Precise understanding of basic mechanisms underlying the global brain dysfunction that follows subarachnoid hemorrhage (SAH) is needed. Pathophysiological and experimental data provide significant clinical implications for the management of patients with aneurysms and for assessing the efficacy of new pharmacological approaches.

In the experimental setting, the hydroxyl radical scavenger AVS ([±]-N,N′-propylenedinitramide; nicaraven) has been shown to suppress cerebral arterial spasm after SAH in dogs, to terminate the evolution of brain edema after middle cerebral artery (MCA) occlusion in rats, and to reduce the occurrence of delayed hypoperfusion after cerebral ischemia in cats. Furthermore, in a multicenter placebo-controlled double-blind clinical trial, AVS significantly ameliorated delayed neurological deficits resulting from ischemia in patients after aneurysmal SAH, with a marked improvement in the Glasgow Outcome Scale scores at 1 month and a 20.3% decrease in the overall incidence of death. These results have been attributed to the central role that free radicals play in the occurrence of ischemic brain damage, particularly after SAH.

We have developed an experimental model of SAH in rats that has produced extensive information about changes induced by intracisternal blood injection on hemodynamic, angiographic and biochemical, pathophysiological, and acute and chronic behavioral parameters, paralleling those seen in humans after SAH.

The purpose of the present study was to evaluate the antivasospastic and brain-protective effects of AVS after induction of experimental SAH and provide support for observations of beneficial effects of AVS made in the clinical setting.

KEY WORDS • behavioral deficit • blood-brain barrier • free radical scavenger • nicaraven • subarachnoid hemorrhage • vasospasm • rat
assessed diameter of basilar arteries (BAs) 2 days after SAH, and in the third experiment, to assess the effects of AVS on blood-brain barrier (BBB) permeability changes 2 days after SAH, we quantified the microvascular extravasation of protein-bound Evans Blue dye by using a spectrophotofluorimetric technique.

Materials and Methods

Experimental Design and Induction of SAH

Studies were conducted using 112 male albino Sprague–Dawley rats (Crl:CD [SD] BR), each weighing approximately 250 g. Animals were housed at a constant temperature of 22 °C (four per 580 × 385 × 200-mm cage), under a 12-hour light/dark cycle (light switched on at 6 a.m.), with free access to food and water. The procedures used in this study were based on the guidelines of the ethical committee on the care and use of laboratory animals at our institution.

All surgical procedures were performed in these animals after induction of ketalar anesthesia (150 mg/kg in a volume of 3 ml/kg) via the intraperitoneal route.

The jugular vein was exposed through a midline linear 2-cm vertical neck incision, and a PE50 catheter filled with saline was inserted and exteriorized at the nape of the neck. The wounds were then infiltrated with bupivacaine (0.25 mg/kg/500 μl). The catheter exiting from the neck was connected with a Tether steel spring, secured to a custom-designed jacket, and connected to a single-channel swivel and to an infusion pump set at a rate of 0.2 ml/hour for saline or AVS administration to maintain continuous intravenous infusion.

The rats were divided into four experimental groups: Groups I and II (sham operation + saline continuous infusion and sham-operated + AVS continuous infusion, respectively) were used to evaluate the possible effects on investigational parameters of AVS administration in sham-operated rats. Groups III and IV (SAH + saline continuous infusion and SAH + AVS continuous infusion, respectively) were used to compare the effect of AVS administration on SAH-induced changes in investigational parameters. The experiments were conducted as follows: on Day −1, baseline preassessments for behavioral tasks were performed. On Day 0, SAH or sham operation procedures were performed.

Subarachnoid hemorrhage was induced in 56 rats by injection of autologous blood into the subarachnoid space via the cisterna magna. Details of the procedure have been published previously.10,14,15 Briefly, the atlantooccipital membrane was exposed through a midline occipital incision. For the simulated SAH, 0.4 ml of autologous arterial nonheparinized blood was injected into the cisterna magna over a period of approximately 30 seconds via a 30-gauge needle fitted to a 500-μl Hamilton syringe. The sham-operated group consisted of 56 rats in which the atlantooccipital membrane was exposed through a midline occipital incision and punctured as described previously. No intracisternal injection was given, because in previous studies performed in our laboratory in which this rodent model was used, we demonstrated that rats mock injected with cerebrospinal fluid (CSF) did not display any lasting behavioral detriment.18 In addition, mock CSF-injected animals there was no appreciable change in the BBB quantitative assessment10 or significant angiographically confirmed changes compared with uninjected control rats.

