Identification of genes involved in mucosal defense and inflammation associated with normal enteric bacteria

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INFLAMMATORY BOWEL DISEASE (IBD) is a disease of unknown etiology. Current theories suggest that both Crohn’s disease and ulcerative colitis are multifactorial entities conditioned by genetic, environmental, microbial, and immunological factors (7). It is widely accepted that luminal bacteria and their products play a role in both forms of IBD (3) and that components of the normal intestinal flora can trigger, initiate, or contribute to IBD pathogenesis (7). This concept is supported by the demonstration of IBD-like lesions in various animal models of experimental IBD, such as indomethacin-induced enterocolitis or carrageenan-induced or dextran sulfate sodium-induced colitis (21, 25, 35). Commensal intestinal bacteria appear to play a key role in the development and exacerbation of chemically induced colitis. Moreover, chronic intestinal inflammation also develops in genetically engineered animals such as interleukin (IL)-2, IL-10 knockout mice or HLA-B27 transgenic rats only in the presence of luminal bacteria and not under germ-free (GF) conditions (11, 26, 30). Together, these reports clearly show that commensal intestinal bacteria are intimately involved in the development of mucosal inflammation when inciting or genetic abnormalities are present. However, in such models, the molecules that are responsible for the development and perpetuation of mucosal inflammation, and the cellular source of these molecules, have not been identified. It is reasonable to assume that the mechanism of mucosal inflammation depends, at least in part, on the interaction of commensal luminal bacteria associated with mucosal cells, particularly epithelial cells, which are in close physical contact with the bacteria. Therefore, identification and characterization of the molecules associated with conventional bacteria-induced inflammation and recovery is critically important for the understanding of any type of experimental, or perhaps clinical, form of IBD. Identification of essential molecules involved in the mucosal defense system would also be indispensable in establishing whether abnormalities found in IBD mucosa are primary or secondary events caused by the existence of bacteria associated with IBD pathogenesis. This could lead to a better therapeutic approach by controlling secondary inflammatory responses even if the etiology of IBD has not been fully elucidated.

Because of the lack of experimental systems, only limited information is available regarding normal antibacterial responses in the gut. Mucosal cells in genetically “normal” animals without obvious inflammation-inducible agents may develop antibacterial responses via a well-balanced expression of specific gene products to create and maintain a mucosal defense system. Molecules participating in mucosal defense against luminal bacteria are probably multiple, including cytokines, receptors, cytokine proteins, and enzymes, among others. In addition, it is likely that differential antibacterial mechanisms are present in the colon and small intestine.

In this study, we have established a novel approach for analysis of mucosal defense mechanisms against nonpathological luminal microorganisms. This ap-
proach enables us to understand better the epithelial cell response induced by the enteric flora via the examination of the induction or suppression of gene products not previously known to be specifically involved in gut mucosal defense and inflammation.

MATERIALS AND METHODS

Animals. GF and specific pathogen-free (SPF) ICR mice were purchased from Clea Japan (Tokyo, Japan). Fecal suspensions were freshly prepared from SPF mice by 10-fold dilution of colonic content with saline. GF mice at 6, 7, and 8 wk of age (n = 8 per age group) were given intestinal microorganisms via oral administration of fecal suspensions and were reared with SPF mice under SPF conditions. Bacteria-reconstituted 8-wk-old mice were killed after 3, 7, and 14 days by cervical dislocation. Non-bacteria-reconstituted GF (n = 8) and SPF (n = 8) mice were used as controls and were also killed at the age of 8 wk. This protocol was approved under the regulations in the Guide for the Care and Use of Laboratory Animals of Tohoku University School of Medicine.

Isolation of intestinal and colonic epithelial cells in mice. Tissues from the ileum, cecum, and rectum were obtained in each group, immediately fixed in 10% buffered formalin, and embedded in paraffin wax. Three-micrometer-thick sections were stained with hematoxylin-eosin. Albumin concentration in serum, glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), and lactate dehydrogenase (LDH) activity and C-reactive protein (CRP) were determined using Spotchem (KDK, Kyoto, Japan). The small intestine and colon were treated separately and mined using Spotchem (KDK, Kyoto, Japan). 

