Phosphatidylinositol 3–Kinase Inhibitor Failed to Reduce Cerebral Vasospasm in Dog Model of Experimental Subarachnoid Hemorrhage

Hitoshi Kimura, MD; Kenroh Sasaki, PhD; Toshinari Meguro, MD; John H. Zhang, MD, PhD

Background and Purpose—Phosphatidylinositol 3–kinase (PI3-kinase) is involved in smooth muscle contraction induced by growth factors and/or G protein–coupled receptor agonists. To evaluate the role of PI3-kinase in the pathogenesis of delayed vasospasm, we applied 2 PI3-kinase inhibitors to an established canine double-hemorrhage model of experimental subarachnoid hemorrhage.

Methods—Twenty-four dogs underwent double blood injections via the cisterna magna on days 0 and 2. The dogs were killed on day 7. Dogs were treated with either vehicle (dimethyl sulfoxide), wortmannin, or LY294002 once per day on day 2 through day 6. Angiography was performed before blood injection and before the dogs were killed. The basilar arteries were collected for morphology, Western blot analysis, and PI3-kinase activity.

Results—The residual diameter of the basilar arteries in the dimethyl sulfoxide treatment group, which was compared with day 0 angiogram, decreased markedly on day 7 (the percentage of the residual diameter was 47.8 ± 0.8%). Wortmannin and LY294002 did not significantly change residual diameter on day 7. Both PI3-kinase inhibitors abolished PI3-kinase activity compared with the vehicle treatment group. However, both PI3-kinase inhibitors failed to significantly attenuate PI3-kinase protein expression (Western blot) (P > 0.05, ANOVA).

Conclusions—Delayed treatment, which was to mimic the clinical situation, with PI3-kinase inhibitors failed to reverse vasospasm. PI3-kinase may not play an important role in the delayed vasospasm. The possible effect of PI3-kinase inhibitors in the early stage of vasospasm was not investigated in the present study. (Stroke. 2002;33:593-599.)

Key Words: 1-phosphatidylinositol 3-kinase ■ subarachnoid hemorrhage ■ vasospasm, intracranial ■ wortmannin ■ dogs
biphasphate [Pi(4,5)P2] were purchased from AVANTI. The compound γ(32)P-ATP (3000 Ci/mmoll) was purchased from Amersham. The silica gel 60 thin layer chromatography (TLC) plates were purchased from VWR Scientific Products. All other chemicals were purchased from Sigma.

**SAH Model**

Twenty-four adult mongrel dogs of either sex, weighing 18 to 24 kg, were used in this canine double-hemorrhage model. The dogs were anesthetized with thiopental (10 mg/kg) and mechanically ventilated during the procedures. Each dog’s body temperature was maintained at 37°C with a heating blanket, and a 4F catheter was inserted into the femoral artery to monitor mean arterial blood pressure and blood gases. Experimental SAH was induced according to the method described by Varsos et al. and has been described previously. Cerebral angiography was performed with the use of the Exposcop 7000 (Ziehm International Medical Systems). One of the vertebral arteries was catheterized with a 4F catheter via the femoral artery to obtain the baseline vertebrobasilar angiogram. The cisterna magna was punctured transcutaneously, and 0.4 mL/kg of cerebrospinal fluid (CSF) was withdrawn. An equivalent amount of arterial blood was withdrawn from the femoral artery and immediately injected into the cisterna magna. This first injection was considered the day 0 SAH. On day 2, this blood injection procedure was repeated without angiography. All dogs were killed on day 7 after angiography.

Three groups of dogs were treated with the vehicle (dimethyl sulfoxide [DMSO]) (n=6), wortmannin (n=6), and LY294002 (n=6). Treatment began on day 2 and ended on day 6 by intracisternal injection. The first injection of drugs was conducted 1 hour after the second blood injection. The doses of the inhibitors were calculated individually for each dog to approximate similar drug levels in the CSF, taking into account the relative size of their respective CSF spaces. Wortmannin and LY294002 were diluted in DMSO to 10 mmol/L and 30 mmol/L. Drug solutions (approximately 40 µL) were diluted with 1 mL of CSF and injected intracisternally to obtain a final concentration in the CSF of 10 and 30 µmol/L, with the assumption that the canine CSF volume was 2.0 mL/kg.21 The final concentration of DMSO was approximately 0.1%. Serving as an SAH control, a fourth group of dogs (n=6) underwent double-hemorrhage injections but were not treated. All dogs were killed by an overdose of pentobarbital (120 mg/kg) on day 7, including a fifth group of untreated dogs (n=4) for tissue harvesting to serve as a normal control in the Western blotting analysis and the PI3-kinase activity measurement.

