Gastrin induces the interaction between human mononuclear leukocytes and endothelial cells through the endothelial expression of P-selectin and VCAM-1

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Ibiza S, Álvarez Á, Romero W, Barrachina MD, Esplugues JV, Calatayud S. Gastrin induces the interaction between human mononuclear leukocytes and endothelial cells through the endothelial expression of P-selectin and VCAM-1. Am J Physiol Cell Physiol 297: C1588–C1595, 2009. First published October 7, 2009; doi:10.1152/ajpcell.00082.2009.—Gastric mucosal inflammation is frequently associated with hypergastrinemia, and a correlation exists between the level of gastrin and degree of gastritis. We have previously observed that gastrin promotes leukocyte-endothelial interactions and contributes to the inflammation observed in these circumstances (2, 3). This experimental evidence demonstrates a proinflammatory activity of gastrin in humans. The interaction between human leukocytes [U-937 cells, peripheral blood polymorpho-nuclear (PMN), and peripheral blood mononuclear (PBMC) cells] and human umbilical vein endothelial cells (HUVEC) was analyzed in static and dynamic conditions. The endothelial expression of adhesion molecules [P-selectin, E-selectin, intercellular adhesion molecule-1, vascular cell adhesion molecule (VCAM)-1] was analyzed by flow cytometry and fluorescent microscopy screening. Gastrin increased the static adhesion of U-937 cells to HUVEC (1 h; 10^{-9} M: 122 ± 9%; 10^{-8} M: 143 ± 17%; 10^{-7} M: 162 ± 14% vs. control, all P < 0.05). Incubation of HUVEC with gastrin (4 h) also increased PBMC rolling (vehicle: 63 ± 12; 10^{-9} M: 109 ± 29; 10^{-8} M: 141 ± 24; 10^{-7} M: 261 ± 16 leukocytes/mm, P < 0.05) and adhesion (vehicle: 3 ± 2; 10^{-9} M: 11 ± 4; 10^{-8} M: 17 ± 5; 10^{-7} M: 15 ± 5 leukocytes/mm², all P < 0.05) in the parallel-plate flow chamber. Treatment of PBMC with gastrin had no effects. The cholecystokinin (CCK)-2 receptor antagonist (L-365,260, 10^{-3} M) prevented the proinflammatory effects of gastrin on the interactions between human leukocytes and endothelial cells through the activation of CCK-2 receptors and the enhancement of endothelial P-selectin and VCAM-1.

cholecystokinin-2 receptors; endothelial adhesion molecules; leukocyte-endothelial cell interactions

GASTRIN IS CONSIDERED A MAJOR physiological regulator of gastric acid secretion and growth, and these effects are known to be mediated through gastrin/cholecystokinin-2 (CCK-2) receptors (36). Gastrin is secreted from antral G cells in response to food intake, and this release is influenced by local conditions. In particular, there is an increase of gastrin secretion in circumstances of chronic mucosal inflammation, such as Helicobacter pylori infection and autoimmune gastritis. The hypergastrinemia related with these conditions correlates with the degree of gastritis (12, 22, 35) and has been proposed as a marker of gastric inflammation (25). Chronic gastritis predisposes to mucosal atrophy and cancer, and gastrin and its receptors are expressed in many gastrointestinal and nongastrointestinal tumors (29, 37).

The abovementioned pathological alterations in gastrin secretion have led to research aimed at detecting the possible contribution of this hormone to disease (7). Gastrin hypersecretion may modify the epithelial structure by its well-known ability to promote cellular proliferation (36), and also by regulating migration (26), invasion (39) and apoptosis (6, 19, 28, 33) processes in epithelial cells. A new field of research is currently exploring the relevance of gastrin in inflammatory/immune responses. In this context, CCK-2 receptors have been detected in key cellular players in these processes [leukocytes (8) and endothelial cells (21)], and in two studies significant changes have been observed in the expression of some inflammation-related genes in the gastric mucosa of gastrin knockout mice (10, 15). Gastrin induces the expression of proinflammatory molecules such as cyclooxygenase-2 and interleukin (IL)-8 in epithelial cells (13, 19), and the equilibrium between gastrin and somatostatin release seems to be essential for defining the host lymphocytic response to the Helicobacter infection (31, 40). We have recently demonstrated that the activation of CCK-2 receptors by gastrin promotes leukocyte-endothelial cell interactions in the rat mesentery. Furthermore, hypergastrinemia caused by H. pylori components or by pharmacological treatment with antisecretory drugs seems to contribute to the inflammation observed in these circumstances (2, 3). This experimental evidence demonstrates a proinflammatory effect of gastrin in rodents. However, to our knowledge, the possibility that gastrin exerts an analogous effect in humans is yet to be explored. Thus we have analyzed the effects of gastrin on the interactions between human leukocytes and endothelial cells in vitro and the molecular mechanisms involved in the effects observed.

