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Recombinant human lactoferrin prevents NSAID-induced intestinal bleeding in rodents

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Abstract

Recombinant human lactoferrin (RHLF) was tested for its ability to prevent non-steroidal anti-inflammatory drug (NSAID)-induced intestinal injury in rats and mice. Acute and chronic models using indometacin, naproxen and diclofenac were used. Measurements were made of intestinal bleeding and inflammation. Orally administered RHLF was effective at preventing acute NSAID-induced increases in gut bleeding and myeloperoxidase activity. Oral RHLF was also effective at blocking some chronic manifestations of indometacin usage. Protection by RHLF of the intestinal tract from NSAIDs appears to be linked to attenuation of neutrophil migration to the intestine, and is independent of prostaglandins and nitric oxide. RHLF does not bind to the NSAID or interfere with the NSAID biological activity. We conclude that orally administered RHLF is effective at preventing NSAID-induced intestinal injury in rodents and should be investigated for this potential therapeutic use in man.

Introduction

Recombinant human lactoferrin (RHLF) is a glycoprotein with antibiotic, anti-inflammatory and immune-modulating activity that is identical to endogenous lactoferrin in its biological effects (Ward et al 1995; Conneely 2001; Brock 2002). To understand some of the biological actions of lactoferrin that may be important for its use as a pharmaceutical agent, our laboratory has tested RHLF for activity within the gastrointestinal tract. There are reported to be receptors for endogenous lactoferrin on the intestinal brush border of several mammalian species, including man (Mazurier et al 1985; Davidson & Lonnerdal 1988; Hu et al 1988; Kawakami & Lonnerdal 1991; Gislason et al 1993), although their function in the adult is not clear (Suzuki & Lonnerdal 2002). In the infant, lactoferrin in milk may promote growth and maturation of the gastrointestinal tract (Zhang et al 2001). In adult mice, we have found that RHLF can reduce gastritis induced by the bacterium *Helicobacter felis* (Dial et al 2000), an effect that may be related to its ability to inhibit *Helicobacter* growth (Miehlke et al 1996). The aforementioned properties of RHLF suggest it may be useful in protecting against challenges to the stomach or intestines.

The use of non-steroidal anti-inflammatory drugs (NSAIDs) carries with it an approximately three times greater risk of gastrointestinal bleeding and ulceration than non-use (Gabriel et al 1991; Wallace 1997). To further characterize the ability of RHLF to protect the gastrointestinal tract, we have utilized the rodent NSAID-injury model. In this model an NSAID induces enteropathy by direct topical injury to the gut, as well as by removal of cytoprotective prostaglandins through cyclooxygenase inhibition. If RHLF can protect the gastrointestinal tract against NSAID-induced injury, it may be clinically useful for chronic consumers of NSAIDs. Indeed, one clinical report (Troost et al 2003) suggests that RHLF can prevent an increase in intestinal permeability due to acute indometacin ingestion in man. Therefore, we have tested RHLF against three different NSAIDs using two animal species, to characterize the nature and the extent of protection against this drug-induced enteropathy.

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Materials and Methods

Materials

Purified recombinant human lactoferrin (5.5% iron content) was supplied by Agennix Inc. (Houston, TX). All other chemicals were purchased from Sigma Chemical Co. (St Louis, MO).

Animals

Adult male Sprague-Dawley rats, 150–225 g, were used. They were obtained from Harlan Sprague-Dawley Inc. (Houston, TX) and acclimated at least one week before use. Adult male CD-1 mice, 25–30 g, were obtained from Charles River Laboratories (Wilmington, MA). The rodents were housed in approved facilities with a 12-h light–dark schedule. All animal manipulations were approved by The University of Texas Health Science Center at Houston Animal Welfare Committee.