The Animal Care and Use Committee at the University of Mississippi Medical Center evaluated and approved this protocol.

**Measurements of Arterial Diameter**

Arterial diameters were measured in a double-blind fashion on magnified angiograms. To eliminate magnification differences on the angiograms, a penny was placed on the dog’s chin during the angiography run. The same penny was always used and put at the same point of the dog’s chin. Relative to the size of this coin as a standard, all arterial diametric values were adjusted. Two researchers independently measured the arterial diameters on the magnified angiograms at 3 points: the distal, central, and proximal portions of the basilar artery. The mean of these 3 measurements was calculated to yield the arterial diameter. The mean of the values measured in both studies was taken as the final diameter of the basilar artery. The caliper of the basilar artery on day 7 was calculated as the percentage of the mean basilar artery diameter on day 0 in each dog.

**Morphology**

The basilar arteries were fixed in 10% buffered formalin and carefully removed from the brain stem. The basilar arteries were stained with hematoxylin and eosin for light microscopy.

**Western Blot Analysis**

The basilar arteries were carefully removed from the brain stem. Branches, excess tissue, and blood were carefully removed from the basilar artery. The basilar arteries were immediately frozen in liquid nitrogen and stored at −80°C until used. The frozen arteries were homogenized for 20 minutes at 4°C with an ultrasonic wave (10 seconds, 3 times) in 100 µL of an extraction buffer containing 50 mmol/L Tris-HCl (pH 7.5); 1% nonylphenol ethoxylate (Igepal); 0.25% sodium deoxycholate; 150 mmol/L NaCl; 1 mmol/L EGTA; 1 mmol/L phenylmethylsulfonyl fluoride; 1 µg/mL aprotinin, leupeptin, pepstatin; 1 mmol/L Na3VO4; and 1 mmol/L NaF. The insoluble material was removed by centrifugation at 16 000g at 4°C for 15 minutes. The samples (20 to 30 µg protein) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis with 7.5% polyacrylamide gel. After electrophoretic transfer of the separated polypeptides to nitrocellulose membranes, the membranes were blocked with 3% nonfat milk. The membranes were then washed and incubated with PI3-kinase antibodies (p85 or p110γ) at 4°C. Nitrocellulose membranes were later washed with 3% nonfat milk and incubated with an anti-rabbit IgG (horseradish peroxidase conjugate) antibody at room temperature. An enhanced chemiluminescence kit (Amersham) was used to visualize the protein bands. The results were quantified by Quantity One Software (Biorad).

**Immunoprecipitation**

The immunoprecipitation with PI3-kinase antibodies (p85 or p110γ) was performed for PI3-kinase activity according to previously described methods22,23 with some modifications. The basilar arteries were carefully removed from the brain stem. Branches, excess tissue, and blood were carefully removed from the basilar artery. The basilar arteries were then immediately frozen in liquid nitrogen and stored at −80°C until analyzed. The frozen basilar arteries were homogenized for 20 minutes at 4°C in the lysis buffer (buffer A; 137 mmol/L NaCl, 20 mmol/L Tris-HCl [pH 7.4], 1 mmol/L CaCl2, 1 mmol/L MgCl2, 1 mmol/L sodium orthovanadate, containing 1% NP-40 and 1 mmol/L phenylmethylsulfonyl fluoride). The lysates were centrifuged for 10 minutes at 16 000g at 4°C. The protein concentration was measured with a kit from Bio-Rad. Only the supernates of the lysates were used in the protein measurements. The protein concentration was adjusted to 500 µg/mL, with the total protein amount at 500 µg.23 A combination of 1.0 mL lystate (500 µg of protein) and 5 µL anti-PI3-kinase antibody p85 or 10 µL anti-PI3-kinase antibody p110γ was incubated with rocking for 1 hour at 4°C. After the addition of 30 µL protein A–agarose and rocking for 1 hour at 4°C, the antibody-enzyme immunoprecipitates were collected by centrifugation. The immunoprecipitates were washed 3 times with buffer A containing 1% NP-40, washed 3 times with 0.1 mol/L Tris-HCl (pH 7.4) and 0.1 mol/L sodium orthovanadate, and washed 2 times with TNE (10 mol/L Tris-HCl [pH7.4], 150 mmol/L NaCl, 5 mol/L EDTA) that contained 0.1 mol/L sodium orthovanadate.