Determination of LDH activity and C-reactive protein (CRP). Serum was obtained by cardiac puncture at the end of the experimental day, and LDH activity in serum, glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), and lactate dehydrogenase (LDH) activity were determined using Spotchem (KDK, Kyoto, Japan).

Tissues sampled for histology and serological assessment. Tissues from the ileum, cecum, and rectum were obtained in each group, immediately fixed in 10% buffered formalin, and embedded in paraffin wax. Three-micrometer-thick sections were stained with hematoxylin-eosin. Albumin concentration in serum, glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), and lactate dehydrogenase (LDH) activity and C-reactive protein (CRP) were determined using Spotchem (KDK, Kyoto, Japan).

Isolation of intestinal and colonic epithelial cells in mice. The small intestine and colon were treated separately and washed with Hanks’ balanced salt solution (HBSS). Epithelial cells were isolated as previously described elsewhere with minor modifications (13). Briefly, small intestine or colon tissue was cut into small pieces (~2–3 cm in length and incubated in HBSS with 1.5 mg/ml dithiothreitol (DTT) for 10 min at room temperature. Tissue pieces were then put into HBSS with 1 mM EDTA at room temperature for three 10-min periods. At the end of each period, they were shaken rapidly with a glass rod. The supernatants were collected by centrifugation, and the resulting pellets were washed twice in RPMI 1640. Viability of purified epithelial cells was assessed using Trypan blue dye exclusion and Trypan blue dye exclusion counts.

Differential display. Differential display was performed as previously described (14). cDNA subpopulations were generated from 2.5 μg of epithelial total RNA using reverse transcriptase (SuperScript II; Gibco, Grand Island, NY) with oligo GT15X (X = A, G, or C) primers in 20 μl of reverse transcription buffer supplied by the manufacturer. Oligo GT15X primers were synthesized with DNA/RNA synthesizers (Applied Biosystems, Foster City, CA). The resulting cDNA solutions were diluted fivefold in Tris-EDTA buffer. PCR was performed on each of the cDNA subpopulations with 10-bp upstream primers (Operon 10-mer kits; Operon, Alameda, CA). Two microliters of diluted cDNA solutions were added to a mix with 0.5 units of Taq DNA polymerase (Amersham Japan, Tokyo, Japan), 0.5 units of Gene Taq (Nippon Gene, Toyama, Japan), 0.2 mM nucleotides (dATP, dGTP, dCTP, and dTTP), 0.5 μM appropriate oligo GT15X, 0.5 μM upstream primers, and Gene Taq buffer supplied by the manufacturer in a total volume of 20 μl for 30 cycles with a Robocycler thermocycler (Stratagene, La Jolla, CA). Each cycle consisted of 94°C for 30 s, 42°C for 2 min, and 72°C for 1 min. PCR products were identified by staining with SYBR Green I (FM, Rockland, ME) after electrophoresis on 6% acrylamide gels, and photographs were taken. Bands were selected on the basis of whether 1) bands were induced or suppressed by bacterial reconstitution, 2) bands were differentially modulated between small intestine and colon, and 3) bands were small intestine or colon specific.

Cloning and sequencing. The gel piece containing the band of interest was excised under a ultraviolet illuminator. The DNA was reamplified by the direct addition of the gel to an appropriate 100-μl PCR reaction and 20–25 cycles of PCR. The reamplified fragments of DNA were fractionated on 1% agarose gels, excised, and purified by PCR preps DNA purification system (Promega, Madison, WI). The cDNA was subcloned into a T vector (pGem-T Easy Vector System; Promega) and sequenced using a Cy5 Auto Cycle sequencing kit (Pharmacia Biotech, Tokyo, Japan) and an ALFRed DNA sequencer (Pharmacia Biotech). The subcloned cDNA was used as a probe for Northern blot analysis.

Northern blot analysis. Total RNA (20 μg) was fractionated by electrophoresis on 1% agarose-formaldehyde gels and transferred onto a nylon transfer membrane (Amersham, Tokyo, Japan). The protocol for Northern blotting was described previously (9).

