Role of *Listeria monocytogenes* Exotoxins Listeriolysin and Phosphatidylinositol-Specific Phospholipase C in Activation of Human Neutrophils

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Polymorphonuclear leukocytes (PMN) are essential for resolution of infections with *Listeria monocytogenes*. The present study investigated the role of the listerial exotoxins listeriolysin (LLO) and phosphatidylinositol-specific phospholipase C (PlcA) in human neutrophil activation. Different *Listeria* strains, mutated in individual virulence genes, as well as purified LLO were used. Coincubation of human neutrophils with wild-type *L. monocytogenes* provoked PMN activation, occurring independently of phagocytosis events, with concomitant elastase secretion, leukotriene generation, platelet-activating factor (PAF) synthesis, respiratory burst, and enhanced phosphoinositide hydrolysis. Degranulation and leukotriene formation were noted to be solely dependent on LLO expression, as these features were absent when the LLO-defective mutant EGD— and the avirulent strain *L. innocua* were used. These effects were fully reproduced by a recombinant *L. innocua* strain expressing LLO (INN+) and by the purified LLO molecule. LLO secretion was also required for PAF synthesis. However, wild-type *L. monocytogenes* was more potent in eliciting PAF formation than mutants expressing LLO, suggesting the involvement of additional virulence factors. This was even more obvious for phosphoinositide hydrolysis and respiratory burst; these events were provoked not only by INN+ but also by the LLO-defective mutant EGD— and by a recombinant *L. innocua* strain producing listerial PlcA. We conclude that human neutrophils react to extracellularly provided listerial exotoxins by rapid cell activation. Listeriolysin is centrally involved in triggering degranulation and lipid mediator generation, and further virulence factors such as PlcA apparently contribute to trigger neutrophil phosphoinositide hydrolysis and respiratory burst. In this way, listerial exotoxins may influence the host defense against infections with *L. monocytogenes*.

The host response to *Listeria monocytogenes*, a facultative intracellular bacterial pathogen, can be divided into two stages (20, 34). Early nonspecific resistance is thought to be mediated primarily by resident macrophages, in particular Kupffer cells of the liver. A subsequent resistance-specific stage, required for the complete resolution of infection, depends on the generation of specific T-cell-mediated immunity. Several studies have, however, demonstrated that polymorphonuclear neutrophilic granulocytes (PMN) are essential for both stages of host defense. PMN function to lyse *Listeria*-infected parenchymal cell, thereby exposing the bacteria to professional phagocytes such as the neutrophils themselves, and T-cell-mediated immunity to *Listeria* organisms is incomplete in the absence of PMN (2, 12, 14, 15, 33, 35).

Neutrophils are well equipped for acute lysis of infected parenchymal cells and destruction of listeriae (25, 38). Their NADPH oxidase system generates superoxide anion and derived oxygen radicals, their granules contain a variety of proteolytic enzymes capable of attacking cellular proteins and connective tissue components, and they release different lipid mediators, such as leukotriene B4 (LTB4) and platelet-activating factor (PAF), known to effect further PMN recruitment. Previous studies addressing PMN- *L. monocytogenes* interaction in vitro noted that upregulation and secretion of the pro-inflammatory cytokines interleukin-1β, interleukin-6, and tumor necrosis factor occurred within hours in the neutrophils in contact with listeriae (3).

This investigation addressed the acute phase of PMN activation by *L. monocytogenes*, with particular focus on the role of listerial exotoxins. Among the latter, listeriolysin (LLO), a member of sulfhydryl-activated pore-forming toxins and thus far the best-characterized virulence factor of listeriae, and phosphatidylinositol-specific phospholipase C (PlcA) were recently recognized to be potent inducers of endothelial cell signalling events (36, 37) prior to cell invasion. The pathogenic wild-type *L. monocytogenes* is here reported to provoke oxidative burst, degranulation, generation of leukotrienes and PAF, and enhanced phosphoinositide hydrolysis-related signalling events in human neutrophils within a few minutes, independent of phagocytosis events. Employing genetically engineered strains of *L. monocytogenes* and *L. innocua*, used as the host for selective expression of exotoxins, as well as purified exotoxins, we identified listeriolysin as a key agent in human neutrophil activation. Efficacious stimulation of these phagocytes, however, requires additional listerial virulence factors, of which PlcA may play a prominent role.