**PI3-Kinase Activity**

The immunoprecipitates obtained with the PI3-kinase antibodies (p85 or p110γ) were assayed according to previously described methods23–26 with some modifications. The last wash was removed as completely as possible, and to each immunoprecipitate was added 50 µL of TNE, 10 µL (20 µg) PIP(4,5)P2, and 10 µL 100 mmol/L MgCl2. The reaction was initiated by adding 5 µL of the ATP working solution (0.88 mmol/L ATP, 3000 Ci/mmol γ32P-ATP, and 20 mmol/L MgCl2) that contained 30 µCi γ32P-ATP per sample. After constant agitation for 10 minutes, the reaction was terminated by adding 20 µL of 6H HCl. The radiolabeled lipids were extracted with 160 µL CHCl3:MeOH:H2O:NH4OH (60:47:11:3:2), dried, and visualized by autoradiography. The TLC plates were visualized after development.
Physiological Variables in a Dog Model of Experimental SAH

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>SBP, mm Hg</th>
<th>DBP, mm Hg</th>
<th>Heart Rate, bpm</th>
<th>PaO2, mm Hg</th>
<th>PaCO2, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>6</td>
<td>88.2±5.5</td>
<td>57.7±5.8</td>
<td>125.0±6.9</td>
<td>7.385±0.013</td>
<td>34.8±2.2</td>
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<tr>
<td>Day 0</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Day 7</td>
<td></td>
<td>90.2±2.9</td>
<td>62.0±1.7</td>
<td>117.8±9.7</td>
<td>7.450±0.029</td>
<td>36.7±1.8</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>5</td>
<td>93.8±4.7</td>
<td>61.6±5.9</td>
<td>116.8±6.7</td>
<td>7.393±0.023</td>
<td>37.3±0.9</td>
</tr>
<tr>
<td>Day 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td></td>
<td>84.6±4.8</td>
<td>50.2±6.0</td>
<td>101.4±5.5</td>
<td>7.410±0.015</td>
<td>38.2±0.6</td>
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<tr>
<td>LY294002</td>
<td>6</td>
<td>90.5±2.7</td>
<td>59.0±3.6</td>
<td>100.7±4.4</td>
<td>7.353±0.016</td>
<td>33.6±1.7</td>
</tr>
<tr>
<td>Day 0</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td></td>
<td>91.0±2.8</td>
<td>61.7±1.5</td>
<td>112.0±3.8</td>
<td>7.380±0.016</td>
<td>35.7±2.2</td>
</tr>
</tbody>
</table>

SBP indicates systolic blood pressure; DBP, diastolic blood pressure. Values are mean±SE.

with a PhosphorImager System (Molecular Dynamics). Rate of flow value was calculated in each sample and each experiment. Every TLC was performed under the same conditions. In preliminary experiments, PI alone, PI(4)P alone, or PI(4,5)P2 alone was reacted to obtain each rate of flow value for PI(3)P, PI(3,4)P2, or PI(3,4,5)P3, respectively. On the basis of these rates of flow values, each mass was considered to be each 3-phosphorylated phosphoinositide in this experiment. Additionally, on the basis of the density of the background, the density of mass was revised. Then the density of mass in each group was compared with the control group, and the difference in densitometric analysis was compared. The efficiency of immunoprecipitation was consistent and uniform throughout all measurements.

