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Bactericidal Activity of Human Lactoferrin: Influence of Physical Conditions and Metabolic State of the Target Microorganism

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Lactoferrin is an iron-binding protein that is bactericidal against *Streptococcus mutans* and several other microorganisms. In this study, the influence of several physical conditions as well as the metabolic state of *S. mutans* on lactoferrin susceptibility were investigated. After exposure to lactoferrin, a 15-min lag period occurred before the initiation of killing, indicating that a two-step process is involved in lactoferrin killing. Cultures harvested during the early exponential phase were very sensitive to lactoferrin, whereas cultures harvested in the early stationary phase were markedly more resistant. The rate of killing was dependent on temperature; there was no loss of viability at 2°C. Killing occurred at pH 5.0 to 6.0 in water and 20 mM glycine, but did not occur at any pH in 50 mM sodium phosphate or *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer. Addition of exogenous ferrous or ferric ions did not reverse or prevent lactoferrin killing, nor did addition of 1 mM magnesium chloride.

Recent studies from this group have demonstrated a direct interaction of iron-free (apo-) lactoferrin with a variety of microorganisms that results in an iron-irreversible inhibition of the treated organism (1-3). Likewise, the reversal of the killing capacity of neutrophils by the addition of iron or iron compounds would suggest that iron-binding proteins play an essential role in the bactericidal activity of polymorphonuclear leukocytes (5, 6, 8). These effects are distinct from the traditionally studied bacteriostatic phenomenon of iron deprivation by iron-chelating molecules (reviewed in reference 12).

The present studies investigated the influence of a variety of factors on the bactericidal activity of purified human lactoferrin on the oral bacterium *Streptococcus mutans*. In other studies, we have demonstrated susceptibility to lactoferrin killing of a wide range of microorganisms, including gram-negative and gram-positive bacteria, aerobic and anaerobic bacteria, as well as yeast (1). Apolactoferrin is capable of binding to the cell surface of *S. mutans*, a susceptible bacterium; however, growth of inhibited *S. mutans* is not restored by removal of cell-associated lactoferrin (3). Although the growth of *S. mutans* ceases in an iron-free environment, its viability is retained for long periods under conditions of iron deprivation (3). This is in marked contrast to the rapid loss of recoverable colony-forming units (CFU) observed with lactoferrin treatment. It has, therefore, been concluded that the irreversible inhibition observed when bacteria are treated directly with apolactoferrin is not due to simple iron deprivation. There is a total shutdown of all cell functions with lactoferrin treatment, including the incorporation of deoxyribonucleic acid and ribonucleic acid precursors and amino acids, as well as a shutdown in the uptake or metabolism, or both, of carbohydrates (3). Glucose metabolism of *S. mutans* is inhibited by treatment with subcidal concentrations of apolactoferrin and therefore might offer a clue to the initial target for lactoferrin killing. The present studies investigate the influence of the metabolic state of the bacterium, the incubation temperature, pH, and the presence of metal ions on the kinetics of killing of *S. mutans* NCTC 10449 by lactoferrin.

MATERIALS AND METHODS

Lactoferrin preparations. Lactoferrin was purfied from decaseinated human colostral whey by ion-exchange and molecular sieve chromatography (7). Iron-free (apo-) and iron-saturated lactoferrin were prepared by dialysis against either 0.1 M citric acid (pH 2.3) and saturated ferrous ammonium sulfate, respectively. Purity was established by crossed immunoelectrophoresis against rabbit anti-human colostrum and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Chemicals. All chemicals were of reagent grade. Type 1 ferritin from horse spleen was purchased from

Sigma Chemical Co. (St. Louis, Mo.) Apoferritin was prepared by dialysis of the commercial ferritin against 1% thioglycolic acid adjusted to pH 5.0 (9). Water in all experiments was deionized by treatment with a commercial mixed-bed resin and subsequently distilled from a two-stage quartz still.