Acute NSAID injury

For the indometacin model in rats, at zero time and seven hours later, fasted rats were administered a control protein (bovine albumin, orally) or RHLF at 200mg kg^{-1}, orally or intraperitoneally. This dose of RHLF was found previously to be effective in-vivo (Dial et al 2000). This was followed one, three and seven hours later by an intraperitoneal injection of L-NAME (20 mg kg^{-1}), a non-specific nitric oxide synthase inhibitor, to enhance the NSAID injury (Lichtenberger et al 1995). At two hours after the first dosing, the rats were given indometacin at 10 mg kg^{-1}, orally. Rats were euthanized 24 h after the initial dosing. At that time the distal half of the small intestine was excised and flushed with 10 mL of water that was later analysed for haemoglobin content as an index of bleeding. Ileal tissue (a 10-cm segment starting 10 cm proximal to the caecum) was dissected, frozen and later analysed for myeloperoxidase (MPO) activity as an index of inflammation. For alternate NSAID models using naproxen and diclofenac, identical treatments as above were performed except that naproxen (40 mg kg^{-1}) and diclofenac (30 mg kg^{-1}) were substituted for indometacin.

For the indometacin model in mice, at zero time the mice were administered vehicle (phosphate-buffered saline (PBS), orally) or RHLF at 200 mg kg^{-1}, orally or intraperitoneally. Two hours later they were injected with indometacin at 10 mg kg^{-1}, orally. They were euthanized 24 h after the first dosing. The distal half of the small intestine was excised and flushed with 2 mL of water for analysis of haemoglobin. The small intestine from 2 to 7 cm proximal to the caecum was excised and analysed for MPO activity.

Chronic NSAID injury

A chronic model of indometacin injury in rats was utilized. Rats were treated daily for four days with either vehicle (PBS) or RHLF (30, 100, 200 or 400 mg kg^{-1}, orally), followed by indometacin (5 mg kg^{-1}, i.p.). On the fifth day, the rats were weighed and euthanized, blood was collected for haematocrit readings, the intestines were examined for the presence of adhesions and the tail region was visually examined for faeces as evidence of diarrhoea. Surface hydrophobicity of ileal mucosa was estimated by contact angle analysis as previously described (Lichtenberger et al 1995). Briefly, the tissue was collected, rinsed in saline, spread on a glass slide with the mucosal side up and air-dried for 30 min before reading the contact angle on a Rame-Hart Inc. goniometer (Mountain Lakes, NJ). Because of limitations on the number of rats that could be handled at one time, the study was divided into two parts with the lower and higher doses of RHLF being used in the first and second parts, respectively.

Binding to indometacin

Potential binding between RHLF and indometacin was detected by incubation of PBS (vehicle), 15 μM RHLF or 15 μM bovine albumin (control protein) with 70 μM indometacin spiked with 40 000 counts min^{-1} of ^{14}C-indometacin (New England Nuclear, Boston, MA). After 16 min (the approximate time for fluid to empty from the stomach), the solutions were centrifuged at 5000 *g* in YM-30 filter units (Amicon/Centricon, Beverly, MA) to separate smaller molecules with a molecular weight < 30 000 daltons (i.e., PBS and free indometacin) that appear in the filtrate from larger molecules (i.e., RHLF and albumin). The filtrate was counted for radioactivity and the results were expressed as percent of PBS control (100% unbound).

Analgesia test

Pain was assessed in rats that had had an inflammation induced in one hind paw by injection of 0.2 mL of Freund's Complete Adjuvant under the skin of the paw three days earlier. On the day of the pain test, rats were treated with either saline (control) or RHLF at 200 mg kg^{-1}, orally. Then 30 min later, they received either saline or indometacin at 10 mg kg^{-1}, orally. After another two hours the rats were placed in a Randall-Sellito apparatus (IITC Inc., Woodland Hills, CA) to detect the pressure threshold (in mmHg) at which they experienced discomfort (vocalization or paw extension or withdrawal) on their inflamed and control non-inflamed paws. This technique has been described in more detail in a previous publication (Lichtenberger et al 1996).

Biochemical assays

Haemoglobin (Hb) was assayed by a modification of the method of Crosby & Furth (1956), as described previously (Lichtenberger et al 1983). All samples were in a volume of 10 mL, and results are expressed as a concentration ((mg Hb) mL^{-1}). Myeloperoxidase activity (MPO) was detected by absorbance changes in the substrate tetramethylbenzidine as previously described (Lichtenberger

et al 1996). Results are expressed as units of MPO per mg of protein. Protein was assayed by dye binding with Bio-Rad protein reagent (Bio-Rad, Hercules, CA) using bovine albumin as a standard.