Detection of murine cryptdin isoform. cDNA was synthesized using oligo (dT) 12–18 (GIBCO BRL) as a primer for the reaction. Cryptdin-1, -4, and -5 cDNAs were amplified using the consensus forward primer Defcrp130 (5'-AGAGACTAAACCTGGAGGACGC-3') paired with cryp1 (5'-CGACAGCAGACGGTGTA-3') for cryptdin-1, cryp4 (5'-CCGGCGGGCGGCGAAGTA-3') for cryptdin-4, and cryp5 (5'-ACGACGAAATCCGATGTA-3') for cryptdin-5. cryp4 and crypt5 oligonucleotides do not anneal to known defensin sequences, but cryp1 is an exact complement to the sequence for cryptdin-6, -9 to -11, -15, and -17 cDNAs; therefore, these isoforms would be detected under the assay conditions described (4). PCR were performed using a program of 15–23 cycles at 94°C for 40 s, 60°C for 40 s, and 72°C for 40 s. Murine β-actin cDNA was also amplified as an internal standard using the primer sets (5'-CAACTGGGACGATGGAGAAGA-3', 3'-TTATAGGGATTGATTCTG-5'). PCR amplification products were subjected to 2% agarose gel electrophoresis, stained with ethidium bromide, and viewed under ultraviolet light, and photographs were taken.

In situ hybridization. Ileal tissues were obtained from GF, SPF, and 3-day bacteria-reconstituted mice, fixed in 4% paraformaldehyde solution containing 0.5% glutaraldehyde, and embedded in paraffin wax. Cryptdin-5 cDNA was obtained by RT-PCR as described in Detection of murine cryptdin isoform and subcloned into pGEM-T Easy Vector (Promega). Digoxygenin-labeled antisense and sense cRNAs were synthesized from the subcloned plasmid after linearization using a DIG RNA labeling kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer’s instructions. Because cryptdin-5 cDNA is highly (>80%) homologous to other cryptdin cDNAs, it is possible for the antisense cRNA probe to hybridize to other cryptdin mRNAs (23). Two-micrometer-thick sections were covered with hybridization buffer containing antisense or sense probe (0.5 μg/ml). Hybridization was al-
allowed to proceed at 45°C for 16 h, and then sections were washed twice for 60 min in 50% formamide containing 2× saline sodium citrate (SSC) at 45°C and once for 60 min in 50% formamide containing 1× SSC at 45°C. The hybridized digoxigenin-labeled probes were visualized using antidigoxigenin Fab fragment (Boehringer Mannheim) and BCIP-NBT substrate. The sections were developed in substrate solution for 3 h, washed in PBS buffer, and then counterstained with methyl green.

**Immunohistochemistry.** Three-micrometer-thick sections were mounted on silane-coated glass slides and incubated with a monoclonal antibody against murine serum amyloid A (SAA) at a dilution of 1:800 overnight at 4°C. This antibody is a rat-mouse chimeric antibody (kindly provided by Dr. T. Yamada, Juntendo Medical School, Tokyo, Japan) obtained by immunizing BALB/c mice with recombinant mouse SAA1 followed by fusion to mouse myeloma cell SP2. The reacting epitope of the antibody was suspected to be somewhere between residue 76 and the carboxyl terminus of SAA (36). Biotin-labeled anti-rat IgG (Dako Japan, Tokyo, Japan) was used at a dilution of 1:400 as a second antibody. Peroxidase-labeled avidin was obtained from Nichirei (Tokyo, Japan), and the antigen-antibody complex was visualized with 3,3'-diaminobenzidine (DAB) solution and counterstained with hematoxylin. The specificity of the immunohistochemical staining was confirmed by replacing the primary antibody with normal mouse IgG or PBS.

**RESULTS**

**Serological and general assessment.** Bacteria-reconstituted mice gained weight similarly to their control SPF counterparts. No mice died. There was no significant abnormality in serum total proteins, albumin, GOT, GPT, or LDH except for CRP among GF, conventionalized, and SPF mice (Table 1). Stool was softer in mice 3 days after bacterial reconstitution compared with mice in other groups. Bloody stool was not noticed in any mouse.