Within 5 minutes of SAH or sham operation, AVS and vehicle (sterile saline) were continuously administered intravenously in 56 rats each for 2 days (angiography and BBB experiments) or 5 days (behavioral experiments) through the cannulated jugular vein. The rate of infusion was 1 mg/kg/minute (daily dose 1.44 g/kg).

Behavioral Assessment Protocol

Three assessment tasks were used to characterize lasting behavioral deficits over a 5-day period after procedures in the four experimental groups. Ten animals per group were preassessed the day before the procedure (Day −1), and tests were conducted daily from the day after the procedure through the following 5 days. The beam balance test is a task that is used to assess both motor and vestibular functioning by quantifying the animal’s ability to balance on a narrow wooden beam (1 cm wide) for up to 60 seconds. For measuring beam balance capacity, rats were assigned a score, according to the beam balance rating scale of Levin, et al.20,21 The time the animals remained on the beam was also recorded. Data for each daily session consisted of the mean ± standard deviation (SD) of three consecutive trials. The beam walking test is a learned avoidance test similar to that used by Feeney, et al.12,13,15 This task is used to evaluate the somatomotor, motivational, and attention functions, together with memory and locomotor activities. The rats were trained with a negative reinforcement paradigm in which termination of the adverse stimuli (noise and light) served as a reinforcement reward. The animals had to traverse the top of an elevated (1-m) narrow wooden beam (120 × 5 cm), with the reward consisting of the cessation of a loud white noise and a bright light. During both training and testing animals were placed on an end of the beam across from the source of noise and light, and the difficulty of the task was increased by placing four equally spaced pegs (height 5 cm) along the top of the beam. The noise and light were turned off immediately after the animal had traversed the beam and had entered a darkened box (10 × 15 × 10 cm) with its two forelegs. The time taken to traverse the beam was recorded. Data for each daily session represented the mean ± SD of three consecutive trials. Body weight, a gross measure of food and water intake, is a parameter that is used to assess the appetite drive and the occurrence of motivational deficits. Rats were preweighed on Day −1 and their weight was recorded daily for the following 5 days.

Angiography Protocol

Assessment of spasm in the BA was conducted with the aid of angiography performed 48 hours after the SAH or sham procedures in 10 animals per group. The left common carotid artery was surgically exposed by means of a midline skin incision and ligated cautiously; a polypropylene PE50 catheter was then inserted into the vessel. The external carotid artery was ligated to divert the flow of contrast medium into the intracranial circulation. The tip of the catheter was pushed to within 5 mm of the carotid bifurcation. Cerebral angiography studies were performed with the aid of an x-ray machine and MR1 mammography medical x-ray film, with iopamidol (400 mg/ml, 100 μl/rat) used as contrast medium. Each angiogram was examined with the aid of a computerized image analysis system to measure the diameters of BAs. The mean vessel caliber (in millimeters ± SD) was calculated for each experimental group.

Blood-Brain Barrier Evaluation Protocol

The BBB assessment protocol was conducted in eight animals per group 48 hours after the SAH or sham procedures. The method we used involved the quantitative evaluation of the vascular permeation of Evans Blue dye by means of a fluorescence spectrophotometer technique, according to the measurement protocol of Uyama, et al.,24 and the extraction technique of Rössner and Tempel.25 Briefly, 2% Evans Blue in saline (5 ml/kg) was administered intravenously through the cannulated jugular vein as a blood-brain permeability tracer and was allowed to circulate for 60 minutes. To remove the intravascularly localized dye, the rats’ chests were opened and the animals were perfused with saline through the left ventricle at a pressure of 110 mm Hg until colorless perfusion fluid was obtained from the right atrium. The whole brain was removed, and measurement of vascular permeability was made by comparing its weight with preweighed loci in the frontal, temporal, parietal, occipital, and cerebellar cortices, and subcortical and cerebellar gray matter and brainstem nuclei, as defined by the Paxinos and Watson atlas.26 Each brain area was homogenized in 1 ml of 50% trichloroacetic acid (weight/volume) and centrifuged (10,000 rpm, 20 minutes). One milliliter of the supernatant was added to 1.5 ml of the solvent (50% trichloroacetic acid/ethanol, 1:3). A fluorescence detector was used at an excitation wavelength of 420 nm (bandwidth 10 nm) and an emission wavelength of 680 nm (bandwidth 10 nm). Calculations were based on external standards in the solvent (10–500 ng/ml). Data are expressed as the mean ± SD (in micrograms) of extravasated Evans Blue dye per gram of tissue.
Effects of AVS after experimental SAH