Statistical methods

The differences between experimental groups were evaluated by analysis of variance followed by the Fisher LSD test. For comparison of ratios, the Fisher exact test was used. The level of significance was set at $P < 0.05$ for all tests.

Results

Acute NSAID injury

In the rat, a single dose of indometacin coupled with nitric oxide synthase inhibition induced acute, significant bleeding into the gastrointestinal lumen and an increase in neutrophil migration (MPO activity) to the intestine (Figures 1A and 1B). These effects were almost completely prevented by pretreatment of the rats with orally administered RHLF. Intraperitoneally administered RHLF was less effective than the oral dosing and, unlike orally administered protein, the differences did not reach statistical significance versus BSA/indometacin values. Use of other NSAIDs, naproxen and diclofenac, as injurious agents induced significant bleeding into the intestine similar to indometacin (Table 1), but without a detectable effect on MPO activity (not shown). Again, orally administered RHLF reduced the bleeding induced by those NSAIDs, and was more effective than intraperitoneally administered RHLF.

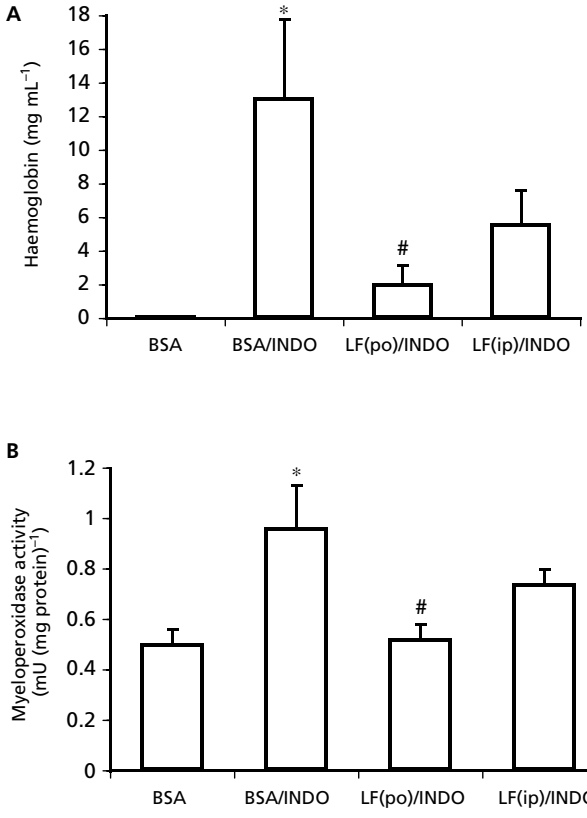

Figure 1 Effect of RHLF on acute indometacin-induced intestinal injury in rats. Rats were treated as described in the Methods. Rinses of the distal small intestinal lumen were analysed for haemoglobin (A) and ileal tissue was analysed for myeloperoxidase (MPO) activity (B). BSA, bovine serum albumin; INDO, indometacin; LF(po), oral RHLF; LF(ip), intraperitoneal RHLF. Values are expressed as the mean ± s.e.m. for 4–8 rats per group. $^{*}P < 0.05$ vs BSA control; $^{\#}P < 0.05$ vs BSA/INDO.

Table 1 Effect of RHLF on naproxen and diclofenac-induced intestinal bleeding in the rat

Treatment	Naproxen	Diclofenac
Saline/saline	0.4 ± 0.4	0.5 ± 0.5
Saline/NSAID	17.7 ± 3.6*	53.1 ± 16.3*
RHLF (p.o.)/NSAID	5.4 ± 3.6$^{\#}$	20.9 ± 5.5
RHLF (i.p.)/NSAID	7.7 ± 3.2	34 ± 4.3

Rats were treated orally with either saline or RHLF (200 mg kg^{-1}), followed by the NSAID as described in Materials and Methods. Flushes of the distal small intestine were analysed for haemoglobin. Values are expressed as the mean ± s.e.m. in units of (mg Hb) mL^{-1}. All rats had the same total flush volume of 10 mL. $^{*}P < 0.05$ vs saline/saline; $^{\#}P < 0.05$ vs saline/NSAID, n = 8 or 9 per group for naproxen and 2–4 per group for diclofenac.

