Effect of azelastine on platelet-activating factor-induced microvascular leakage in rat airways

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Tamaoki, Jun, Isao Yamawaki, Etsuko Tagaya, Mitsuko Kondo, Kazutetsu Aoshiba, Juno Nakata, and Atsushi Nagai. Effect of azelastine on platelet-activating factor-induced microvascular leakage in rat airways. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L351–L357, 1999.—To determine the effect of the antiallergic drug azelastine on airway mucosal inflammation, we studied airway microvascular permeability in response to platelet-activating factor (PAF) in pathogen-free rats. Vascular permeability and neutrophil accumulation were assessed by the percent area occupied by Monastral blue-labeled blood vessels and by myeloperoxidase-containing granulocytes, respectively, in whole mounts of the trachea and main bronchus. Intravenous PAF caused dose-dependent increases in the area density of Monastral blue-labeled vessels and neutrophil influx, and the former effect was inhibited by depletion of circulating neutrophils by cyclophosphamide or treatment with the neutrophil elastase inhibitor ONO-5046. Pretreatment with azelastine inhibited PAF-induced vascular leakage without affecting neutrophil accumulation. This inhibitory effect of azelastine was not seen in neutropenic rats and ONO-5046-treated rats. PAF increased neutrophil elastase contents in bronchoalveolar lavage fluid, an effect that was inhibited by azelastine. Therefore, azelastine attenuates PAF-induced airway mucosal vascular leakage, probably involving inhibition of the release of neutrophil elastase from activated neutrophils. Antiallergic drug; vascular permeability; asthma

AZELASTINE [4-(p-chlorobenzyl)-2-(hexahydro-1-methyl-1H-azepine-4-yl)-1-(2H)-phthalazine] is an orally effective and long-lasting antiallergic drug that belongs to a new class of drugs and has no structural similarity to other agents currently being used to treat asthma. The AZELASTINE-Asthma Study Group (2) has recently shown that oral administration of azelastine to patients with chronic asthma produces stability or a decrease in clinical symptoms and an improvement in pulmonary function. Although the mechanism of drug action is not fully understood, previous in vitro studies and clinical trials have demonstrated that azelastine inhibits the release of histamine from mast cells and basophils (5) and attenuates allergen-induced early bronchoconstriction and late reactions in atopic subjects (23). These late reactions are characterized by an increase in airway microvascular permeability, infiltration of inflammatory cells into the airway mucosa, and airway hyperresponsiveness. Based on the finding by Tani-gawa et al. (32) that azelastine possesses an inhibitory action on antigen-induced vascular leakage in the rat skin, we hypothesized that this drug could also affect airway inflammatory responses.

Platelet-activating factor (PAF) is a potent mediator of airway inflammation and can mimic several features of asthma, including bronchoconstriction, airway microvascular leakage, and inflammatory cell infiltration (15, 24). Because isolated airway smooth muscle preparations do not respond to PAF (26) and because inhaled PAF potently induces airway vascular leakage with comparatively little increase in lung resistance, it seems that PAF causes airway narrowing through mechanisms other than airway smooth muscle contraction, such as airway wall edema and liquid filling of the airway lumen resulting from an increase in airway microvascular permeability. In fact, PAF increases cytosolic Ca^{2+} in microvascular endothelial cells, an effect generally accepted to be "proinflammatory" (17). Therefore, the objective of the present study was to determine whether azelastine inhibits PAF-induced airway inflammation and, if so, to elucidate its mechanism of action. To accomplish this goal, the amount of vascular leakage was assessed by measuring the percent area occupied by Monastral blue-labeled blood vessels in the mucosa of the rat trachea and bronchus with a stereological point-counting method. The influx of granulocytes into the airway mucosa was determined by counting peroxidase-containing cells present in the whole mounts stained specifically for this enzyme.

