 hours, an apparent increase in mean fraction P-selectin/CD31 was noted (Fig 5).

A simple assessment of the intensity of microvessel-associated peroxidase stain was made to further indicate the responses of P-selectin to ischemia/reperfusion. The intensity of P-selectin—associated stain in the area of the post-ischemic/reperfused zone with type IV neuron injury was generally greater (level 2) than that of the non-ischemic/reperfused zone and of the control subjects at all time points (Table 3). Lighter stained microvessels (level 1) were found scattered in the peripheral area.

To further estimate the distribution of total microvascular endothelial P-selectin, sections were fixed with acetone, which served to increase endothelial cell permeability to the anti-P-selectin antibodies.30 The proportion of total endothelial P-selectin (acetone) available as surface-expressed P-selectin (methanol) was significantly greater ($P<.05$) in the post—ischemic/reperfused basal ganglia than in the nonischemic basal

### Table 2. Fraction of CD31-Positive Microvessels Expressing P-Selectin or Intercellular Adhesion Molecule-1

<table>
<thead>
<tr>
<th></th>
<th>P-selectin</th>
<th>GPIIb/IIIa</th>
<th>Δ(P-selectin-GP)</th>
<th>ICAM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>Non-I/R</td>
<td>Post-I/R</td>
<td>Non-I/R</td>
<td>Post-I/R</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>0.006±0.004</td>
<td>0.000±0.000</td>
<td>0.006±0.004</td>
</tr>
<tr>
<td>MCA:O</td>
<td>3</td>
<td>0.013±0.015</td>
<td>0.108±0.079*</td>
<td>0.001±0.026</td>
</tr>
<tr>
<td>MCA:O/R</td>
<td>1 h</td>
<td>3</td>
<td>0.036±0.019</td>
<td>0.096±0.031*</td>
</tr>
<tr>
<td></td>
<td>4 h</td>
<td>3</td>
<td>0.014±0.007</td>
<td>0.134±0.132</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>3</td>
<td>0.012±0.012</td>
<td>0.223±0.141*</td>
</tr>
</tbody>
</table>

Values are mean±SD per 1000 fields. ICAM-1 indicates intercellular adhesion molecule-1; non-I/R, non-ischemia/reperfusion zone; post-I/R, post-ischemia/reperfusion zone; MCA:O, 2-hour middle cerebral artery occlusion; and MCA:O/R, 3-hour middle cerebral artery occlusion with indicated periods of reperfusion.

### Table 3. Intensity Scores of Microvessel-Associated P-Selectin and Intercellular Adhesion Molecule-1

<table>
<thead>
<tr>
<th>Duration</th>
<th>Non-I/R</th>
<th>Peripheral</th>
<th>Center</th>
<th>Post-I/R</th>
<th>Non-I/R</th>
<th>Peripheral</th>
<th>Center</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1 (100)</td>
<td>1 (100)</td>
<td></td>
<td>1 (100)</td>
<td>1 (100)</td>
<td>1 (100)</td>
<td></td>
</tr>
<tr>
<td>MCA:O</td>
<td>1 (100)</td>
<td>1 (95)</td>
<td>2 (98)</td>
<td>0</td>
<td>1 (100)</td>
<td>1 (100)</td>
<td>1 (100)</td>
</tr>
<tr>
<td>MCA:O/R</td>
<td>1 h</td>
<td>1 (100)</td>
<td>1 (78)</td>
<td>2 (77)</td>
<td>1 (100)</td>
<td>1 (88)</td>
<td>1 (93)</td>
</tr>
<tr>
<td></td>
<td>4 h</td>
<td>1 (97)</td>
<td>1 (69)</td>
<td>2 (98)</td>
<td>1 (100)</td>
<td>1 (100)</td>
<td>1 (100)</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>1 (100)</td>
<td>1 (87)</td>
<td>2 (93)</td>
<td>1 (100)</td>
<td>1 (100)</td>
<td>1 (100)</td>
</tr>
</tbody>
</table>

1ICAM-1 indicates intercellular adhesion molecule-1; non-I/R, non-ischemia/reperfusion zone; post-I/R, post-ischemia/reperfusion zone; MCA:O, 2-hour middle cerebral artery occlusion; and MCA:O/R, 3-hour middle cerebral artery occlusion with indicated periods of reperfusion. Number in parentheses is mean percentage of positive microvessels with intensity level indicated: 0, no stain; 1, lightly positive stain; and 2, strongly positive stain (see Figs 1 and 2 and text). Center refers to region with type IV neuron damage; peripheral area had predominantly type II and III injury, without type IV.