In the mouse, a single dose of indometacin without nitric oxide synthase inhibition induced bleeding into the gastrointestinal lumen (Figure 2A). While MPO activity increased after indometacin, the value did not reach statistical significance (Figure 2B). Both orally and intraperitoneally administered RHLF significantly prevented the indometacin-induced bleeding, and had a tendency to blunt the increase in intestinal MPO activity.

Chronic NSAID injury

In the chronic model of indometacin-induced injury, the NSAID caused different types of injury to be manifested compared with the acute model (Table 2). There was an indometacin-induced reduction in body weight, and appearance of intestinal adhesions and diarrhoea in all rats. The effects of indometacin on haematocrit as evidence of bleeding and on ileal contact angle as evidence of surface damage were variable, and generally these parameters were reduced. Co-treatment with RHLF resulted in a dose-dependent reversal of body-weight loss and a reduction in the incidence of diarrhoea. RHLF returned ileal contact angles to normal and modestly reversed haematocrit changes at higher RHLF doses. There was no apparent effect of RHLF to block formation of intestinal adhesions.

Binding to indometacin

Duplicate tubes, containing radiolabelled indometacin and either PBS, RHLF or BSA, were incubated and passed

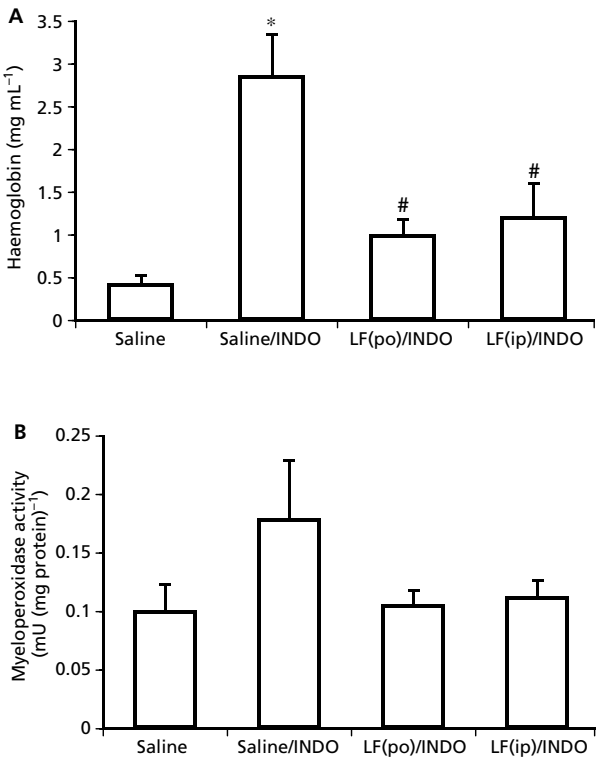

Figure 2 Effect of RHLF on acute indometacin-induced intestinal injury in mice. Mice were treated as described in the Methods. Rinses of the distal small intestinal lumen were analysed for haemoglobin (A) and ileal tissue was analysed for myeloperoxidase (MPO) activity (B). INDO, indometacin; LF(po), oral RHLF; LF(ip), intraperitoneal RHLF. Values are expressed as the mean ± s.e.m. for 7–10 mice per group. $^{*}P < 0.05$ vs saline control; $^{\#}P < 0.05$ vs saline/INDO.

through molecular sizing filters. The filtrates were counted and the results showed less than 3% variability between duplicates. The results showed that, with PBS (control) set at 100%, RHLF = 97% and BSA = 30% of control. These data suggest that indometacin did not bind to RHLF, whereas 70% of the NSAID did bind to BSA.

Analgesia test

The Randall-Sellito apparatus demonstrated that the inflamed rat paw was significantly more sensitive to nociceptive effects of pressure than the un-inflamed paw (Figure 3). Treatment with RHLF alone did not significantly affect the pain threshold of inflamed paws when compared with those of saline-treated rats. However, indometacin alone had a prominent and statistically significant effect in increasing the pain sensitivity threshold of inflamed paws, signifying the expected analgesic effect. The combination of RHLF with indometacin also provided pain relief, similar to indometacin alone. In other words, RHLF did not interfere with indometacin's analgesic activity.