**Histological assessment.** The villi in the small intestine of GF mice were short and slender, and the lamina propria was underdeveloped compared with that of SPF mice (Fig. 1, A and D). After bacterial reconstitution, histological findings of small intestinal mucosa were comparable to those of SPF mice (Fig. 1, B and C). Paneth cells were recognized as granulated epithelial cells in the small intestine and were found at 0–5 cells per crypt throughout the series of experiments. Colonic mucosa of GF mice was characterized by atrophy of the epithelium and minimal infiltration of cells into the lamina propria (Fig. 2A). In contrast to the case of minor morphological alteration in the small intestine, an acute inflammatory change developed in the colonic mucosa.

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**Table 1. Serological analysis of bacterial reconstitution model**

<table>
<thead>
<tr>
<th></th>
<th>Days After Bacterial Reconstitution</th>
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<tbody>
<tr>
<td></td>
<td>GF</td>
</tr>
<tr>
<td>Total protein, g/dl</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>Albumin, g/dl</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>GOT, IU/l</td>
<td>122 ± 25</td>
</tr>
<tr>
<td>GPT, IU/l</td>
<td>&lt;5</td>
</tr>
<tr>
<td>CRP, mg/dl</td>
<td>&lt;30</td>
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</table>

Data are expressed as means ± SE. GF, germ free; SPF, specific pathogen free; GOT, glutamic-oxaloacetic transaminase; GPT, glutamic-pyruvic transaminase; CRP, C-reactive protein.
mucosa 3 days after bacterial reconstitution, characterized by hyperplastic changes of epithelial cells, infiltration of mono- and polymorphonuclear cells, and edema in both the mucosa and submucosa (Fig. 2B). Mucosal inflammation was remarkable at 7 days and reduced at 14 days, exhibiting histological findings similar to those obtained in SPF (Fig. 2C). No ulceration was observed in any lesion of any group.

Summary of differential display. A total of 60 displays with different primer sets were carried out. The reproducibility of the displayed pattern was confirmed by three individual amplifications using the same RNA source (data not shown). Most of the bands appeared in both small intestine and colon regardless of bacterial reconstitution. Forty-three bands were selected based on the criteria described and categorized in Table 2.

Detection of small intestine-specific gene induction in differential display. In the display with the combination of the primer GT15C and GTTGCGATCC, small intestine-specific bands, with enhancement at 3 days, were detected (Fig. 3A). The cDNA was 180 bp in length and was 99% homologous to nucleotides 259-438 of cryptdin-related sequence 4C (CRS4C). Northern blotting confirmed the preferential expression of CRS4C in the small intestine and induction at 3 days (Fig. 3B). In another display, with the combination of the primer GT15C and TGCTCTGCCC, a pattern similar to CRS4C was noticed. The cloned cDNA had a 99% homologous sequence to nucleotides 20-265 of cryptdin-17 (data not shown).

Cryptdin isoform mRNAs in murine intestinal epithelial cells. The amplified product for the cryptdin-4 isoform was of the expected size and was most strongly detected at 3 days, as demonstrated by semiquantitative RT-PCR (Fig. 4). Similar results were obtained from the gene amplification for cryptdin-1 and -5 isoforms (data not shown).

In situ study for cryptdin mRNA expression in ileum. When the ileum from GF mice was examined, a few cells, which were present at the bottom of the crypt, exhibited weak but positive staining for cryptdin-5 (Fig. 5A). In contrast, the number of positive cells increased and was approximately four or five per crypt, when we examined the ileum from 3-day bacteria-

Table 2. Preliminary results of differential display

<table>
<thead>
<tr>
<th>Gene Expression Site</th>
<th>Small Intestine-Specific</th>
<th>Colon-Specific</th>
<th>Both Small Intestine and Colon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Induction</td>
<td>3</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Suppression</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Constitutive</td>
<td>7</td>
<td>12</td>
<td>Many</td>
</tr>
</tbody>
</table>

Values are number of bands.