MATERIALS AND METHODS

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical cords according to the method of Jaffe et al. (14). In short, the umbilical vein was cannulated, rinsed with phosphate buffer, and filled with collagenase A (1 mg/ml) for 5 min at 37°C. Endothelial cells were eluted with medium 199, centrifuged, washed, pelleted once more, and then seeded with endothelial cell growth medium (EGM-2) in T25 culture flasks. After reaching confluence, the primary cultures were detached from the flask with trypsin and transferred to culture dishes. Experiments were performed when the cells had once again attained confluence, and performed in cells from passage 1.

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Human monocyte-like U-937 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 2 mM L-glutamate.

Leukocyte Isolation

Citrated blood samples from healthy donors were incubated with dextran (3%) for 45 min. Peripheral blood mononuclear cells (PBMC) and polymorphonuclear leukocytes (PMN) in the supernatant were separated by gradient density centrifugation (250 g, 25 min) with Ficoll-Paque Plus. After red blood cell lysis, leukocytes were washed Hanks’ balanced salt solution (HBSS, without Ca²⁺ or Mg²⁺) and resuspended in complete RPMI media.

Static Endothelial-Leukocyte Attachment Assay

To analyze leukocyte adhesion to endothelial cells in static conditions, leukocytes were incubated with calcine-AM (20 μM, 20 min, 37°C) and, after washing, were poured over confluent HUVEC in 24-well plates at a concentration of 10⁶ cells/ml. The plates were incubated for 1 h at 37°C and 5% CO₂. Nonadherent leukocytes were subsequently removed by means of thorough washing, cells were lysed with zwittergent (4 mM), and the number of adherent leukocytes was determined by measuring the fluorescence emitted by the cell lysate. The experiment was performed in the presence or absence of different concentrations of gastrin (10⁻¹¹ to 10⁻⁷ M). Cells incubated with tumor necrosis factor (TNF)-α (10 ng/ml, 4 h) were used as positive controls.

In a further set of experiments, HUVEC and U-937 were treated separately with gastrin for 1 h. Attachment assays were performed following washing to remove the drug and avoid it exerting an action on the other cell type.

Parallel Plate Flow Chamber Assays of Leukocyte Binding to HUVEC

Interactions between leukocytes and HUVEC under flow conditions were analyzed as described previously (23). In brief, HUVEC were plated in glass microscope slides coated with human fibronectin (2 μg/cm²) and immersed in six-well plates. Experiments were performed once the cells had reached confluency (24–48 h). Confluent endothelial monolayers on glass cover slips were incubated for 4 h with culture media containing different concentrations of gastrin (10⁻⁹ to 10⁻⁷ M) or TNF-α (10 ng/ml) and were then carefully positioned in a circular recess in the bottom plate of the chamber, where a portion (5 × 25 mm) of the monolayer was exposed to flow. The flow apparatus with the HUVEC monolayer was mounted on an inverted microscope (Nikon Eclipse TE 2000-S) equipped with ×10 and ×40 phase contrast objectives. A circular glass window in the top plate allowed direct live microscopic examination of the monolayer exposed to flow. The monolayer was perfused with the leukocytes (PBMC or PMN) suspended in Dulbecco’s PBS (DPBS) + albumin (10⁶ cells/ml), which moved through the chamber at a controlled flow rate of 0.36 ml/min (estimated shear stress = 0.7 dyne/cm²). Images were recorded during a 5 min-period with a video camera (Sony Exwave HAD). Leukocyte adhesion was determined by counting the number of leukocytes that maintained stable contact with the monolayer for 30 s. Leukocyte rolling was calculated by counting the number of leukocytes that maintained stable contact with the monolayer for 100 s. The number of leukocytes rolling over 100 μm was counted by a blinded experimenter. The velocities of 20 consecutive leukocytes in the field of focus were determined by measuring the time required to travel a distance of 100 μm. In some cases, HUVEC monolayers treated with gastrin (10⁻⁷ M) were coincubated with rhodamine green heptagastatin (RG-7P), and the expression of the respective epitope. Nonspecific fluorescence was detected using isotype-matched nonbinding antibodies, and its value was subtracted from that obtained in each experimental group.