Data were statistically analyzed using parametric or nonparametric methods; an analysis of variance or Kruskal–Wallis one-way analysis was performed. Post hoc Steel–Dwass and Tukey multiple comparison tests were used. Differences were accepted as being significant at a probability of less than 0.05 after Bonferroni adjustment. Sham + saline = Group I; sham + AVS = Group II.

Sources of Supplies and Equipment
The AVS (lot no. R5L02) was generously provided by Chugai Pharmaceutical Co., Ltd., Tokyo, Japan. The Sprague–Dawley rats were purchased from Charles River Italia SpA, Como, Italy. The ketamine was obtained from Parke-Davis Italia SpA, Milan, Italy, the PE50 catheters were acquired from Clay Adams, Parsippany, NJ, and the bupivacaine (Marcaina 0.5%) from Pierrel SpA, Capua, Italy. The x-ray machine (model SAI5 SM6) and medical x-ray film were obtained from Sais, Bolologna, Italy, and Eastman Kodak, Rochester, NY, respectively. The iopamidol (iopamiro 400) was purchased from Bracco Industria Chimica SpA, Milan, Italy. The fluorescence detector (model FP-920) was obtained from Jasco Corp., Tokyo, Japan.

Results
General observations and systemic physiological evaluations in this model (including mean arterial blood pressure, arterial blood gas levels, blood pH, plasma glucose levels, and body temperature as monitored throughout the experimental procedure) have been described in detail elsewhere.\textsuperscript{5–8,13,15} Briefly, rats tolerated the procedure well and no signs of acute neurological dysfunction were noted. In rats killed on the 2nd day after SAH, a blood clot was still clearly identifiable in the cisterna magna and in the basal cisterns: no extradural hemorrhages were found, whereas the presence of blood in the ventricles was demonstrated in 25% of cases. In rats killed on Day 5 post-SAH no blood clot was visible in the cisterna magna or in other brain loci.

Behavioral Assessment
None of the experimental groups differed significantly from the others in baseline preinjection assessments used to measure behavioral changes over the 5-day observation period (Figs. 1–4).

Beam Balance. The blood-injected saline-treated rats (Group III) exhibited significant deficits in score (Days 1–2, \( p < 0.01 \)) and time (Day 1, \( p = 0.05 \)), as compared with sham-operated saline-treated animals (Group I). When compared with Group III, AVS-treated animals with SAH (Group IV) exhibited significantly improved score (Days 1–2, \( p < 0.01 \)) and time (Days 1–4, \( p < 0.01 \)) performances. As compared with Group I, AVS administration in sham-operated animals (Group II) did not induce any significant beam balance alteration (Figs. 1 and 2).

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Beam Walking. The time taken to traverse the beam remained significantly higher on Days 1 to 4 (p < 0.01) in Group III as compared with Group I animals. In Group IV rats the time animals took to traverse the beam was significantly reduced on Days 1 to 4 (p < 0.01) as compared with Group III rats. The administration of AVS did not induce a significant alteration in beam walking performance in sham-operated animals (Group II) when compared with Group I rats (Fig. 3).

Body Weight. The body weights had significantly decreased after SAH in Group III rats by Days 4 and 5 (p < 0.05) as compared with Group I animals. The administration of AVS did not significantly influence the SAH-related loss in body weight. As compared with Group I rats, AVS administration in Group II animals did not result in any body weight change (Fig. 4).

Angiographic Assessment

Good and reproducible filling of the vertebrobasilar system was achieved in all groups of rats. In saline-treated animals intracisternal blood injection produced a significant angiographically confirmed constriction of the BAs at 2 days after SAH (p < 0.01). In Group IV rats a significant reduction in BA vasoconstriction was observed (p < 0.05). Administration of AVS did not induce any significant alteration in BA caliber in Group II animals when compared with Group I rats (Fig. 5).