Microorganism. *S. mutans* NCTC 10449 (Bratthall serotype *c*) was maintained as lyophilized stock cultures. Cultures were grown in a partially defined medium at 37°C to either early exponential phase (absorbancy at 660 nm [A_{660}] ≃ 0.2) or late exponential/early stationary phase (A_{660} ≃ 0.8). Cells were harvested by centrifugation, washed twice, and suspended to a known concentration in either sterile deionized, distilled water, 0.15 M saline, buffers, salts, or 1 mM $MgCl_2$. Viability determinations were performed by serial 10-fold dilutions and plating on brain heart infusion agar (Difco) containing 1.0% sucrose. Plates were incubated at 37°C for 48 h before colony counts were made.

Treatment of *S. mutans* with lactoferrin. In all experiments, washed cells of *S. mutans* were incubated with an appropriate concentration of apolactoferrin. A concentration of 100 μg (≃1.2 μM) of lactoferrin per 10^7 CFU in 1.0 ml of distilled water or saline gave complete killing in 1 h at 37°C. Controls were incubated under identical conditions with an equivalent concentration of iron-saturated lactoferrin or in distilled water. Equal portions were removed at various intervals for viability analysis. Data presented are representative of at least three separate experiments. The temperature of incubation was varied to include 2, 23, and 37°C. The reaction mixtures were incubated in temperature-controlled Tecam Dri-Block DB-1 (Techne, Cambridge, England), and the tubes were not removed throughout the incubation. A Dri-Block was place in an ice bath to achieve the 2°C temperature.

RESULTS

Kinetics of killing of *S. mutans* with lactoferrin. The bacteria were harvested in early exponential phase, washed in distilled water, and resuspended to approximately 10^8 CFU/ml. A portion (10^7 CFU) of bacterial suspension was incubated at 37°C with 2.0 μM apolactoferrin or iron-saturated lactoferrin in 1.0 ml of distilled water. The reaction mixture was incubated at 37°C in a temperature-controlled Dri-Block. Equal portions were removed, and viability was determined at 5-min intervals. The data indicated that there was approximately a 15-min period during lactoferrin exposure before the initiation of cell death (Fig. 1). The loss in viability after this initial lag period was logarithmic. With increasing concentrations of apolactoferrin, the shape of the kinetics curve remained essentially the same, with no effect on the length of the lag period. However, with decreasing concentrations of lactoferrin, the period before the initiation of detectable killing was prolonged (Table 1; R. R. Arnold, J. E. Russell, W. J. Champion, and J. J. Gauthier, unpublished data).

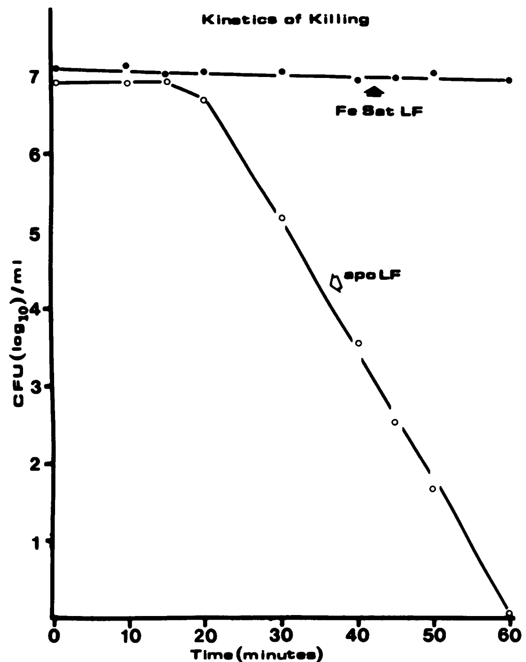

FIG. 1. *Viability of S. mutans NCTC 10449 harvested in early exponential phase (A_{660} ≃ 0.2) incubated at 37°C with either 1.2 μM apolactoferrin (ApoLF) or iron-saturated lactoferrin (Fe Sat LF).*

Effect of bacterial growth phase on sensitivity to lactoferrin killing. The bacteria were harvested during either early exponential (A_{660} ≃ 0.2) or early stationary phase (A_{660} ≃ 0.8). Portions (10^7 CFU) of each were incubated with 4.2 μM apolactoferrin in 1.0 ml of distilled water of 37°C. The viability of bacteria harvested during exponential growth was totally lost within 1 h of incubation (Fig. 2). Though lactoferrin was at a concentration more than twofold greater than that necessary to give total killing of exponentially harvested cells, no loss in viability could be detected in 2 h with late exponential cells. When the cell numbers were reduced 100-fold, some sensitivity to lactoferrin killing of cells harvested in early stationary growth could be detected (Fig. 2).