MATERIALS AND METHODS

Protocols. The research protocols were approved by the Animal Care and Use Committee of the Tokyo (Japan) Women's Medical College. Pathogen-free male Sprague-Dawley rats weighing 180–200 g were obtained from SLC Jap (Hamamatsu, Japan) for use in this study. The rats were anesthetized with an intraperitoneal injection of urethan (500 mg/kg) and α-chloralose (50 mg/kg). The larynx and upper trachea were exposed, the trachea just below the larynx was incised, an intubation tube was inserted 2 mm into the trachea, and the rats were artificially ventilated (frequency, 60 breaths/min; tidal volume, 10 ml/kg with a constant-volume ventilator (model SN-480-7, Shinano, To-kyo, Japan). All reagents and tubes used in the present experiments contained <0.1 ng/ml of detectable bacterial lipopolysaccharide as measured by the Limulus amebocyte lysate assay (SRL Laboratories, Tokyo, Japan).

To determine the magnitude of increase in airway vascular leakage, we used Monastral blue pigment as a tracer to identify the abnormally permeable blood vessels. The particles of Monastral blue are too large to cross the endothelium of tracheal and bronchial blood vessels with normal permeability but, when the blood vessels become abnormally permeable, the pigment passes through gaps in the endothelium...
and is trapped by the basal lamina, where it remains and thus labels the sites of extravasation (18). The Monastral blue suspension in saline was sonicated in an ultrasonic cleaner for 5 min, passed through a filter (pore size, 5 µm; Millipore, Bedford, MA), and injected into a femoral vein (30 mg/kg). Immediately after the dye injection, the rats were given graded doses of PAF (0.1–10 µg/kg) or an equivalent volume of the vehicle through a femoral vein. Five minutes after the PAF injection, fixative (1% paraformaldehyde and saline in 50 mM phosphate buffer, pH 7.4) was perfused, with a mean arterial pressure of 120 mmHg (37), via a cannula inserted through the left ventricle into the ascending aorta to wash the blood vessels is expressed as a percentage of the total area of the vascular space. This time point was chosen because the response of airway microvascular leakage to intravenous PAF has been shown to reach a plateau within 4–5 min after the injection (4). Then the trachea and left main bronchi were removed, opened longitudinally along the ventral midline, and incubated in the fixative for 3 h at room temperature.

To determine the effect of azelastine on PAF-induced microvascular leakage and neutrophil recruitment, the rats were given various doses of intravenous azelastine (1–30 µg/kg), and 5 min later, PAF (10 µg/kg) was administered intravenously.

Neutrophil recruitment. To stain peroxidase-containing granulocytes, including neutrophils and eosinophils that contain myeloperoxidase and eosinophil peroxidase, respectively, tracheal and bronchial sections were incubated for 7 h at 4°C in a solution consisting of 25 mg of 3,3’-diaminobenzidine dissolved in 50 ml of 0.05 M Tris·HCl buffer, pH 7.6, containing 0.5 ml of 100% Triton X-100 and 0.5 ml of 1% hydrogen peroxide. After incubation, the tissues were washed with distilled water and placed in glycerol for 12 h at room temperature. The glycerol was replaced by successive changes of 100% ethanol, 100% tolue, and 100% ethano. The tissues were then placed in distilled water, cleaned of connective tissues under a dissecting microscope, and flattened between glass slides. Finally, the trachea and bronchi were dehydrated in 100% ethanol for 24 h, cleared in 100% tolue for 15 min, and mounted on a glass slide. This procedure produced tracheal and bronchial whole mount sections in which neutrophils and eosinophils were identified. Neutrophils, which appeared in whole mounts as small cells stained a faint yellow-brown, were distinguishable from eosinophils, which appeared in whole mounts as small cells stained intensely brown to black (18, 33). In our preliminary experiments, intravenously administered PAF (10 µg/kg) had no significant effect on the number of eosinophils observed in the tracheal mucosa (242 ± 64 cells/mm² before PAF, n = 5 rats, vs. 270 ± 51 cells/mm² 5 min after PAF, n = 6 rats). Thus we estimated only neutrophils in the present experiments. Neutrophils were counted at a magnification of ×400 in 20 square regions on the right side of the trachea and main bronchus, totaling ~4 mm² in each whole mount. These values are expressed as the number of neutrophils per square millimeter of the mucosal surface area.

Vascular permeability. Airway vascular permeability was assessed by using stereological point counting to determine the area density of the blood vessels labeled with Monastral blue in the whole mount sections. Point counting was performed in the same 20 regions as used in the quantitation of neutrophils, and the area occupied by Monastral blue-labeled blood vessels is expressed as a percentage of the total area of tracheal and bronchial mucosae measured.