**MATERIALS AND METHODS**

**Materials.** Arachidonic acid, superoxide dismutase, cytochrome c type IV, bovine serum albumin (BSA), and HEPES were purchased from Sigma (Deisenhofen, Germany). The protein kinase C inhibitor staurosporine was obtained from Calbiochem (Giessen, Germany). The PAF antagonist BN50727 was kindly provided by P. Braquet (Henri Beaufour Institute, Paris, France). S-2484, a substrate for neutrophil elastase, was purchased from Kabi-Vitrum (Stockholm, Sweden).

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Sweden). RPMI 1640 medium, Hank’s balanced salt solution (HBSS), brain heart infusion (BHI), erythromycin, and fetal calf serum were from Gibco Laboratories (Grand Island, N.Y.). Percoll was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). The leukotrienes LTC₄, LTD₄, LTE₄, LTB₄, OH-LTB₄, and COOH-LTB₄ and the synthetic LTα were a generous gift from J. Rokach, Merck Frosst (Toronto, Ontario, Canada). Additions of hemolytic toxins were graciously supplied by W. Bartmann, Hoechst AG (Frankfurt, Germany). Purified LLO migrated as a 58-kDa band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and was judged to be greater than 95% pure.

Bacterial strains. Table 1 describes the Listeria strains used in this study. Recombinant strains of L. monocytogenes and L. innocua were obtained as previously described (9). The apathogenic L. innocua strain was used as the host for selective expression of the LLO (hly) or plcA gene. To induce high levels of either protein from the recombinant strain, the hly and plcA genes were cloned onto a plasmid also harboring the prfA regulator. Bacteria were grown in BHI broth at 37°C, and erythromycin (5 μg/ml) was used where appropriate. The hemolysin assay was performed as described previously (9) except that human erythrocytes were incubated with 40 mM phosphate buffer (pH 5.0). LLO eluted as a sharp peak at 200 to 260 m with a Mono S HR5/5 column and eluted with a linear gradient of 50 to 500 mM NaCl in 20 mM sodium phosphate buffer to obtain PMN concentrations of 10⁶ cells/ml. Thus, approximately 3.5 × 10⁶ bacteria were obtained as 10⁶ bacteria were obtained (27) to yield a PMN fraction containing 2% fetal calf serum plus 40 mM HEPES buffer (pH 7.4). Then bacteria were spun at 3,000 × g for 2 min. Serumotonia was determined by liquid scintillation counting and related to that released from the same volume of platelet suspension after cell lysis with Triton X-100 (final concentration, 0.85% [v/vol]). Known quantities of PAF were used to establish a calibration curve for the bioassy. Counts of each sample were used to ascertain the specificity of platelet secretion by the inhibitory effect of the PAF receptor antagonist BN50727 (1 μM).

Release of granule constituents and LDH. Elastase was taken as marker for neutrophil degranulation and enzyme activity in the cell supernatant was measured according to standard procedures (26, 29). LDH, as a marker for overt cytotoxicity, was quantified by a colorimetric technique. Enzyme release was expressed as percentage of total enzyme activity liberated in the presence of 100 μg of superoxide dismutase per ml.

Superoxide generation. PMN O₂⁻ generation was measured as superoxide dimutase-inhibitable reduction of cytochrome c as described elsewhere (10). Duplicate reaction mixtures containing neutrophils (10⁶ to 10⁵ PMN/ml) and 75 μg ferricytochrome c were incubated at 37°C in the presence or absence of 10 μg of superoxide dismutase per ml.