Discussion

These studies support the possible use of recombinant human lactoferrin to prevent non-aspirin NSAID-induced intestinal bleeding, the major and limiting side effect of this class of drugs. RHLF was very effective against acute indometacin-, naproxen- and diclofenac-induced injury. RHLF was also effective against most of the manifestations of chronic indometacin injury, even though this model expressed more severe injury than the acute model. Whether RHLF will be effective against aspirin also, which typically injures the gastric mucosa, will remain for future studies to determine.

The mechanism by which RHLF protects the gut against these NSAIDs appears to be related to several factors. First, RHLF is more active when administered orally than parenterally in our studies, suggesting that RHLF may be acting directly or indirectly on the mucosal rather than the serosal surface. It could do this in several ways. RHLF could be acting as an antibiotic in the gut and prevent the overgrowth of Gram-negative bacteria that can colonize an injured area and exacerbate damage and prolong healing (Elliot et al 1998). This would be an indirect action on the mucosa by a direct action on the

Table 2 Effect of RHLF on chronic indometacin-induced injury in the rat

Treatment	Body weight (g)	Haematocrit	Intestinal adhesions	Ileal contact angle	Diarrhoea
Saline	256 ± 4	0.54 ± 0.01	0/10 (0%)	37 ± 2	0/10 (0%)
Indometacin	239 ± 2*	0.37 ± 0.02*	9/9 (100%)*	25 ± 3*	9/9 (100%)*
RHLF30/indometacin	244 ± 4	0.37 ± 0.02*	8/10 (80%)*	36 ± 2$^{\#}$	4/10 (40%)*$^{\#}$
RHLF100/indometacin	240 ± 6*	0.37 ± 0.02*	9/10 (90%)*	35 ± 4$^{\#}$	7/10 (70%)*
Saline	208 ± 2	0.39 ± 0.05	0/5 (0%)	33 ± 2	0/5 (0%)
Indometacin	143 ± 6*	0.26 ± 0.05	4/4 (100%)*	31 ± 3	4/4 (100%)*
RHLF200/indometacin	173 ± 7*$^{\#}$	0.32 ± 0.04	4/5 (80%)*	30 ± 3	2/5 (40%)
RHLF400/indometacin	173 ± 4*$^{\#}$	0.33 ± 0.06	4/5 (80%)*	40 ± 6	1/5 (20%)$^{\#}$

Rats were treated for four days with saline, RHLF and indometacin as described in the Materials and Methods. The doses of RHLF as indicated are in units of mg kg^{-1}. Values are expressed as the mean ± s.e.m. or as the number positive/total and its percentage. $^{*}P < 0.05$ vs saline; $^{\#}P < 0.05$ vs indometacin.

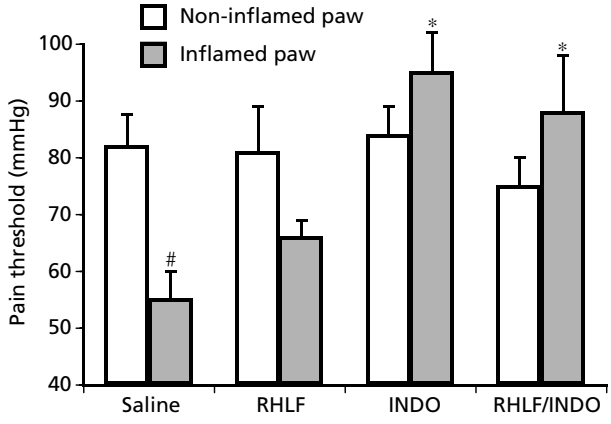

Figure 3 Effect of RHLF on indometacin-induced analgesia in rats. Rats with one inflamed hind paw were treated orally with either saline, RHLF (200 mg kg^{-1}), indometacin (10 mg kg^{-1}) or RHLF plus indometacin and tested for sensitivity to pressure/pain on their non-inflamed and inflamed paws. INDO, indometacin. Values are expressed as the mean ± s.e.m. for 4 or 5 rats per group. $^{*}P < 0.05$ vs saline control inflamed paw; $^{\#}P < 0.05$ vs saline control non-inflamed paw.

microbial flora. An example of such antibiotic activity was reported by Nebermann et al (2001), who showed that bovine lactoferrin administered directly to the duodenum prevented *E. coli* translocation to the peritoneum and mesenteric lymph nodes. RHLF could also be acting directly on intestinal crypt cells to induce growth, as this action has been previously ascribed to this protein (Nichols et al 1987, 1989).