Fluorescent microscopy screening. Cell monolayers were incubated under different conditions in 48-well plates, and, after being washed with HBSS, were incubated with Hoescht plus the corresponding antibody (20 min, 37°C, in darkness) conjugated with FITC or PE. After washing was completed, plates were analyzed in a modular microscope-based imaging platform (microscope Olympus IX81 coupled to a Hamamatsu camera) designed for fully automated image acquisition and data analysis of adherent cell cultures (scan®R screening station). Data relating to the fluorescent signal emitted by the objects (identified by Hoescht staining) were analyzed in a cytometry-oriented manner by multiparameter data schemes, gating, and classification. Experiments were performed in duplicate wells, with between four and five thousand cells being analyzed in each well. Nonspecific fluorescence was detected using isotype-matched nonbinding antibodies, and its value was subtracted from that obtained in each experimental group.

Analysis of the Expression of Adhesion Molecules in Peripheral Blood Leukocytes

Leukocyte adhesion molecules were analyzed in citrated blood samples from healthy donors (40 μl). These samples were treated for 15 min at 37°C and were then incubated (30 min, in ice and darkness) with saturating concentrations of the corresponding FITC-conjugated antibody. Red blood cell lysis and leukocyte fixation was carried out automatically with the system EPICS TQ-PREP (Coulter Electronics). Neutrophils, monocytes, and lymphocytes were identified by forward- and right-angle light scatter analysis. The median of the specific fluorescence intensity was used as a marker of the expression of the respective epitope. Nonspecific fluorescence was detected by employing isotype-matched nonbinding antibodies, and its value was subtracted from that obtained in each experimental group. All experiments were performed in duplicate, and ten thousand events were analyzed in each case. Analyses were carried out in an EPICS XL-MCL cytometer (Coulter Electronics).

Gasrin Binding to HUVEC

The interaction between gastrin and HUVEC was detected by measuring the binding of a fluorescently labeled gastrin derivative [rhodamine green heptagastatin (RG-7P)]. HUVEC were incubated with RG-7P (10⁻⁷ M) for 30 min in the presence or absence of unlabeled gastrin (10⁻⁷ to 10⁻⁵ M) or the CCK-B/gastrin receptor antagonist L-365,260 (10⁻⁶ to 10⁻⁸ M).

Measurement of Cell Death

Cellular respiration, assessed by mitochondrial-dependent reduction of 3-[(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan, was used as an index of cell viability. HUVEC were treated with different concentrations of gastrin (10⁻⁹ to 10⁻⁷ M), and 1 or 4 h later, the medium was removed and replaced with a
warm (37°C) medium containing 0.2 mg/ml of MTT. After incubation at 37°C for a further 30-min period, the medium containing the MTT was removed, and the cells were dissolved in dimethyl sulfoxide. The extent of the conversion of MTT to formazan was quantified by measurement of optical density at 550 nm with a wavelength correction of 650 nm.