Phosphoinositide metabolism. The phosphodiesterase activity of stimulated phospholipids was investigated by measuring the incorporation of inositol phosphate into isolated phospholipids. LLO was admixed to the HBSS containing 100 mM HEPES and 0.5% BSA, Bacteria were washed twice and suspended in HBSS containing 100 mM HEPES and 0.5% BSA, followed by a modification of the method of Nadeau et al. (31). The mobile phase used for reverse-phase HPLC was methanol-water-acetic acid, 66:34:0.16 (pH 4.9) for 5 min, followed by a linear gradient to 50 to 500 mM NaCl in 20 mM sodium phosphate buffer to obtain PMN concentrations of 10⁶ cells/ml. Approximately 3.5 × 10⁶ bacteria were obtained by centrifugation in a discontinuous Percoll gradient (27) to yield a PMN fraction containing 2% fetal calf serum plus 40 mM HEPES buffer (pH 7.4). Then bacteria were spun at 3,000 × g for 2 min. Serumotonia was determined by liquid scintillation counting and related to that released from the same volume of platelet suspension after cell lysis with Triton X-100 (final concentration, 0.85% [v/vol]). Known quantities of PAF were used to establish a calibration curve for the bioassy. Counts of each sample were used to ascertain the specificity of platelet secretion by the inhibitory effect of the PAF receptor antagonist BN50727 (1 μM).

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Preparation of human granulocytes. Heparinized blood from healthy donors was centrifuged in a discontinuous Percoll gradient (27) to yield a PMN fraction of approximately 97% purity. Prior to experiments, PMN were kept in RPMI 1640 with 10% fetal calf serum for 30 to 60 min at 37°C. Immediately before stimulus application, cells were washed twice and suspended in HBSS-HEPES buffer to obtain PMN concentrations of 10⁶ to 10⁵ PMN/ml. Cell viability, as assessed by trypan blue exclusion, ranged above 96%, and lactate dehydrogenase (LDH) release was consistently below 3%. The apathogenic L. innocua strain was used as the host for selective expression of the LLO (hly) or plcA gene. To induce high levels of either protein from the recombinant strain, the hly and plcA genes were cloned onto a plasmid also harboring the prfA regulator. Bacteria were grown in BHI broth at 37°C, and erythromycin (5 μg/ml) was used where appropriate. The hemolysin assay was performed as described previously (9) except that human erythrocytes were incubated with 40 mM phosphate buffer (pH 5.0). LLO eluted as a sharp peak at 200 to 260 m with a Mono S HR5/5 column and eluted with a linear gradient of 50 to 500 mM NaCl in 20 mM sodium phosphate buffer to obtain PMN concentrations of 10⁶ cells/ml. Thus, approximately 3.5 × 10⁶ bacteria were obtained.

Incubation of PMN with purified LLO. LLO was admixed to the HBSS containing 20 mM HEPES and 100 mM LiCl (10⁵ PMN/ml). At different times after stimulus application, samples were quenched with trichloroacetic acid (final concentration, 7.5%), kept on ice for 15 min, and extracted four times with dichloromethane. Neutral lipids were then washed with sodium sulfate, 80 and processed to separate neutral lipids on Dowex anion-exchange columns as described by Berridge et al. (41). Under these assay conditions, cyclic nucleotides were quantitatively separated on HPLC, and the peak representing cAMP was used as index of the total adenylate cyclase activity in the samples.

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TABLE 1. Listeria strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>relevant genotype</th>
<th>Abbreviation used</th>
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<tbody>
<tr>
<td>L. monocytogenes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGD</td>
<td>1/2a; wild type; +</td>
<td>EGD</td>
<td></td>
</tr>
<tr>
<td>EGDhlyl1</td>
<td>1/2a; hly; --</td>
<td>EGD</td>
<td></td>
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<tr>
<td>L. innocua</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11288/pERL3</td>
<td>6a; wild type;</td>
<td>INN</td>
<td></td>
</tr>
<tr>
<td>11288/pERL3</td>
<td>6a; plcA hly; +</td>
<td>INN</td>
<td></td>
</tr>
<tr>
<td>11288/pERL3</td>
<td>6a; plcA hly; +</td>
<td>INN</td>
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* Hemolytic phenotypes observed on sheep blood agar plates were scored as follows: +, strongly hemolytic; +, weakly hemolytic; --, nonhemolytic.
barbed end-capping protein and effecting selective loss of the nonmuscle F-actin content (27), was graciously provided by K. Aktories, Freiburg, Germany. It was provided to the neutrophils at concentrations of 400 (C2I) and 800 (C2II) ng/ml 30 min before granulocyte-bacterium coincubation. Pilot experiments ascertained that any phagocytosis is fully inhibited in these cells.