Effect of temperature. Washed cells from an early exponential culture of *S. mutans* were incubated with an optimal concentration of apolactoferrin at various temperatures and plated for viability determinations (Fig. 3). It should be noted that, since samples were tested for viability at 1-h intervals, the precise kinetics of killing by lactoferrin are not depicted in the figure. At 37°C there were no detectable CFU after 1 h of

TABLE 1. *Effects of pH on the bactericidal activity of lactoferrin on S. mutans*

Buffer[a]	pH	Culture	Mean $\log_{10}$ CFU/ml			
			0[b]	1	2	3
Water[c]	5.0	LF[d]	6.96 ± 0.12	4.95 ± 0.16	3.97 ± 0.21	2.48 ± 0.24
		Control	6.98 ± 0.09	6.95 ± 0.11	6.97 ± 0.08	6.98 ± 0.11
	5.5	LF	6.91 ± 0.06	5.81 ± 0.20	4.55 ± 0.18	2.94 ± 0.31
		Control	6.82 ± 0.31	6.75 ± 0.26	6.74 ± 0.21	6.83 ± 0.23
	8.0	LF	6.76 ± 0.15	6.71 ± 0.09	6.72 ± 0.19	6.76 ± 0.17
		Control	6.79 ± 0.13	6.76 ± 0.02	6.77 ± 0.07	6.76 ± 0.04
20 mM glycine	5.0	LF	6.77 ± 0.15	5.23 ± 0.21	3.79 ± 0.19	<1.0
		Control	6.79 ± 0.11	6.77 ± 0.10	6.76 ± 0.05	6.74 ± 0.08
	6.0	LF	6.79 ± 0.08	5.28 ± 0.11	3.97 ± 0.09	1.32 ± 0.21
		Control	6.74 ± 0.02	6.70 ± 0.10	6.69 ± 0.08	6.70 ± 0.12
	7.0	LF	6.80 ± 0.13	6.11 ± 0.12	5.85 ± 0.14	4.97 ± 0.09
		Control	6.77 ± 0.09	6.80 ± 0.11	6.79 ± 0.09	6.77 ± 0.08
	8.0	LF	6.91 ± 0.17	6.89 ± 0.21	6.83 ± 0.16	6.63 ± 0.11
		Control	6.93 ± 0.14	6.91 ± 0.11	6.90 ± 0.13	6.90 ± 0.14

[a] No killing was apparent in either 50 mM phosphate or HEPES buffer at any pH tested.
[b] Hours of treatment.
[c] Bactericidal activity was equivalent to water in 0.15 M NaCl.
[d] Lactoferrin (LF) concentration of 0.12 μM.

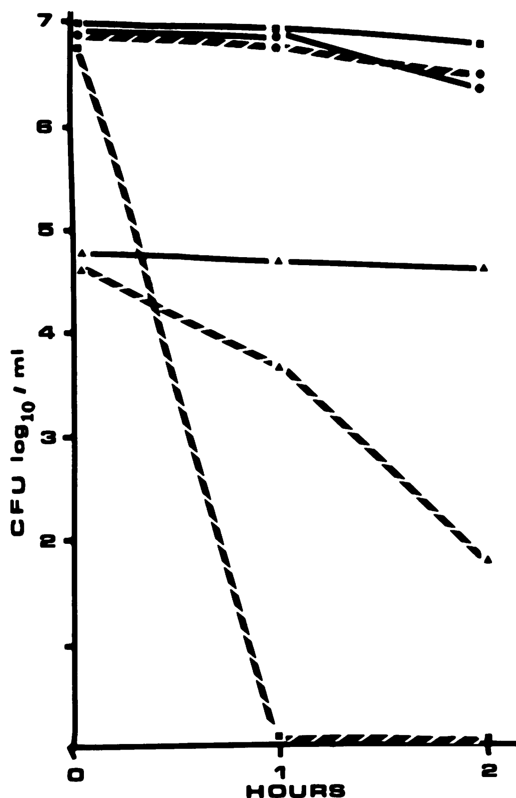

FIG. 2. *Recoverable colony-forming units of S. mutans NCTC 10449 harvested during early exponential growth (A_{660} ≃ 0.2 [■]) or late exponential/early stationary growth (A_{660} ≃ 0.8 [●, ▲]) after incubation at 37°C with 4.2 μM concentrations of either apolactoferrin (——) or iron-saturated lactoferrin (• - - -).*