*P-selectin is a polyclonal preparation; ICAM-1, monoclonal preparation.

1Note that in the non-I/R zone microvessels without stain (level 0) accounted for 98.6±1.1% of the total vessel number.
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Fig 3. Bar graph shows distribution of mean vessel diameter range in P-selectin (methanol)-positive microvessels. At all time points, the microvascular distribution of surface P-selectin was seen mainly in the 7.5- to 30.0-µm-diameter range in both the non-ischemia/reperfusion (I/R) and post-I/R zones. MCA indicates middle cerebral artery; C, control cohort; a, 4.0 to 7.5 µm; b, 7.5 to 30.0 µm; c, 30.0 to 50.0 µm; d, 50.0 to 100.0 µm; and e, > 100.0 µm. There were no significant differences in vessel diameter distribution between the non-I/R and the post-I/R data sets or among the time points within either set.

The appearance of the PMN leukocyte CD11b/CD18 counterreceptor ICAM-1 was also studied. Anti-ICAM-1 MoAb was detected on the endothelium of affected microvessels (Fig 2). In the post-ischemic/reperfusion basal ganglia, the fraction of ICAM-1-positive microvessels increased significantly (P<.05) at 1 hour and 4 hours after reperfusion over the contralateral non-ischemic/reperfused and post-ischemic/reperfused sides in the acetone-fixed specimens (data not shown). The intensity of endothelium-associated ICAM-1 signal in the post-ischemic/reperfused zone was no different than that of the few positive microvessels in the non-ischemic/reperfused zone (Table 3), although the number of positive vessels was significantly greater at 1 and 4 hours of reperfusion in the post-ischemic/reperfused zone (Fig 8). ICAM-1 presented mainly in the noncapillary 7.5- to 30.0-µm-diameter microvessels (Fig 4).

Discussion

There are few studies of P-selectin34-36 and ICAM-137-38 expression in vivo after focal ischemia/reperfusion and none so far in cerebral vessels during ischemia and reperfusion. The endothelial expression of P-selectin in microvessels of the LSA territory increased significantly in frequency after 2-hour MCA occlusion and then at 3-hour MCA occlusion/1-, 4-, and 24-hour reperfusion in the awake baboon, suggesting persistent upregulation of ischemia. The frequency of ICAM-1 expression appeared maximal at 1 to 4 hours after reperfusion and gradually decreased with duration of reperfusion.
of P-selectin after ischemia and reperfusion. In contrast, ICAM-1 expression appeared transiently at 1 and 4 hours after reperfusion in this model.

Here, the vascular distribution of P-selectin was primarily in the 7.5- to 30.0-µm-diameter group, consistent with the distribution of precapillary arterioles and postcapillary venules. The known preferential distribution of P-selectin and ICAM-1 expression in postcapillary venules is consistent with the favored site of ischemia/reperfusion-related PMN leukocyte adhesion. Moreover, P-selectin functions as a procoagulant factor. Therefore, the vascular and cellular distribution of P-selectin and ICAM-1 in the post-MCA occlusion/reperfusion zone may be critical to the generation of cerebrovascular complications. Thus, the appearance of P-selectin and ICAM-1 expression in the post-MCA occlusion/reperfusion zone may be critical to the generation of cerebrovascular complications.

It should be noted that the vascular and cellular distribution of P-selectin and ICAM-1 in the post-MCA occlusion/reperfusion zone may be critical to the generation of cerebrovascular complications. Thus, the appearance of P-selectin and ICAM-1 expression in the post-MCA occlusion/reperfusion zone may be critical to the generation of cerebrovascular complications. Thus, the appearance of P-selectin and ICAM-1 expression in the post-MCA occlusion/reperfusion zone may be critical to the generation of cerebrovascular complications.
rectly to fibrin deposition through monocyte tissue factor. In vivo this unregulated expression of P-selectin could result in a vicious cycle of persistent neutrophil adhesion and activation, together with platelet activating factor release and platelet aggregation, tissue injury, cytokine release, further ICAM and P-selectin expression, and eventual tissue destruction.