Statistics. For statistical comparison, one-way analysis of variance was performed. A P level of 0.05 was considered significant.

RESULTS

Elastase secretion. Incubation of human neutrophils with wild-type L. monocytogenes (EGD+) caused a rapid secretion of elastase, plateauing after 10 to 15 min (Fig. 1). In contrast, neither the apathogenic strain L. innocua (INN−), which is nonhemolytic and noninvasive, nor an isogenic strain of L. monocytogenes (EGD−), which produces a truncated nonhemolytic 40-kDa polypeptide, provoked substantial elastase secretion. Protease liberation was reproduced by purified LLO in the absence of bacteria, and the most prominent elastase secretion occurred when neutrophils were incubated with L. innocua engineered to overexpress LLO (INN+). Use of L. innocua as a host to express phospholipase C (INN-PlcA) did not result in significant release of elastase. Microscopic examination of the neutrophil-EGD+ cocultures did not reveal substantial listerial phagocytosis within the 15-min coincubation period (ingestion of bacteria estimated to be <5%). To exclude any impact of phagocytosis events on elastase secretion, additional experiments with botulinum C2 toxin-preincubated neutrophils were performed. Coincubation of these cells with EGD+ resulted in elastase concentrations of 3.3 ± 0.2 U/liter (mean ± standard error of the mean [SEM]; n = 5) within 15 min, consistent with corresponding data for non-C2-toxin-treated PMN (3.2 ± 0.2 U/liter). LDH release in response to EGD+, EGD−, INN-PlcA, INN−, and LLO was less than 5%; release in response to INN+ was less than 15%.

Lipid mediator generation. Incubation of PMN with EGD+ induced the synthesis of large amounts of LTB4, 20-OH- and 20-COOH-LTB4, as well as LTA4 decay products (Fig. 2A to C). Product release peaked within 10 min after this challenge. The LLO-negative strains EGD− and INN− failed to activate this metabolic response. Leukotriene formation was fully restored when L. innocua was engineered to express LLO, and it was

FIG. 1. Neutrophil elastase secretion evoked by various bacterial strains. PMN (10⁷) were incubated with EGD+, EGD−, INN+, INN−, or INN-PlcA (for each strain, 3.5 × 10⁶ bacteria/ml) or with purified LLO (1 µg/ml) for 10 min. Means ± SEM of five independent experiments each are given.

FIG. 2. Generation of leukotrienes in response to various bacterial strains. Neutrophils (10⁷ PMN/ml) were simultaneously exposed to free arachidonic acid (10 µM). Incubation was terminated after 10 min. LTB4, 20-OH-LTB4, and 20-COOH-LTB4 are indicated as LTB4 in panel A, nonenzymatic hydrolysis products of LTA4 are summarized as LTA4 decay in panel B, and the sum of data for leukotrienes is shown in panel C. Dose-dependent generation of leukotrienes in response to purified LLO is demonstrated in panel D. Means ± SEM of six independent experiments each are given.
similarly noted in the presence of purified LLO (Fig. 2D), with an optimum concentration of 1 μg/ml. In contrast, no significant leukotriene synthesis was noted in the presence of INN-PlcA.

Incubation of PMN with EGD caused marked PAF liberation (Fig. 3), which plateaued after 5 min. In contrast to elastase secretion and leukotriene formation, this effect was only partially reproduced when PMN were incubated with INN+ or purified LLO. EGD– and INN– did not activate PAF synthesis.

Respiratory burst. Respiratory burst, measured as O₂⁻ release, was induced in PMN incubated with wild-type EGD as well as LLO-expressing L. innocua (Fig. 4). Comparable O₂⁻ release did also occur in the presence of EGD–, which failed to express LLO. INN-PlcA, engineered to produce high levels of PlcA, provoked respiratory burst to a minor extent. In contrast, INN– was largely ineffective. In the presence of the protein kinase inhibitor staurosporine, O₂⁻ release was suppressed to baseline levels under all experimental conditions used.