Data Analysis
Data are expressed as mean±SEM. Statistical differences between the control and other groups were compared by 1-way ANOVA and then the Tukey-Kramer multiple comparison procedure if a significant difference had been determined by ANOVA. A probability value of P<0.05 was considered statistically significant.

Results

Physiological Evaluation
Twenty-three dogs remained healthy. There were no significant differences in physiological variables among groups when compared at day 0 or day 7 (Table). One dog in the wortmannin treatment group had severe neurological deficits after the second blood injection. According to the policy of the University of Mississippi Medical Center Animal Care and Use Committee, that dog was killed on day 2.

Angiography on Day 7
The caliber of the basilar artery was calculated as the ratio of the mean diameter of the basilar artery on day 7, ie, a percentage of which was recorded on day 0. Figure 1A shows the development of severe vasospasm on day 7 in the DMSO, wortmannin, and LY294002 groups.

Effects of PI3-Kinase Inhibitors
The PI3-kinase inhibitor treatment groups were compared with the vehicle (DMSO) group because the PI3-kinase inhibitors were diluted with DMSO. The mean value of the residual diameter of the basilar arteries in the vehicle (DMSO) treatment group on day 7 was 47.8±0.8% and in the SAH control (nontreatment) group was 46.2±6.3%, thus indicating that intrathecal injection of DMSO did not alter the vessel diameter. The residual diameters of the basilar arteries in the wortmannin and LY294002 groups on day 7 were 47.9±8.8% (P=0.966) and 55.8±4.3% (P=0.097), respectively (Figure 1B). These results showed that neither PI3-kinase inhibitor significantly attenuated vasoconstriction (P>0.05, ANOVA) compared with the vehicle group (DMSO).

Morphological Examination
In the DMSO, wortmannin, and LY294002 groups, narrowing of the vessel lumen, corrugation of the lamina elastica and
endothelium, and thickness of the vessel wall were observed under light microscopy (Figure 2).

Western Blotting Analysis
The amounts of PI3-kinase p85 (class IA) and p110γ (class IB) subunits on day 7 in this canine double-hemorrhage model were estimated by Western blotting. The extracts from the basilar arteries were incubated with anti–PI3-kinase p85 or p110γ antibodies. The level of PI3-kinase p85 and p110γ in the basilar artery in normal dogs (non-SAH) was used as 100%. PI3-kinase subunits on day 7 did not change significantly compared with the amount in normal basilar artery (Figure 3A). Wortmannin and LY294002 did not affect the expression of either PI3-kinase subunit.

PI3-Kinase Activity
The in vitro assay of PI3-kinase activity was conducted. Extracts (total protein 500 μg) from basilar arteries were immunoprecipitated with anti–PI3-kinase (p85 or p110γ) antibodies. The level of either PI3-kinase subunit activity in normal control arteries was very low (Figure 3B and 3C). In all groups, narrowing of the vessel lumen, severe corrugation of the lamina elastica and endothelium, and thickness of the vessel wall were observed. A normal basilar artery (B.a) is shown to compare with these spastic arteries. H.E indicates hematoxylin and eosin.

Discussion
In the present study severe vasospasm, demonstrated both angiographically and histologically, was observed in dogs in all groups. PI3-kinase activity but not PI3-kinase protein expression was increased in the basilar arteries collected on day 7 after experimental SAH. PI3-kinase inhibitors abolished PI3-kinase activity but failed to significantly attenuate the vasoconstriction.

**PI3-Kinase Activity**
PI3-kinases are a group of lipid kinases that catalyze the phosphorylation in the D-3 position of the inositol ring of PI.9 Membrane-bound growth factor receptor tyrosine kinase activates class IA PI3-kinase. Growth factors stimulate the SH2 domain with tyrosine-phosphorylated proteins and result in rapidly elevated PI(3,4,5)P3 levels.27 The GTP-bound form of the small G protein Ras28 can also regulate class IA PI3-kinase. The βγ-subunits of heterotrimeric G proteins29,30 directly activate the class IB PI3-kinase P110γ catalytic subunit. The class I PI3-kinases use PI, PI(4)P, and PI(4,5)P2 as substrates that lead to the formation of PI(3)P, PI(3,4)P2, and PI(3,4,5)P3, respectively.31 PI(3)P can be produced in vitro via phosphorylation of PI by class I, II, or III PI3-kinase.9 However, the majority of PI(3)P in mammalian cells is probably produced by class III PI3-kinase.10 The class I PI3-kinases are the only enzymes that can use PI(4,5)P2 as a substrate to synthesize PI(3,4,5)P3.9