Contributions of histamine, oxygen radicals, and neutrophils. Because azelastine can antagonize histamine receptors (6), it is possible that azelastine could have exerted its effect through histamine antagonism. To test this possibility, we studied whether histamine is involved in the PAF-induced extravasation. A combination of intraperitoneal mepramine (10 mg/kg), an H₁-receptor antagonist; cimetidine (10 mg/kg), an H₂-receptor antagonist; and thioperaime (5 mg/kg), an H₃-receptor antagonist, was given, and 30 min later, intravenous PAF (10 µg/kg) was given. In addition, to assess whether the vascular action of PAF is mediated by toxic oxygen metabolites, the rats were pretreated with intravenous superoxide dismutase (SOD; 20,000 U/kg) or catalase (1 x 10⁶ U/kg) 5 min before PAF injection. The doses and route of histamine-receptor antagonists and radical scavengers were chosen according to previous studies (31, 36).

To elucidate the role of neutrophils in the effect of azelastine, the rats were depleted of circulating neutrophils. Cyclophosphamide (100 mg/kg) was injected intraperitoneally 5 days before the experiment. On the day before the PAF injection, a second dose of cyclophosphamide (50 mg/kg) was given. Pretreatment with cyclophosphamide caused a 99.1% decrease in the peripheral neutrophil count, from 8,200 ± 90/mm³ in the untreated rats (n = 16) to 74 ± 5/mm³ in the cyclophosphamide-treated rats (n = 16). Then, the effect of azelastine (30 µg/kg) on the increase in vascular permeability produced by PAF (10 µg/kg) was examined. To assess the contribution of neutrophil elastase, a potent tissue-damaging lysosomal enzyme released from neutrophils, the rats were given intravenous ONO-5046 [N-[2-[4-(2,2-dimethylpropionyloxy)phenylsulfonyl]aminolbenzoy]laminocacetic acid; 30 mg/kg], a selective neutrophil elastase inhibitor (12), and 5 min later, the effect of azelastine on the PAF-induced vascular permeability and neutrophil influx was examined.

Measurement of neutrophil elastase. To further elucidate the role of neutrophil elastase in the observed effect of azelastine, we measured neutrophil elastase activity in bronchoalveolar lavage (BAL) fluid. Because the BAL procedure per se may cause alterations in microvascular permeability, the experiment was conducted separately in a similar manner except that Monastral blue was not injected. After the PAF injection, BAL was performed through the tracheal cannula by three instillations of 5 ml of saline, the BAL fluid was centrifuged, and the activity of tracheal neutrophil elastase in the supernatant was determined with a chromogenic substrate specific for neutrophil elastase (methoxyxycucinyl-L-alanly-L-prolyl-L-valyl-p-nitroanilide) (29). The BAL samples reacted with 1 mM of the substrate dissolved in 1-methyl-2-pyrrolidzone. The change in absorbance per minute was recorded at 410 nm in a spectrophotometer (Ultraspex Plus, Phamacia LKB Biochrom, London, UK). Our preliminary studies with purified neutrophil elastase from human purulent sputum as a standard reference showed that this assay was capable of measuring neutrophil elastase concentrations as low as 10⁻¹¹ M reliability.

Drugs. The following drugs and chemicals were used in the present experiments: azelastine (gift from Eisai, Tokyo, Japan), PAF, Monastral blue dye, cimetidine, thioperaime, SOD, catalase, cyclophosphamide, methoxyxycucinyl-L-alanly-L-prolyl-L-valyl-p-nitroanilide, 1-methyl-2-pyrrolidzone (all from Sigma, St. Louis, MO), mepyramine (Rhone-Poulenc, Dagenham, UK), 3,3′-diaminobenzidine (Wako Pure Chemical, Osaka, Japan), purified neutrophil elastase (Elastin Products, Pacific, MO), and ONO-5046 (gift from Ono Pharmaceutical, Osaka, Japan). PAF was dissolved in 9:1 chloroform-methanol as a stock solution and stored at −70°C. Aliquots were subsequently evaporated under a stream of nitrogen and resuspended in Krebs-Ringer phosphate dextrose buffer containing 0.25% bovine serum albumin.
albumin. Before infusion, the PAF was further diluted in sterile normal saline.