Fig. 3. Detection of cryptdin-related sequence 4C (CRS4C). A: differential display with the combination of primer GT15C and GTTGCGATCC using RNA from epithelial cells of the small intestine and the colon. The arrow indicates small intestine-specific bands, with enhancement at 3 days. Cloning and sequence analysis revealed that cDNA was homologous to CRS4C. 3, 7, 14: 3, 7, and 14 days after bacterial reconstitution. B: Northern blot analysis using the cloned cDNA as a probe. C: the blot was rehybridized with β-actin cDNA.
reconstituted mice (Fig. 5C). Similar results were obtained using the ileum from SPF mice. Positive cells were found only at the bottom of the crypt in the series of experiments. When the sense probe was used, positive staining was not detected in any of the samples (Fig. 5, B, D, and F).

Detection of colon-specific gene induction by differential display. In the display with the combination of primer GT15C and TCTGTGCTGG, colon-specific bands, with enhancement at 3, 7, and 14 days, were detected (Fig. 6A). Cloning and sequencing revealed that the cDNA was 160 bp in length and was 99% homologous to nucleotides 371–530 of murine serum amyloid A 1 (SAA1). Northern blotting using this clone as a probe confirmed preferential and induced expression of SAA1 gene in the colon (Fig. 6B).

Immunoreactivity for SAA in colon. To investigate the expression of SAA protein, an immunohistochemical technique was used. Only weak staining was observed in epithelial cells at the luminal surface of the
Fig. 6. Detection of serum amyloid A1 (SAA1). A: differential display, with the combination of primer GT15C and TCCTGTGCTGG, using RNA from epithelial cells of the small intestine and the colon. The arrow indicates colon-specific bands, with enhancement at 3, 7, and 14 days. Cloning and sequence analysis revealed that cDNA was homologous to SAA1. 3, 7, 14: 3, 7, and 14 days after bacterial reconstitution. B: Northern blot analysis using the cloned cDNA as a probe. C: the blot was rehybridized with β-actin cDNA.

rectum from GF and SPF mice (Fig. 7, A and C). In contrast, when GF mice were given luminal bacteria and reared under SPF conditions for 7 days, strong immunoreactivity was observed in the cytoplasm of the entire epithelium, from the top to around the bottom of the crypts (Fig. 7B). Similar results were obtained when the cecum and the transverse colon were examined (data not shown).

DISCUSSION

Bacterial reconstitution resulted in an acute, severe, but self-limited mucosal inflammation in mice. The trigger of mucosal inflammation was the first exposure of conventional bacteria in GF mice. This suggests that constitutively expressed molecules in GF mice failed to prevent inflammatory responses and maintain homeostasis. Although the ratio of each bacterial species in colonic microflora is different between mice after bacterial colonization and in SPF counterparts, the bacterial composition immediately becomes comparable within a few weeks (8, 19). Our preliminary data exploring four representative bacterial species have also shown transient increase of Enterobacteriaceae and Streptococcus in feces 3 and 7 days after bacterial reconstitution. In addition, Lactobacillus and Bacteroides were increased in number in the process (8). Conventionalized mice no longer exhibited mucosal inflammation at 2 wk regardless of the presence of conventional bacteria. This is critical because conventionalized mice with a genetically normal background could develop and maintain a protective system against luminal bacteria, although we did not identify the bacterial species responsible for triggering the mucosal inflammation and recovery.

This functional alteration possibly requires induction or suppression of genes to synthesize functional proteins, which are essential for the establishment of mucosal defense. To analyze this process, we applied differential display by using separately isolated small intestinal and colonic epithelial cells as RNA sources. Differential display is a recently described tool that detects altered gene expression (18). This approach has been quite successful in identifying a number of new genes (1, 5, 17, 27, 33, 37). Advantages of this method are the requirement of a small amount of RNA and the ease of visual comparison among many samples. This

Fig. 7. Immunohistochemical staining with anti-SAA antibody in the rectum (×100). 7 days, 7 days after bacterial reconstitution. Weak immunoreactivity was only observed in superficial epithelial cells at the luminal surface of the rectum in GF and SPF mice. Positive cells were observed in epithelial cells from the surface to the bottom of crypts at 7 days after bacterial reconstitution.
enables us to analyze at one time the RNA expression in small intestinal and colonic epithelial cells harvested at different time intervals. Although another novel approach, which uses cDNA array, is applicable for gene screening, this method does not easily compare gene composition in many samples (15).