ICAM-1 supports ischemia/reperfusion-induced granulocyte endothelial adhesion and is necessary for transmigration. In this study, ICAM-1 was detected early after reperfusion (ie, 4 to 7 hours after the initiation of ischemia), after the appearance of P-selectin. Because mRNA synthesis is required, the initial appearance of ICAM-1 occurs 30 minutes after α-thrombin exposure and may persist for up to 120 minutes in HUVECs. Sobel et al reported the presence of ICAM-1 antigen at 2 and 4 days in vessels of the infarct zone of two patients suffering stroke. Inflammatory stimuli including endotoxin, IL-1, and TNF-α lead to ICAM-1 expression. In primary cultures of human brain microvessel endothelium, upregulation of ICAM-1 expression to continuous LPS or cytokine exposure was concentration and time dependent, appearing as early as 4 hours after incubation and persisting for up to 72 hours. The transient appearance of ICAM-1, located on the endothelial cell surface, after MCA occlusion/reperfusion in this model implies that either the stimuli for expression (eg, cytokines) appear transiently or may be limited by reperfusion. Rapid postexpression metabolism of ICAM-1 may produce circulating products, which may be removed during reperfusion. Alternatively, superoxide release from granulocytes trapped in the postcapillary venule during focal ischemia/reperfusion may stimulate ICAM-1 expression locally, and for a limited period in this model.

These observations raise the possibility that agents that block P-selectin and/or ICAM-1 function may attenuate edema formation and/or ischemic injury. Palabrica et al noted that the anti-P-selectin MoAb GA6 could inhibit leukocyte-platelet binding and fibrin deposition in a baboon arteriovenous shunt. Another anti-P-selectin MoAb (PB1.3) significantly attenuated myocardial infarct size after 10 minutes of occlusion/reperfusion in a feline myocardial ischemia/reperfusion model. Harlan reported that treatment with PB1.3 resulted in significantly less edema and tissue necrosis after 6-hour occlusion/reperfusion in a rabbit ear model.

Few in vivo studies of anti–ICAM-1 strategies on ischemia/reperfusion injury have been reported. Ma et al noted that cats exposed to the anti–ICAM-1 MoAb RR 1/1 developed significantly less myocardial necrosis and had enhanced endothelial-dependent relaxation to acetylcholine relative to those receiving nonbinding control antibody after myocardial ischemia/reperfusion. In the central nervous system, Clark et al found no improvement in the neurological deficit after anti–ICAM-1 MoAb R6.5 infusion in irreversible brain ischemia in a rabbit thromboembolism model. Neither study examined the appearance of ICAM-1 during ischemia/reperfusion.

After focal cerebral ischemia and reperfusion, cerebral microvascular endothelia display a prolonged expression of P-selectin and a transient appearance of ICAM-1 on their surface. In other systems, the coordinated appearance of both adhesion receptors is required for “rolling” of circulating granulocytes, their endothelial adhesion in postcapillary venules, transmigration, and eventual tissue destruction. The appearance of both adhesion receptors and PMN leukocyte adhesion in postcapillary venules after MCA occlusion/reperfusion is consistent with those findings. Such findings suggest that the effect of anti–P-selectin and of anti–ICAM-1 strategies on the microvascular consequences of focal cerebral ischemia and reperfusion should be carefully studied in appropriate models.

Acknowledgments

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References

In this issue of *Stroke*, Okada and coworkers report that middle cerebral artery occlusion/reperfusion alters the surface expression of intercellular adhesion molecule-1 (ICAM-1) and P-selectin on microvessel endothelium in regions of the brain subjected to ischemia. The expression of these molecular determinants of leukocyte/endothelial cell adhesion was not detected on capillary endothelium but was noted in approximately 10% of microvessels with diameters ranging between 7.5 and 30 μm. Although the type of vessel expressing ICAM-1 and P-selectin was not characterized, the fact that leukocyte adhesion occurs preferentially in postcapillary venules during inflammation suggests that leukocytes may also become sequestrated in regions of the brain subjected to ischemia.
P-selectin and intercellular adhesion molecule-1 expression after focal brain ischemia and reperfusion.
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