Statistics. All values are expressed as means ± SE. One-way analysis of variance and Fisher’s multiple range test were used to determine significant differences between groups, and P < 0.05 was considered significant.

RESULTS

As demonstrated in Fig. 1, intravenous administration of PAF increased both vascular permeability and neutrophil influx in the airway mucosa of pathogen-free rats, whereas the vehicle alone had no such effects. Neutrophils observed in this series of experiments were migrating through Monastral blue-labeled postcapillary venules or were located in close contact with the vascular endothelium. The area occupied by Monastral blue-labeled blood vessels was increased by PAF in a dose-dependent fashion in the trachea and main bronchus, the observed maximal response being from 0.4 ± 0.3 to 17.4 ± 4.0% (P < 0.001; n = 5 rats) and from 0.5 ± 0.3 to 26.9 ± 4.5% (P < 0.001; n = 5 rats), respectively (Fig. 2). Similarly, PAF dose dependently increased the number of neutrophils present in the tracheal and bronchial mucosae where the threshold dose was less than that for Monastral blue extravasation.

Administration of azelastine inhibited the PAF-induced increase in microvascular permeability in a dose-dependent manner (Figs. 1 and 3). The area occupied by Monastral blue-labeled blood vessels in the PAF (10 µg/kg)-treated rats was reduced by azelastine (30 µg/kg) from 17.7 ± 2.9 to 6.6 ± 1.2% in the trachea (P < 0.05; n = 8 rats) and from 28.0 ± 3.9 to 7.2 ± 0.8% in the main bronchus (P < 0.05; n = 8 rats). In contrast, azelastine did not alter the PAF-induced increase in the number of neutrophils in the tracheal and bronchial mucosae (Figs. 1 and 4).

Pretreatment of the rats with a combination of histamine-receptor antagonists or with SOD had no effect on the vascular leakage induced by PAF (Table 1). Pretreatment with catalase slightly attenuated the PAF action, but the inhibition did not reach a significant level. In the neutropenic rats treated with cyclophosphamide, PAF (10 µg/kg) increased the area occupied by Monastral blue-labeled blood vessels in both the tracheal and bronchial mucosae, but the magnitude of each increase was about three times less compared with the rats without cyclophosphamide treatment. These responses were not significantly inhibited by 30 µg/kg of azelastine. Pretreatment with ONO-5046 did not alter the PAF-induced neutrophil influx (216 ± 38 cells/mm² for PAF alone; 198 ± 42 cells/mm² for ONO-5046 plus PAF; n = 8 rats/group) but reduced the increase in the area density of Monastral blue-labeled blood vessels, and azelastine did not further inhibit the response of vascular leakage as in the case of neutropenic rats.

As shown in Fig. 5, neutrophil elastase activity in the BAL fluid was markedly increased by PAF (10 µg/kg), from 10 ± 3 to 112 ± 29 nM (P < 0.001; n = 8 rats), and this increase was inhibited by 72% with prior administration of azelastine (30 µg/kg; P < 0.01; n = 8 rats).
DISCUSSION

Our present studies demonstrate that PAF increases microvascular permeability in the airway mucosa of pathogen-free rats, presumably through a release of neutrophil elastase from activated neutrophils, and that the antiasthma drug azelastine attenuates the PAF-induced vascular leakage.