In our 60 differential displays, most of the bands appeared in both small intestine and colon regardless of bacterial reconstitution. A total of 43 bands were selected as candidates for differentially expressed genes, and differential expression of CRS4C and SAA1 genes was confirmed in this study.

CRS4C was detected as a small intestine-specific gene. This molecule is one of the cryptdin-related sequences of unknown function (6, 12, 23). Cryptdins are constitutive members of the defensin family, and their selective expression in Paneth cells has been identified (24). To our knowledge, antimicrobial activities of cryptdins have been shown only in vitro (6, 23), and critical factors that induce cryptdin genes are still unknown. In particular, microbial antigens have not been considered as activators (4) because cryptdin mRNA in the small intestine of GF mice was as abundant as in conventional mice (22). In contrast, CRS4C and cryptdin-4 mRNAs were clearly induced at 3 days after bacterial reconstruction in small intestinal epithelial cells. The enhanced expression of cryptdin mRNA was caused by the increased number of expressing cells, demonstrated in part by in situ hybridization. Because the number of cryptdin mRNA-expressing cells was comparable between SPF and bacteria-reconstituted mice, differential mRNA expression detected by Northern blot and RT-PCR suggests that the degree of cryptdin mRNA expression in each cell may be different between the two groups. Although induction mechanisms of cryptdins remain unknown, antibacterial peptide genes were actually inducible in association with luminal bacteria in vivo. This observation also suggests that our approach of displaying time-dependent gene expression provides more information than a random comparison of gene expression in GF and SPF conditions.

SAA1 was detected as a colon-specific gene with enhancement of its mRNA level at 3 and 7 days after bacterial reconstitution. Induction of SAA was also observed at the protein level, as demonstrated by immunohistochemistry. SAA1 is an acute-phase protein mainly produced in the liver (32). Because IL-1β, IL-6, and lipopolysaccharide (LPS) induced SAA gene in hepatic and other cells (16, 31), SAA1 might be induced locally by direct exposure to these cytokines and/or LPS. Therefore, this observation indicates that epithelial gene expression modulated by soluble factors is also detectable by the approach described here. Additionally, if SAA induction was mediated by soluble factors such as IL-1β, IL-6, and LPS, enhancement of epithelial SAA mRNA level might be relevant to other experimental colitis and human IBD because enhanced gene and protein expression of proinflammatory cytokines have been well established in mucosal inflammation (7). The functional properties of this molecule still remain unclear; however, the chemotactic functions of T cells, monocytes, or neutrophils have been reported (2, 34). Furthermore, the binding capacity of SAA to LPS may suggest a possible defensive role of the mucosa to a bacterial insult. In fact, the recombinant SAA1 inhibits cytokine secretion in LPS-stimulated mononuclear cells (unpublished data).

Our approach highlights several significant future directions. Although how the detected genes contribute to mucosal defense and the inflammatory process remains unknown, investigation of the expression of these genes in other experimental colitis models will likely be able to demonstrate the relevance and significance of the genes involved in mucosal inflammation. Gene expression of human homologues of selected genes may be easily investigated using epithelial RNAs from human IBD patients to show possible modulation of mucosal defense-associated genes (10, 20). We inoculated multiple bacterial species prepared from SPF mice into GF mice; however, it is also important to determine which bacterial species are essential for the induction or modulation of selected genes. Inoculation of a single species such as Bacteroides may contribute to an answer to this fundamental question. Moreover, this bacterial reconstitution model is also applicable to analysis of immune activation and the anergy of mucosal lymphocytes and macrophages against normal enteric bacteria.

In conclusion, CRS4C, cryptdin, and SAA1 have been identified as molecules associated with the mucosal defense system against luminal microorganisms and the mucosal inflammation triggered by them through the new approach described here. Further investigation should lead to a better understanding of the role of luminal bacteria in the generation and amplification of mucosal inflammation in experimental and human IBD.

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