Evaluation of the BBB

Table 1 summarizes the concentration of extravasated Evans Blue dye expressed as micrograms per gram of brain tissue for all loci examined in the four experimental groups. In sham-operated rats, baseline levels of Evans Blue ranged from 1.959 ± 0.322 to 4.182 ± 0.465 in Group I rats and from 1.917 ± 0.32 to 4.111 ± 0.481 in Group II animals. In Group III rats Evans Blue dye extravasation was significantly increased (p < 0.01) as compared with sham-operated saline-treated animals in the frontal, temporal, parietal, occipital, and cerebellar cortices, and in subcortical gray matter (caudate-putamen-thalamus), and cerebellar and brain stem nuclei. As compared with the Group III rats, AVS (in Group IV) administration significantly decreased the SAH-induced BBB permeability changes in the frontal, temporal, parietal, occipital, and cerebellar cortices, and the subcortical (caudate-putamen-thalamus) and cerebellar gray matter and brainstem nuclei (p < 0.01). In Group II rats AVS administration induced no significant BBB alteration as compared with Group I animals.

Discussion

The purpose of this investigation was to evaluate the antivasospastic and brain-protective effects of AVS in a well-characterized rodent model of experimental SAH. In this study we evaluated the effects of AVS on: 1) the functional behavioral consequences of SAH over a 5-day observation period; 2) the diameter of the BAs visualized on angiographic studies obtained 2 days after SAH; and 3) the BBB permeability changes that occur on the 2nd day after SAH. In Group IV rats significant protection against the SAH-related behavioral alterations, angiographically confirmed BA spasm, and BBB functional alterations was demonstrated. These results demonstrate useful antivasospastic and brain-protective actions of AVS after experimental SAH and provide support to observations of the beneficial effects of AVS in the clinical setting.

Investigational Parameters

Considerable clinical and experimental work has shown that SAH induces focal and generalized disturbances of several brain functions. The experimental parameters investigated in the present study may reflect the effect of SAH on global brain performance, as well as on gross macro- and microvascular systems.

A simple and inexpensive animal model of SAH was used in this study, which has already been extensively studied in our laboratory. Using this model we
Effects of AVS after experimental SAH

TABLE 1

<table>
<thead>
<tr>
<th>Area</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>frontal cortex</td>
<td>4.182 ± 0.465</td>
<td>4.111 ± 0.481</td>
<td>9.141 ± 1.692†</td>
<td>6.137 ± 0.634</td>
</tr>
<tr>
<td>temporal cortex</td>
<td>3.667 ± 0.401</td>
<td>3.923 ± 0.175</td>
<td>8.772 ± 1.732†</td>
<td>6.022 ± 0.554</td>
</tr>
<tr>
<td>parietal cortex</td>
<td>3.675 ± 0.387</td>
<td>3.670 ± 0.146</td>
<td>8.405 ± 1.141†</td>
<td>5.183 ± 0.411</td>
</tr>
<tr>
<td>occipital cortex</td>
<td>3.839 ± 0.294</td>
<td>3.747 ± 0.196</td>
<td>8.207 ± 1.087†</td>
<td>6.022 ± 0.404</td>
</tr>
<tr>
<td>subcortical GM (CPT)</td>
<td>2.365 ± 0.435</td>
<td>2.240 ± 0.396</td>
<td>4.261 ± 0.899†</td>
<td>3.004 ± 0.397</td>
</tr>
<tr>
<td>cerebellar cortex</td>
<td>4.156 ± 0.365</td>
<td>4.004 ± 0.267</td>
<td>6.974 ± 0.775†</td>
<td>5.205 ± 0.345</td>
</tr>
<tr>
<td>cerebellar nuclei</td>
<td>1.959 ± 0.322</td>
<td>1.917 ± 0.320</td>
<td>3.772 ± 1.167†</td>
<td>2.577 ± 0.396</td>
</tr>
<tr>
<td>brainstem</td>
<td>3.009 ± 0.678</td>
<td>3.071 ± 0.499</td>
<td>6.047 ± 0.643†</td>
<td>4.001 ± 0.181</td>
</tr>
</tbody>
</table>

* The BBB permeability changes were measured 2 days after procedures in all groups of rats. Values are expressed as the means ± SD for 10 rats in each group. Abbreviations: CPT = caudate-putamen-thalamus; GM = gray matter.
† p < 0.01 in Group III compared with Group IV. Statistical analysis completed using the Tukey multiple comparison test.