incubation, and killing was initiated within 15 min. When the incubations were carried out at 23 or 4°C (refrigerated), the rate of killing was markedly reduced when compared with that observed at 37°C. At 2°C there was no detectable loss in viability. The significant loss in viability observed at 4°C (rate of killing intermediate between 2 and 23°C; data not shown) compared with 2°C may be attributed to fluctuations in the temperature during the sampling procedure rather than to the apparently small difference in temperature.

Effects of pH on bactericidal activity. Bacteria harvested during exponential growth were washed and suspended (10^8 CFU/ml) in either distilled water, 20 mM glycine, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), or 50 mM potassium phosphate buffer adjusted to pH 5, 6, 7, or 8. Portions (100 μl) of the bacteria were incubated with 0.12 μM apolactoferrin (suboptimal concentrations) at 37°C.

In initial experiments, the pH of water was adjusted to either 5.0 or 8.0 by the addition of either 10 mM NaOH or 10 mM HCl. There was a significant loss in viability at pH 5.0 that was equivalent to that seen at pH 5.5 with nonadjusted distilled water (Table 1). The bactericidal activity was lost under slightly alkaline conditions (pH 8.0).

Optimal bactericidal activity was observed in 20 mM glycine buffered to pH 5.0, with negligible reduction in activity at pH 6.0, marginal activity at pH 7.0, and no detectable activity at pH 8.0. However, no detectable loss in viability could be detected over a 4-h period in HEPES or phos-

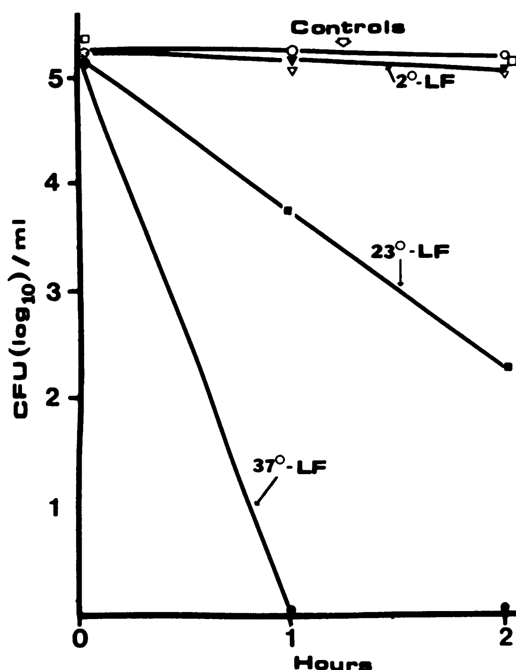

FIG. 3. *Effects of temperature on the bactericidal activity of lactoferrin (LF). Washed cells of S. mutans NCTC 10449 harvested in early exponential phase were incubated with 1.2 μM apolactoferrin or in distilled water at either 2, 23, or 37°C.*

phate buffer at any of the pH's tested. This lack of lactoferrin activity in these buffers was probably not due to pH variations. Since lactoferrin binds to the bacterial surface in both of these buffers as determined by indirect immunofluorescence (Arnold et al., unpublished data), the inhibition of activity is probably not due to blocking of binding. It is possible that there is a conformational change of lactoferrin in these buffers or that there is a competitive inhibition of the active site.

Bactericidal activity in the presence of iron. Bacteria were washed and suspended in either 2.8 μM $Fe(NH_4)_2SO_4$, 5.6 μM $FeCl_3$, 16 mM ferritin, or 16 mM apoferritin and incubated with 1.2 μM apolactoferrin at 37°C for 2 h (Tables 2 and 3). Sterile saline was substituted for lactoferrin in controls. The presence of iron either as ferric or ferrous ions or in association with ferritin had no effect on the bactericidal activity of lactoferrin, as there was greater than 99.9% cell death within 1 h in both the presence and absence of iron.