The membrane-derived phospholipid PAF is released by a variety of cell types in response to antigen challenge. The role of PAF in the pathogenesis of human asthma remains in question (9, 14), but under experimental conditions, this mediator produces several of the features characteristic of asthma, such as airway inflammation, airway hyperresponsiveness, and bronchoconstriction (15, 24). In the present study, we found that PAF dose dependently increased extravasation of Monastral blue from postcapillary venules in the trachea and main bronchus, which was accompanied by an increase in the number of neutrophils adhered to the vascular walls and migrated to the mucosal surface. Surgical trauma and tracheal intubation per se may have caused the release of inflammatory mediators, leading to neutrophil accumulation and increased vascular permeability. However, in these experiments, both control and experimental groups were prepared identically, and administration of PAF produced clear alterations in both neutrophil accumulation and vascular permeability. PAF thus may play a role in the initiation and development of inflammatory processes in the central airways. These results are consistent with the finding by Brokaw et al. (4) that intravenously administered PAF induced the leakage of protein-bound Evans blue dye and an influx of neutrophils within 5 min into the rat trachea. Furthermore, in contrast to the effect on neutrophils, PAF did not increase the infiltration of eosinophils. This finding is in agreement with the previous results obtained in rats (4), dogs (7), and humans (34). In addition, a low dose of azelastine inhibits airway inflammation.

Fig. 2. Dose-dependent effect of PAF on vascular permeability (top) and neutrophil recruitment (bottom) in trachea (●) and main bronchus (▲) of pathogen-free rats. Control, vehicle alone in trachea (○) and main bronchus (△). Vascular permeability and neutrophil recruitment were assessed by measuring area density of Monastral blue-labeled blood vessels and counting number of neutrophils, respectively, in airway whole mounts. Responses are means ± SE; n = 5 rats/point. Significantly different from corresponding vehicle alone: *P < 0.05; **P < 0.01; ***P < 0.001.

Fig. 3. Effect of azelastine on PAF (10 µg/kg)-induced increase in vascular permeability in trachea (top) and main bronchus (bottom). Rats received various doses of intravenous azelastine 5 min before PAF injection, and area density of Monastral blue-labeled blood vessels was determined. Responses are means ± SE; n = 8 rats/bar. Significantly different from PAF alone: *P < 0.05; **P < 0.01.
PAF (0.3 µg/kg) had no effect on extravasation of Monastral blue but produced neutrophil accumulation. This finding suggests that, on stimulation with PAF, neutrophils may migrate through endothelial cells without necessarily causing increased permeability.

Although the mechanisms by which PAF increases vascular permeability are unclear, evidence suggests that PAF may have the ability to induce contraction of microvascular endothelial cells via activation of phospholipase C and intracellular Ca²⁺ mobilization (10, 17), and it may also cause direct injury to endothelial cells when examined ultrastructurally (19). In addition to these direct mechanisms, PAF may act indirectly through the activation of inflammatory cells such as platelets and neutrophils. It is well known that PAF is a potent chemotaxant for neutrophils and can stimulate the cells to release several mediators (27). In the present experiments, the accumulation of neutrophils coincided with the increase in Monastral blue-labeled blood vessels, and severe depletion of circulating neutrophils by cyclophosphamide caused a marked reduction in the permeability change. This reduction was to a level midway between that in the control and PAF-treated animals, suggesting that the increase in vascular permeability depends, at least partially, on these inflammatory cells. It is conceivable that in this process activated neutrophils may injure endothelium directly by the physical process of migration through vascular walls and/or indirectly via the release of vasoactive substances. In contrast, other studies have shown that PAF does not require circulating neutrophils to exert its vascular effects in hamster cheek pouch (3) and rat skin (22), indicating that there may be species differences as well as variations from tissue to tissue in the same animal.

Clinical trials have shown that azelastine is active in mild and moderate asthma (2, 21). The efficacy of this drug may be derived from the interference with airway responses to chemical mediators, an effect that is associated with the inhibition of Ca²⁺ influx and elevation of intracellular cAMP, followed by stabilization of the cell membrane (5, 30). Furthermore, Ciprandi et al. (8) have recently shown that azelastine reduces aller-

Table 1. Effects of pharmacological blocking agents on PAF-induced airway vascular leakage