The spectrum and time course of changes in intracisternal thromboxane, prostaglandin, and prostaglandin concentrations after SAH, the occurrence of angiographically confirmed arterial spasm in both the vertebralbasilar and internal carotid system 48 hours after intracisternal blood injection, the induction of quantitative and qualitative marked regional alterations in BBB permeability on the 2nd day after SAH, and the occurrence of a widespread depression of brain metabolism in the acute stage after SAH together with the induction of lasting behavioral deficits over a 5-day observation period. The specificity of extravasated blood for causing these pathophysiological changes was demonstrated. These findings substantiated previous observations reported by others who used the same investigational parameters and a similar experimental model, with which they demonstrated the occurrence of pathophysiological changes that parallel those seen in humans after SAH. The results of these studies have led to the hypothesis that the blood itself and/or active substances derived in part from the degradation of the extravasated blood are centrally involved in the pathogenesis of these phenomena.

Studies of post-SAH cerebral vasospasm are important because, despite the recent development of novel pharmacological agents, it remains the leading cause of death and disability following SAH. In humans, the late spasm appears on angiographic studies 2 to 4 days after the hemorrhage and reaches a maximum intensity at approximately Day 7. This angiographically visible arterial spasm appears earlier in the rat and other animal species and its occurrence is limited to the period when blood is found in the subarachnoid space. We chose a 2-day post-SAH interval for angiographic studies based on previous observations made by ourselves and others that the maximal degree of spasm occurs in the rat at Day 2 post-SAH, a time when, in this model, a blood clot is still clearly identifiable in the cisternal subarachnoid spaces. Also, it has been demonstrated that the development of pathophysiological changes affecting the media of rodent cerebral arteries were maximal at 2 days post-SAH. In our study a marked constriction of the BAs was observed at 2 days after cisternal blood injection. The AVS-treated rats in Group IV demonstrated a significant blockade of SAH-induced BA spasm. The results of this study are in agreement with a previous investigation in which AVS has been demonstrated to suppress, in a dose-dependent manner, the occurrence of chronic arterial vasospasm in a canine model.

Early changes in the BBB function have been suspected as important causative factors for post-SAH cerebral dysfunction. An impairment of BBB function after SAH has been demonstrated in humans and has been correlated with the development of delayed cerebral ischemia and poor clinical outcome, post-SAH brain swelling, and microcirculatory disturbances as a consequence of the permeability disruption. Studies concerning barrier disruption associated with SAH are relatively few and there is lack of information with regard to the time course of barrier alterations. In the clinical setting, a BBB impairment was found in nearly 40% of patients within 5 days of SAH. In the present study we chose a 2-day post-SAH time interval on the basis of previous observations made by ourselves and others that the BBB dysfunction is operational in the rat on Day 2 post-SAH and correlates with increased vasoactive eicosanoid CSF levels seen within 2 days posthemorrhage. In these experiments, AVS administration attenuated SAH-induced BBB microvascular changes in all the examined cerebral loci (frontal, temporal, parietal, occipital, and cerebellar cortices, as well as in the subcortical and cerebellar nuclei and brainstem).

The neuropsychological outcome after SAH is being given increasing attention by the neurosurgical community. In a multicenter placebo-controlled double-blind clinical trial AVS has been shown to produce a marked improvement in the Glasgow Outcome Scale scores at 1 month. In previous investigations our laboratory has provided normative values depicting the spectrum and time course of SAH-induced behavioral alterations in the rat as peaking on Days 1 to 4 and normalizing by day 5 after SAH. These behavioral changes were similar to deficits observed in other brain injury models and may parallel those seen in humans after SAH. In the present study behavioral evaluations were focused on the examination of somatomotor performance in the light of the consistent representation in the rodent brain of motor functions and the availability of well-characterized measures to assess motor functions in rats. Treatment with AVS led to significant improvement in the beam walking performance dur-
ing a 4-day period, while also improving scores and time on the beam balance test on Days 1 and 2.