In this study we used both PI and PI(4,5)P2 as a direct indication of PI3-kinase activity. We implemented an in vitro assay of PI3-kinase activity. The activities of both class IA and class IB PI3-kinase were enhanced during vasospasm in the basilar arteries on day 7, and the activities of class IA and class IB PI3-kinase were abolished by PI3-kinase inhibitors. Wortmannin binds covalently to the 110-kDa subunit of PI3-kinase and has been shown to inhibit PI3-kinase irreversibly.12 In the present study wortmannin was added on day 2 through day 6 and inhibited PI3-kinase activity on day 7. It is to be expected that because of the irreversible inhibition and the repeated administration of wortmannin, the PI3-kinase activity would have been inhibited on day 7.

In contrast, LY294002 is a selective PI3-kinase inhibitor (IC50=1.4 μmol/L) that acts on the ATP binding site of the enzyme and has no inhibitory effect on PI4-kinase or other ATP-requiring protein kinases and lipid kinases.18 LY294002 does not affect the activities of epidermal growth factor receptor kinase, mitogen-activated protein (MAP) kinase, protein kinase C, and c-Src even at a higher dose such as 50 μmol/L. Vlahos et al18 observed that incubating LY294002 for 7 days prevented proliferation in cultured aortic smooth muscle cells without causing cell death. In the present study 30 μmol/L LY294002 was added on day 2 through day 6 and inhibited PI3-kinase activity on day 7.

In the vehicle (DMSO) treatment group, the spots corresponding to PI(3)P and/or PI(3,4,5)P3 were present on the TLC plates. In class IB PI3-kinase, the spot assigned to PI(3,4,5)P3 demonstrated a significant difference compared with that of PI(3)P. Maier et al12 observed that PI3-kinase γ (class IB) is assumed to occur as P110γ/P101 heterodimer, and Gβγ enhances formation of PI(3,4,5)P3 but not PI(3)P. The density of spots in the vehicle (DMSO) treatment group increased significantly (P<0.05) compared with the density of spots in the control group. Shigematsu et al13 reported the increase of protein kinase B phosphorylation (the downstream target of PI3-kinase) after arterial injury. Shigematsu et al13 also stated that balloon injury of rat arteries stimulated a marked increase in protein kinase B phosphorylation (the downstream target of PI3-kinase) after 30 and 60 minutes, respectively, followed by...
still smaller increases observed after 1 day and 2 days, respectively. 33 Gaetani et al 34 and Zimmermann and Seifert, 35 respectively, commented that the cerebral arteries are subject to continuous exposure from growth factors and many G protein–coupled receptor agonists, such as platelet-derived growth factor 34 and ET-1, 35 in CSF after SAH. The continuous exposure of the basilar artery might maintain PI3-kinase activity until day 7 in the basilar artery, as studied in this model.

PI3-Kinase and Vasospasm
PI3-kinase is an important component of the signal transduction systems activated by the tyrosine kinase receptor.

Scharenberg and Kinet 36 suggested that PI3-kinase is involved in the regulatory processes that produce IP₃ accumulation and then control Ca²⁺ influx via Ca²⁺-induced Ca²⁺ release. In addition, the PI3-kinase inhibitor wortmannin has been reported to inhibit Ca²⁺ entry in porcine aortic endothelial cells. 37 PI3-kinase inhibitor also reduces angiotensin AT₁A receptor–stimulated and Gβγ complexes–stimulated L-type Ca²⁺ channel current in venous myocytes. 38 PI3-kinase inhibitors attenuate smooth muscle contraction induced by many G protein–coupled receptor agonists 12,14,16 and growth factors. 16