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<th>No Blocker</th>
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<td>CPM</td>
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<td>Trachea 10 µg/kg</td>
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<td>14.8±2.9</td>
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<td>Bronchus 10 µg/kg</td>
<td>26.3±3.1</td>
<td>25.8±4.1</td>
<td>27.4±5.0</td>
<td>23.3±4.1</td>
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Values are means ± SE in percent of Monastral blue-labeled area in airway mucosa; n = 8 rats. PAF, platelet-activating factor; Hist blockers, histamine blockers (mepyramine, cimetidine, and thioperamide); SOD, superoxide dismutase; CPM, cyclophosphamide. Significantly different from response to no blocker (PAF alone): *P < 0.05; †P < 0.01.
gen-induced eosinophilic and neutrophilic infiltration into the nasal mucosa, presumably by downregulating intercellular adhesion molecule-1 expression in patients with nasal allergy. We found that pretreatment with intravenous azelastine reduced the PAF-induced extravasation of Monastral blue dye in a dose-dependent fashion, whereas it did not alter the number of neutrophils present in the airway mucosa. Similar to our findings, Lima et al. (16) have shown that azelastine inhibits allergen-induced formation of pleurisy without altering pleural cellular influx. These results imply that the observed effect of azelastine may not be related to the inhibition of neutrophil accumulation and not to the direct antagonism of PAF as has been previously shown under different experimental conditions (1). Another possibility is that azelastine could have exerted its effect through the inhibition of biosynthesis and/or release of histamine (2) as well as through the antagonism of histamine (6). However, pretreatment with a combination of mepyramine, cimetidine, and thioperamide to block H1-, H2-, and H3-receptors, respectively, was without effect on the increased vascular permeability induced by PAF, implying that, in our experimental condition, histamine may not be involved in the PAF action and the above-mentioned possibility seems unlikely. Moreover, in neutropenic rats, azelastine failed to further reduce the PAF-induced microvascular leakage. Therefore, the possible involvement of direct inhibition of endothelial gap formation by azelastine is also unlikely.

It is known that the vascular actions of PAF in intestinal capillaries are mediated by neutrophils (13) and that exposure of isolated neutrophils to PAF leads to degranulation, aggregation, and enhanced production of oxygen radicals and proteases (11), but precisely how neutrophils mediate the microvascular alterations induced by PAF remains uncertain. Pretreatment with SOD or catalase at a dose sufficient to attenuate lung injury (36) did not significantly alter the vascular responses to PAF. On the other hand, neutrophil elastase can injure vascular endothelial cells (28) and degrade several components of vascular basement membrane matrix (35), thereby producing a strong tissue damage during the acute inflammatory process. We thus tested the involvement of neutrophil elastase in PAF-induced vascular leakage and found that ONO-5046, a specific and intravenously active inhibitor of human neutrophil elastase as well as of rat neutrophil elastase (12), markedly reduced the increase in Monastral blue extravasation produced by PAF. These results suggest that neutrophil elastase may be an important mediator of the vascular action of PAF. Although it is possible that neutrophil elastase might facilitate neutrophil migration via inactivation of the tissue inhibitor of metalloproteinases and destruction of basement membrane (20), ONO-5046 had no effect on neutrophil accumulation in our experiment. Similar to our finding, Sakamaki et al. (25) have recently shown that pretreatment of guinea pigs with ONO-5046 inhibits endotoxin-induced acute lung injury without affecting neutrophil influx. Furthermore, we found that, in ONO-5046-treated rats in which neutrophil elastase activity had been already blocked, azelastine did not further inhibit the PAF-induced Monastral blue leakage. These results suggest that azelastine reduces airway microvascular permeability through inhibition of the release and/or action of neutrophil elastase. This contention is further supported by the finding that PAF caused an increase in the concentration of neutrophil elastase in BAL fluid and that this effect was inhibited by pretreatment with azelastine. Because azelastine does not possess antielastase activity (personal communication from K. Hirano, Eisai, Tokyo, Japan), inhibition of the release of neutrophil elastase from neutrophils by azelastine may be operating in the present experiments. However, it is still uncertain whether the observed effect of azelastine is solely attributable to the specific prevention of elastase release or is associated with the inhibition of steps for activation of multiple neutrophil effector functions, such as adhesion molecule-mediated neutrophil adhesion and the ability of neutrophils to crawl.

In conclusion, neutrophil activation and the subsequent release of neutrophil elastase may play an important role in the airway microvascular leakage produced by PAF, and the antiasthma drug azelastine reduces vascular permeability, probably involving inhibition of the release of neutrophil elastase. Therefore, azelastine might be of value in the prevention of neutrophil-mediated airway mucosal inflammation, but further studies are needed to determine whether this drug is similarly active in humans.

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