A major factor involved in the gastrointestinal protective actions of RHLF is its ability to prevent neutrophil migration to the area of injury. This was seen as a reduction in MPO activity in ileal tissue. The pathogenesis of indometacin-induced injury to the gastrointestinal tract is thought to involve neutrophil activation and migration (Wallace et al 1990; Nygard et al 1994; Beck et al 2000), so that any interference with this process could be beneficial. A recent report (Stadnyk et al 2002) has shown that rat intestinal epithelial cells are capable of responding to indometacin by producing cytokines that can attract neutrophils. It is also reported that lactoferrin can suppress the pro-inflammatory cytokine tumour necrosis factor-α (TNFα) (Machnicki et al 1993) and promote the anti-inflammatory cytokine IL-18 in the gut (Kuhara et al 2000). We therefore speculate that, when administered orally, RHLF may alter intestinal cytokine production and prevent neutrophil recruitment. Future studies will attempt to address this issue. In addition, RHLF may also stimulate cellular responses such as M cell sampling (Furness et al 1999), mobilization of immune cells such as dendritic cells, or have actions on cell pathways that are yet to be identified. Regardless of how it is acting, the prevention of neutrophil migration by RHLF should prevent much of the reactive oxidant injury that is characteristic of enhanced neutrophil presence.

A number of studies have shown that bovine lactoferrin and RHLF are fully degraded in the adult gastrointestinal tract (Kuwata et al 2001; Troost et al 2002), with no intact lactoferrin reaching either the colon or the portal circulation (Wakabayashi et al 2004). However, it has also been shown that as much as 60% of lactoferrin may survive transit through the stomach and be emptied into the upper small intestine (Troost et al 2001). Any systemic effect of lactoferrin is most likely mediated through changes at the level of the gut. However, we cannot yet discriminate between an effect due to intact lactoferrin or one or more of its peptide fragments.

Another potential factor that must be considered in a question of gastrointestinal protection is the role of prostaglandins and nitric oxide, two endogenous mediators known for their beneficial actions on the gastrointestinal tract, particularly the stomach (Wallace 1997). In the case of NSAID-induced injury, at the doses used in these studies, it can be assumed that all cyclo-oxygenases were inhibited and that endogenous prostaglandin levels were extremely low. Similarly, the use of L-NAME, a non-specific nitric oxide synthase inhibitor, would block all NO synthases and prevent the formation of nitric oxide. Despite the inhibition of these two gastrointestinal protective mediators, RHLF was active and protective to the intestinal tissue. This action implies an effect that is totally independent of prostaglandins and nitric oxide.

Still another factor to consider with regard to RHLF protection is whether the protein simply binds to the NSAID and prevents its action, thereby preventing any side effect. To answer that question, studies on the binding of RHLF to indometacin were performed. No significant binding of RHLF to indometacin was detected when binding was easily detected between the NSAID and albumin, a known NSAID-binding agent (Honore & Brodersen 1984). Thus, it does not appear that there is interference with NSAID action by RHLF. To confirm this supposition, a study in which the biological activity of indometacin was measured (pain relief) was performed in the presence and absence of RHLF. In this study, RHLF did not alter the analgesic action of indometacin. Thus, at the doses tested, RHLF's intestinal protective effect occurs without diminishing the analgesic activity of the NSAID.

We have now demonstrated the same protective effect of RHLF in two animal species, the mouse and the rat. A similar protective effect after oral dosing has also been seen in man (Troost et al 2003). These findings in three different species strongly support the likely efficacy of RHLF in providing protection against gastrointestinal injury due to NSAIDs.

Conclusions

RHLF has the ability to prevent intestinal bleeding induced by NSAIDs in rodents. The mechanism by which RHLF accomplishes this action appears to relate to its ability to prevent neutrophil migration to the gut. In addition, accumulated data suggest that RHLF action could also relate to other properties, including its local antibiotic effect and its possible stimulation of intestinal components of the

immune system. Considering that NSAID usage is rising worldwide as more indications for this class of drugs are found, one would expect the incidence of gastrointestinal bleeding to also increase. Therefore, further investigations are warranted into the use of RHLF to prevent this serious drug-induced complication.

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