Materials

L-365,260 was a gift from ML Laboratories (Liverpool, UK); human gastrin I (gastrin-17), rhodamine green heptagastrin, TNF-α, fibronectin, RPMI culture media, albumin, dextran, t-glutamate, Hoechst, and MTT were from Sigma Chemical (St. Louis, MO); FITC or PE-conjugated control antibodies or antibodies against P-selectin, E-selectin, intercellular adhesion molecule (ICAM)-1, VCAM-1, L-selectin, CD18, CD11a, CD11b, CD11c, or CD49d were from Sero-tech (Burlington, Canada); blocking antibodies against VCAM-1 were from BD Bioscience and against P-selectin from R&D Systems; EGM-2 culture media, DPBS, HBSS, and FBS were from LONZA (Verviers, Belgium); Ficoll-Paque Plus was from GE Healthcare; calcein-AM was from Molecular Probes-Invitrogen (Paisley, UK); and zwittergent was from Calbiochem.

Statistical Analysis

All values are means ± SE. Data within groups were compared using the one-way ANOVA or the repeated-measures one-way ANOVA where appropriate, followed in both cases by a Newman-Keuls post-hoc test. Differences were considered significant when the P value was <0.05.

The study was conducted according to the Declaration of Helsinki Principles. The medical ethical committee of the Hospital Clínico Universitario de Valencia approved all the experiments described.

RESULTS

The static adhesion of U-937 to HUVEC was significantly increased in a concentration-dependent manner by the presence of gastrin in the incubation medium (Fig. 1A). Treatment with gastrin (10⁻⁷ M) also augmented the adhesion of PBMC to HUVEC (137 ± 5% vs. control response, P < 0.05). In contrast, the adhesion of PMN to HUVEC was not modified by gastrin (Fig. 1B).

The static adhesion of U-937 to HUVEC was also stimulated when gastrin treatment was applied only to the endothelial cells (Fig. 2A). However, the adhesion process was not affected when gastrin-treated U-937 cells were dropped on nontreated HUVEC (Fig. 2B).

RG-7P interacted with HUVEC, which was evident in the increased fluorescent signal in cells incubated for 30 min with this gastrin derivative (10⁻⁷ M). RG-7P binding to HUVEC was prevented in a concentration-dependent manner by the presence of nonlabeled gastrin (10⁻⁷ to 10⁻⁵ M) or the CCK-2 receptor antagonist L-365,260 (10⁻⁸ to 10⁻⁶ M) (Fig. 3).

Incubation of HUVEC with gastrin for 4 h significantly increased the number of rolling PBMC (Fig. 4B) and reduced their velocity (Fig. 4A) when analyzed in the parallel plate flow chamber. An increased adhesion was also observed (Fig. 4C). The interactions between PBMC and HUVEC induced by gastrin (10⁻⁷ M) were prevented when HUVEC were cotreated with L-365,260 (10⁻⁷ M) (Fig. 5). As occurred in the static assay, treatment with gastrin (10⁻⁷ M) did not modify the adhesion of PMN to HUVEC (125 ± 30% vs. control response) or rolling velocity (92 ± 8% vs. control response), although it did induce a nonsignificant increase in the number of rolling PMN (171 ± 25% vs. control response).

HUVEC viability was not affected by incubation with any of the concentrations of gastrin used at either 1 h (gastrin 10⁻⁷ M: 99 ± 2% of control value) or 4 h (gastrin 10⁻⁷ M: 102 ± 1% of control value).

Flow cytometry assays revealed that, when HUVEC were treated with gastrin for 1 h, the expression of VCAM-1 was more pronounced than that of control cells, whereas the expression of E-selectin and ICAM-1 was not modified by this treatment (Table 1). Automated fluorescence microscopy experiments gave similar results in HUVEC treated with gastrin for 4 h, revealing a higher expression of VCAM-1 (Fig. 6A) and no significant changes in the expression of E-selectin (gastrin 10⁻⁷ M: 110 ± 23% of control value) or ICAM-1 (gastrin 10⁻⁷ M: 103 ± 15% of control value). An increase in the presence of P-selectin was observed on the cellular surface of HUVEC treated with gastrin for 15 min (vehicle: 119 ± 80; 10⁻⁹ M: 175 ± 75; 10⁻⁸ M: 300 ± 106; 10⁻⁷ M: 538 ± 176 arbitrary units of fluorescence, P < 0.05 vs. vehicle) and 4 h (Fig. 6B). This increment in the endothelial expression of VCAM-1 and P-selectin induced by gastrin (10⁻⁷ M, 4 h) was not detected when HUVEC were cotreated with the CCK-2 receptor antagonist L-365,260 (10⁻⁷ M) (Fig. 7).