It is reported that PI3-kinase may act either downstream 39,40 or upstream from Ras protein in the signal transduction pathways. 41 When PI3-kinase activation occurs

Figure 3. A, Western blot analysis using anti–PI3-kinase p85 antibody (left) and anti–PI3-kinase p110γ antibody (right). The sample was extracted from the basilar artery on day 7 in the DMSO group (D), the wortmannin group (W), the LY294002 group (L), and the normal basilar artery as the control group (C). PI3-kinase subunits on day 7 did not change significantly compared with the amount in normal basilar artery (P > 0.05, ANOVA). Wortmannin and LY294002 did not affect the expression of either PI3-kinase subunit. B, Original TLC plate for PI3-kinase activity assay by anti–PI3-kinase p85 antibody (left) and anti–PI3-kinase p110γ antibody (right). The sample was extracted from the basilar artery on day 7 in the DMSO group, the wortmannin group, the LY294002 group, and the normal basilar artery as the control group. Original TLC plate shows the spots corresponding to PIP₃ and PIP(3,4,5)P₃. In the group of vasospastic arteries treated with DMSO, the levels of PIP₃ and PIP(3,4,5)P₃ in the P85 subunit increased markedly, and only the PIP(3,4,5)P₃ level increased in the P110γ subunit. Wortmannin and LY294002 abolished the enhancement of either the P85 or the P110γ subunit. C, Density of the spots corresponding to PIP₃ and/or PIP(3,4,5)P₃ in each group. PI3-kinase activity in both class IA and class IB was abolished by PI3-kinase inhibitors on day 7 compared with the DMSO treatment group (* P < 0.05, ANOVA).
upstream from Ras activation, PI3-kinase might be involved in the function of Src, Shc, and Grb.41 This PI3-kinase might belong to class IB. We previously reported the inhibitory effect of wortmannin on ET-1 but not on hemolysate-induced contraction in rabbit basilar arteries. In addition, the Src inhibitor damcamanthal also failed to inhibit or relax hemolysate-induced contraction.42 PI3-kinase probably does not act upstream from Ras in hemolysate-induced vascular contraction. Hemolysate-induced vascular smooth muscle contraction is probably involved in other signal pathways, for example, Pyk2 and protein kinase C.

Even though PI3-kinase inhibitors failed to prevent or reverse vasospasm, the possible role of PI3-kinase may not be ruled out completely. One of the reasons is that the statistical power of the comparison of vasospasm among DMSO, wortmannin, and LY294002 groups is only 0.177, which is extremely low and much below the desired power of 0.800. Under such a low statistical power, the negative finding might be changed if the number of samples increased. Increasing the number of animals might yield a statistically significant difference for at least the LY294002 treatment group (versus the DMSO treatment group). However, in the presence of a consistent degree of angiographic vasospasm in most dogs in the present study, an increase in the number of animals might lead to a slight improvement by the absolute measurement of basilar artery diameter but this may be statistically significant in comparison with other values. This result, although positive, will not support an important role of PI-3K in delayed vasospasm to justify additional further animal or clinical studies.

Class IB PI3-kinase γ might partly affect MAP kinase pathway but is not involved in the regulation of delayed vasospasm on day 7. Since class IB PI3-kinase γ regulates Ras-MAP kinase pathway and PI3-kinase inhibitor can attenuate ET-1–induced contraction and ET-1–induced MAP kinase activity,32 class IB PI3-kinase γ may be involved in early vasospasm (which was not studied in the present study) by regulating Ras-MAP kinase. Thus, the time course of PI3-kinase activation during cerebral vasospasm, especially in the early stage, requires further investigation. Since the downstream of class IA PI3-kinase is reported to be Akt/protein kinase D,28,31,40 class IA PI3-kinase might regulate functions other than contraction. The effects of class IA PI3-kinase on the vascular system may be related to proliferation,41 differentiation,42 and replication after arterial injury.33,44

Conclusion
PI3-kinase may not play an important role in delayed cerebral vasospasm. The possible action of PI3-kinase in the early stage of vasospasm requires further investigation.

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References


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