Pathological Mechanisms Involved

The liberation of oxyhemoglobin into the CSF on lysis of the red blood cells within the subarachnoid clot generates activated oxygen species by its oxidation to methemoglobin. There is accumulating clinical and experimental evidence that free radical reactions play a pivotal role in the pathophysiology of secondary brain damage, particularly after SAH. Convincing evidence indicates that lipid peroxide and free radical accumulation in the walls of cerebral arteries exposed to a degrading subarachnoid blood clot are integral components in the development of cerebral vasospasm. There is evidence that oxygen radicals targeting the vascular endothelium participate in the genesis of BBB dysfunction after central nervous system injury. Vasoactive substances originating from the degradation products of extravasated blood might also be associated with BBB dysfunction by acting directly on brain microvessels and/or initiating lipid peroxidation phenomena and free radical reaction cascades. Finally, it is tempting to speculate that the global brain dysfunction produced by the neurotoxic effects of subarachnoid blood might be an important causative factor of the observed alterations in behavior.

A water- and oil-soluble derivative of nicotinamide, AVS (nicaraven) demonstrates a radical scavenging action putatively specific to the hydroxyl radical but has no significant direct action on the vascular smooth-muscle cells. Its pharmacological profile can be summarized as follows: AVS scavenges hydroxyl radicals; exhibits anticontractile activity in isolated BA preparations in which contraction has been induced by the addition of 15-hydroperoxyarachidonic acid (SNaito, personal communication, 1997); suppresses arterial spasm after SAH; abolishes the evolution of ischemic brain edema after MCA occlusion; mitigates derangement of cerebral energy metabolism during ischemia and suppresses delayed hyperfusion following ischemia; inhibits enhanced lipoxygenase activity after MCA occlusion within the brain microvessels; and suppresses enhanced sodium/potassium–adenosine triphosphatase activity after 15-hydroperoxyarachidonic acid application in vitro. Thus, AVS has been shown to exert significant antivasospastic and brain-protective effects in various models of central nervous system injury, ischemia, and SAH. Considering all of its other actions, in the present investigation its cellular mechanism of action can be debated only on a conjectural basis, and a detailed analysis of AVS pharmacodynamic activity is beyond the scope of this work. We speculate, however, that the beneficial effects of AVS evidenced in this investigation could be reasonably explained by inhibition of enhanced lipoxygenase activity and aberrant ion fluxes across the microvascular endothelium, in which oxygen free radical generation is involved. In our study AVS (1 mg/kg/minute, daily dose 1.44 g/kg) was continuously administered intravenously in rats for 2 days (angiography and BBB experiments) or 5 days (behavioral experiments). This dose regimen was selected on the basis of previous experimental evidence delineating the pharmacodynamic, pharmacokinetic, and toxicological profile of AVS. In addition, in dose–response experiments in which the effect of AVS on cerebral vasospasm after SAH in dogs was evaluated, it has been demonstrated that a 1 mg/kg/minute dose of AVS achieves a full relaxing effect on angiographically visualized SAH-induced BA constriction as compared with other dose regimens.

Potential Clinical Implications

Taking into account previous studies of AVS, our results are not unexpected. However, because of significant interspecies differences, a direct extrapolation to humans may be considered inappropriate.

In a recent multicenter, placebo-controlled double-blind clinical trial, AVS was demonstrated to have beneficial effects on the incidence of delayed ischemic neurological deficits after SAH, leading to an improvement in the overall outcome. Although the results of this study did not enable the investigators to discriminate between the antivasospastic and brain-protective actions of AVS, both were postulated to contribute to these beneficial effects.

In accordance with these observations, results from the present study provide further experimental confirmation for the beneficial effects of AVS already observed in the clinical setting. Therefore, we suggest that further investigation of the mechanism of action and the potential clinical benefits of this drug is warranted.

References

Effects of AVS after experimental SAH


Manuscript received May 29, 1997. Accepted in final form February 5, 1998.

This work was partially supported by funds given to the University of Messina from Chugai Pharmaceutical Co., Ltd., Tokyo, Japan.

This paper was presented in part at the Sixth International Conference on Cerebral Vasospasm in Sydney, Australia, May 11 to 15, 1997.

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