The increase in the rolling and adhesion of PBMC to gastrin-treated HUVEC (10⁻⁷ M, 4 h) was significantly lower when endothelial cells were incubated for the final 30 min of the treatment period with a blocking antibody against P-selectin. Incubation with a blocking antibody
against VCAM-1 significantly reduced the adhesion of PBMC and partly reversed the reduction of rolling velocity without significantly modifying the number of rolling PBMC (Fig. 8).

Treatment of PBMC with different concentrations of gastrin did not modify their interactions with untreated HUVEC (gastrin $10^{-7}$ M: rolling velocity 79 ± 8%, rolling flux 91 ± 14%, adhesion 100 ± 10% vs. control). Moreover, flow cytometry assays revealed that treatment of peripheral blood samples with gastrin ($10^{-9}$ to $10^{-7}$ M, 15 min) did not influence the expression of L-selectin, CD11a, CD11b, CD11c, CD18, or CD49 in lymphocytes, monocytes, or PMN (data not shown).

**DISCUSSION**

The present study demonstrates for the first time the capacity of gastrin to induce the interaction between human leukocytes and endothelial cells. Our findings corroborate the proinflammatory action of gastrin previously reported in rats and indicate that this activity may occur in humans. Additionally, our results reveal that this effect of gastrin is a result of the activation of the endothelial CCK-2 receptors and the consequent increase in the expression of the endothelial adhesion molecules P-selectin and VCAM-1.
We have observed the stimulatory effect of gastrin in static and dynamic conditions. In the latter case, leukocytes flow over the endothelial surface with a shear stress similar to that encountered in vivo (24), and the interactions between leukocytes and the endothelial monolayer reproduce those that precede the formation of an inflammatory focus in vivo, i.e., rolling and adhesion. Thus the results obtained in this system imply that gastrin could exert a proinflammatory action in humans. We have seen that gastrin acts specifically on endothelial cells but not on leukocytes. Pretreatment of HUVEC with gastrin was sufficient to increase the rolling and adhesion of untreated leukocytes, whereas treatment of isolated leukocytes did not modify their interactions with resting HUVEC in either static or dynamic conditions. Moreover, gastrin specifically increased the surface expression of P-selectin and VCAM-1 in HUVEC, although it did not alter the pattern of expression of adhesion molecules in leukocytes.

The ability of the CCK-2 receptor antagonist L-365,260 to prevent the binding of rhodamine-gastrin to HUVEC reveals that gastrin binds to these cells through CCK-2 receptors, whose presence in endothelial cells has been demonstrated previously (5). The activation of these receptors is responsible for the observed increases in leukocyte rolling and adhesion, since these effects were blocked by pretreatment with the antagonist L-365,260. This is similar to the situation observed with P-selectin and VCAM-1 in HUVEC, although it did not alter the pattern of expression of adhesion molecules in leukocytes.

Table 1. Effects of treatment with different concentrations of gastrin (1 h) on the surface expression of endothelial adhesion molecules in human umbilical vein endothelial cells, as analyzed by flow cytometry

<table>
<thead>
<tr>
<th>Adhesion Molecule</th>
<th>Gastrin, log[M]</th>
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<tbody>
<tr>
<td></td>
<td>9</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>112±4*</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>94±4</td>
</tr>
<tr>
<td>E-selectin</td>
<td>96±2</td>
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</tbody>
</table>

Results are expressed as percentage vs. value in vehicle-treated cells and represent means ± SE. VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1. *P < 0.05, †P < 0.001, and ‡P < 0.01 vs. corresponding value in vehicle-treated group (ANOVA followed by Newman-Keuls test).