Phosphoinositide metabolism. Neutrophil coincubation with EGD+ caused rapid-onset phosphatidylinositol hydrolysis, with a maximal accumulation of inositol phosphates after approximately 5 min (Fig. 5). Incubation of PMN with INN+ induced a strong phosphatidylinositol hydrolysis response, even surpassing that evoked by EGD+. While INN– was unable to activate this pathway in neutrophils, the LLO-negative isogenic strain of L. monocytogenes, EGD–, evoked inositol phosphate accumulation to the same extent as wild-type L. monocytogenes. A corresponding response was provoked by INN-PlcA.

**DISCUSSION**

Coincubation of human neutrophils with wild-type L. monocytogenes provoked rapid-onset PMN activation, occurring independently of phagocytosis events, with elastase secretion, leukotriene generation, PAF synthesis, respiratory burst, and enhanced phosphoinositide hydrolysis representing prominent features. The use of recombinant listeriae producing different exotoxins and purified toxin allowed us to dissect the role of listerial exotoxin secretion in PMN stimulation. Thus, the processes of degranulation and leukotriene formation were noted to be exclusively dependent on LLO expression. These features were absent when LLO-defective mutants were used, and they were fully reproduced by an avirulent recombinant expressing LLO and by purified LLO in the absence of bacteria. Respiratory burst and neutrophil phosphoinositide metabolism were induced by overexpression of LLO as the sole exotoxic agent. However, triggering of these events by L. monocytogenes was noted to occur also in the absence of LLO, suggesting the role of additional listerial toxins, particularly PlcA. PAF synthesis was intermediate with respect to LLO dependency.

Incubation of human neutrophils with the virulent wild-type L. monocytogenes provoked rapid degranulation, as assessed by elastase secretion. In parallel, extensive leukotriene generation, indicating activation of the PMN 5-lipoxygenase pathway, was noted, with the appearance of both LTB4 and its omega oxidation products as well as the release of the unstable intermediate LTA₄, undergoing decomposition to various decay products in the extracellular space. These events were obviously not triggered by phagocytosis of the bacteria, as (i) hardly any ingestion of listeriae was noted within the short coincuba-
tion periods used, in accordance with previous observations on phagocytosis of *L. monocytogenes* by human neutrophils (15), (ii) *L. innocua* INN−, devoid of exotoxins, provoked no substantial elastase secretion or leukotriene generation within this time period, and (iii) complete inhibition of neutrophil phagocytic capacity due to blockage of its actin-based cytoskeleton by butulinum C2 toxin did not interfere with the elicited by EGD+ secretory responses. That both PMN elastase liberation and leukotriene synthesis were clearly related to LLO and dose-effect curves with purified LLO showed maximum overexpressing LLO compared to the PMN-EGD strain upon coincubation of neutrophils with the absence of bacteria. Third, dose-effect relationships were demonstrated: elastase release was clearly more prominent upon coincubation of neutrophils with the *L. innocua* strain overexpressing LLO compared to the PMN-EGD+ cocultures, and dose-effect curves with purified LLO showed maximum leukotriene generation at 1 µg of this exotoxin per ml.

This study did not address the question of the PMN signaling events via which both degranulation and 5-lipoxygenase activation occurred in the neutrophils in response to extracellularly provided LLO. As previously suggested for pore-forming toxins, the formation of a transmembrane aqueous channel might induce metabolic events by enabling an extracellular calcium shift (5, 6), and pilot experiments indeed showed marked inhibition of the LLO-induced elastase secretion by complexing extracellular calcium with EGTA (data not shown). Alternatively, as demonstrated for the activation of human neutrophils by the *Escherichia coli* hemolysin HlyA (23, 24), strong stimulation of the preformed phosphoinositide hydrolysis-related signal transduction pathway may also be due to LLO-induced activation of endogenous phospholipase C (see below), and this pathway is well known to be linked to degranulation and 5-lipoxygenase activation (18, 19). This view is supported by the fact that, concomitant with the different potencies observed in eliciting degranulation, the maximal accumulation of inositol phosphates was noted in the neutrophils treated with the LLO-overexpressing strain INN+. As this mutant is devoid of listerial phospholipase C and other phospholipases, the phosphoinositide hydrolysis in these experiments must derive from an activation of endogenous phospholipase C targeting phosphatidylinositol. The suggestion that LLO-induced neutrophil stimulation employs intracellular phospholipase C activation is also supported by the finding that the high LLO concentration of 10 µg/ml, which effects the maximum membrane perturbation, was less potent in eliciting leukotriene generation than 1 µg/ml. This observation is reminiscent of the bell-shaped dose-effect curves for *E. coli* HlyA on human neutrophil phosphoinositide hydrolysis (23, 24). Clearly, further studies are required to elucidate the intracellular signalling events in LLO-exposed human neutrophils in more detail.

As found for elastase and leukotriene secretion, neutrophil PAF synthesis was provoked by purified LLO and by the *L. innocua* strain expressing LLO and was absent in studies with the LLO-defective mutant EGD−. However, the maximum PAF response to the challenges using only LLO (INN+, purified toxin) was clearly inferior to the response provoked by the wild-type *L. monocytogenes*, suggesting that additional virulence factors of the wild-type strain cooperate with LLO. The role of such additional factors was even more obvious for the respiratory burst and for neutrophil inositol phosphate accumulation, as both features were provoked by the LLO-defective mutant EGD− to nearly the same extent as by the wild-type *L. monocytogenes* strain. Interestingly, in contrast to degranulation, leukotriene generation, and PAF synthesis, the *L. innocua* strain engineered to express the listerial phospholipase PlcA as the sole exotoxic agent displayed some intermediate potency to provoke both neutrophil inositol phosphate accumulation and respiratory burst. The close relationship between phosphoinositide hydrolysis and superoxide anion generation is also supported by the fact that under all experimental conditions investigated, the respiratory burst was inhibited by the protein kinase C inhibitor staurosporine, suggesting the well-known sequence of phosphoinositide hydrolysis, diacylglycerol formation, protein kinase C activation, and subsequent assembling of the multienzyme complex NADPH oxidase as the underlying sequence of events. Additional studies are required to determine to what extent the appearance of inositol phosphates is due to direct activity of the listerial PlcA activity and to what extent it is caused by stimulation of an endogenous phospholipase C pathway in neutrophils exposed to both LLO and listerial PlcA and possibly to other listerial virulence factors. The listerial PlcA is known to catalyse predominantly the scission of phosphatidylinositol into diacylglycerol and cIMP (8); however, the latter is detected as IP, by the analytical technique used in this study. We recently (37) presented evidence that for endothelial cells, LLO-induced pore formation may facilitate access of the listerial PlcA to the phosphatidylinositol moieties located predominantly or even exclusively in the inner leaflet of the eukaryotic plasma membranes (17). In the present investigation, however, inositol phosphate accumulation by neutrophils was also noted upon challenge with the INN+ mutant expressing listerial PlcA in the absence of LLO. Finally, listerial virulence factors in addition to LLO and PlcA may contribute to the induction of phosphoinositide metabolism and respiratory burst in human neutrophils in contact with *L. monocytogenes*.

In conclusion, our results suggest a hitherto unappreciated role for listerial exotoxins, the provocation of strong and rapid PMN stimulation independent of phagocytosis events. The spectrum of neutrophil metabolic events includes degranulation, the formation of inflammatory lipid mediators such as leukotrienes and PAF, the release of reactive oxygen species, and phosphoinositide hydrolysis. Listeriolysin was noted to be a prerequisite for degranulation and lipid mediator synthesis, but the induction of phosphatidylinositol hydrolysis with the subsequent appearance of inositol phosphates and diacylglycerol and the related respiratory burst is evidently linked to additional listerial virulence factors, among which the PlcA may be of major importance. While the production of exotoxins is important for intracellular survival and cell-to-cell spreading of *L. monocytogenes* (8, 11, 13, 28), the ability of human neutrophils to promptly react with these toxins when extracellularly offered might be important for the host defense response to systemic listerial infection.

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