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in rats in which the leukocyte-endothelial interactions induced by gastrin in mesenteric venules were blocked by two different antagonists of CCK-2 receptors (proglumide and L-365,260) but were unaltered by CCK-1-specific blockade with devazepide (3). We observed that gastrin did not modify the expression of adhesion molecules in rat leukocytes when treated ex vivo, whereas leukocytes from hypergastrinemic animals exhibited changes in the expression of adhesion molecules that were indicative of cellular activation (2). Thus it would appear that gastrin induced the proinflammatory effect by acting on structures that were present only in vivo. We detected the CCK-2 receptor in macrophages of the rat mesentery but not in venular endothelial cells. This difference between rat and human endothelial cells may be due to interspecies variation or to differences between the endothelium of big and small vessels. The first hypothesis is more likely if we consider recent reports of the analogous influence of gastrin in HUVEC and human microvascular endothelial cells of two different territories, namely, the skin and the uterus (5). Whatever the reason, our results indicate that gastrin may induce leukocyte-endothelial cell interactions in both rats and humans, although the mechanism of this action is likely to differ between the two species.

Gastrin specifically stimulates the interaction of PBMC without modifying that of PMN. We have observed this effect with the monocytic cell line U-937 and with PBMC. This selectivity is probably dependent on the pattern of expression of adhesion molecules induced by gastrin. Although P-selectin may sustain the rolling of PBMC and PMN, VCAM-1 binds specifically to α4β1 (CD49d/CD29, VLA-4), an integrin that is present in PBMC but absent in PMN (27). Our functional studies confirm that P-selectin and VCAM-1 are responsible for the interactions induced by gastrin, since blocking these molecules significantly reduced the effects of the hormone. The blocking of P-selectin was especially effective in reducing the number of rolling leukocytes and increasing their velocity, which was to be expected given the predominant role of this molecule in such a process (16). A nonsignificant reduction in leukocyte adhesion was also observed, which is probably a consequence of the reduction in rolling (16, 17). Blocking VCAM-1 significantly reduced leukocyte adhesion, and although it did not modify the number of rolling leukocytes it did increase their velocity. The latter effect may be because of the secondary role that VCAM-1 plays in rolling, which is less important than the one it plays in adhesion (1, 4).

Gastrin appeared to trigger the translocation of P-selectin from the Weibel-Palade bodies to the cellular surface of
HUVEC, since the presence of this molecule was seen to increase 15 min after treatment with the peptide. However, gastrin is likely to exert an additional effect by which it induces P-selectin synthesis de novo, since the translocation of this molecule is usually a transient effect and we also observed an increased expression of P-selectin after 4 h of treatment. The molecular mechanisms responsible for the release of P-selectin from Weibel-Palade bodies remain unclear (34); thus, it is difficult to infer the intracellular events that are triggered by gastrin to induce this rapid surface expression of P-selectin. On the other hand, CCK-2 receptor activation can activate several signaling pathways involved in the transcriptional regulation of endothelial adhesion molecule expression, including mitogen-activated kinase cascades (extracellular signal-regulated kinase 1/2, c-Jun NH2-terminal kinases, p38) and protein kinase C (8). These pathways would prompt the synthesis of endothelial adhesion molecules through nuclear factor-kB and activator protein-1 transcription factors. However, these signaling cascades are likely to induce the expression of ICAM-1 and E-selectin in addition to that of VCAM-1, and would not induce P-selectin expression (20, 27). It is interesting to note that the effects induced by gastrin, which consist of an augmented expression of P-selectin and VCAM-1 and unaltered levels of E-selectin and ICAM-1, coincide with the pattern of endothelial activation induced by the Th2 cytokines IL-4 and IL-13 (38). Both cytokines, which also promote the adhesion of PBMC and not that of neutrophils (32), act through the Jak/STAT pathway to induce the abovementioned changes in the expression of adhesion molecules (18, 30) and the synthesis of chemotactic factors for PBMC (11). Although recent evidence suggests that the CCK-2 receptor is linked to the JAK/STAT pathway (9), new experiments are required to establish whether or not this mechanism is involved in the preferential stimulation of mononuclear adhesion induced by gastrin, which locates the action of gastrin at the scene of chronic inflammatory conditions.

In conclusion, the present study reveals that the proinflammatory action of gastrin previously reported in rats may be relevant in humans and, as demonstrated in these animals, could be implicated in the inflammatory responses observed in patients with hypergastrinemia due to H. pylori infection or chronic treatment with antisecretory drugs.

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