Effects of Mg^{2+} on lactoferrin killing. To determine whether Mg^{2+} would prevent lactoferrin killing by stabilizing the cell membrane, bacteria were washed and suspended in 1 mM $MgCl_2$ in 0.15 M saline and incubated with 1.2 μM apolactoferrin at 37°C for 1 h. Equal portions were plated for viability at 15-min intervals (Fig. 4). The bactericidal activity of lactoferrin was

TABLE 2. *Bactericidal activity of lactoferrin on S. mutans in the presence of ionic iron*

Culture[a]	Mean $\log_{10}$ CFU/ml		
	0[b]	1	2
Saline			
Control	5.82 ± 0.03	5.83 ± 0.08	5.80 ± 0.04
LF	5.83 ± 0.05	<1.0	<1.0
$Fe(NH)_2SO_4$			
Control	5.58 ± 0.13	5.49 ± 0.17	5.69 ± 0.19
LF	5.61 ± 0.09	<1.0	<1.0
$FeCl_3$			
Control	5.82 ± 0.09	5.74 ± 0.11	5.71 ± 0.13
LF	5.82 ± 0.13	1.0	<1.0

[a] Concentrations were: lactoferrin (LF), 1.2 μM; $Fe(NH)_2SO_4$, 2.8 μM; $FeCl_3$, 5.6 μM.
[b] Hours of treatment at 37°C.

TABLE 3. *Bactericidal activity of lactoferrin in the presence of ferritin*

Culture[a]	Mean $\log_{10}$ CFU/ml		
	0[b]	1	2
LF and ferritin	5.54 ± 0.21	<1.0	<1.0
LF and apoferritin	5.62 ± 0.30	<1.0	<1.0
Ferritin control	5.69 ± 0.19	5.62 ± 0.21	5.54 ± 0.18
Apoferritin control	5.48 ± 0.24	5.43 ± 0.22	5.41 ± 0.19

[a] Concentrations were: lactoferrin (LF), 1.2 μM; ferritin and apoferritin, 16 μM.
[b] Hours of treatment at 37°C.

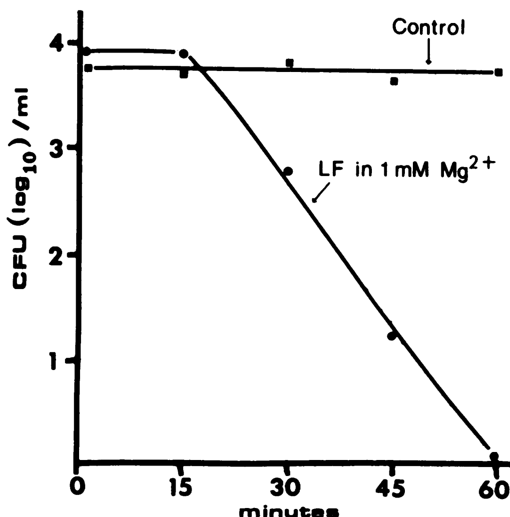

FIG. 4. *Bactericidal activity of apolactoferrin (LF) in the presence of magnesium ions. Early exponential cells of S. mutans NCTC 10449 were washed and suspended in 1 mM $MgCl_2$. The bacteria were then incubated in the presence or absence of lactoferrin.*

not affected by the presence of Mg^{2+}, as a typical killing kinetics was observed.

DISCUSSION

The data presented here support the concept that apolactoferrin is capable of exerting a bactericidal effect on *S. mutans* that is independent of simple iron deprivation. The kinetics of killing (Fig. 1) suggest that the bactericidal event is the result of a two-step process. With an optimal bactericidal lactoferrin concentration, there is a lag of approximately 15 min in the onset of killing that is not detectably diminished by increasing the lactoferrin concentration. Washing before the initiation of death retains cell viability (Arnold et al., unpublished data). This indicates a requirement for exogenous (non-cell-associated) lactoferrin during the lag phase. The fact that viable CFU are recoverable with dilution and plating through 50 min of incubation with lactoferrin indicates the necessity for exogenous lactoferrin throughout the killing process. These data are compatible with the concepts of either saturation by lactoferrin of target sites on the cell surface or active depletion by lactoferrin of an essential component, such as iron, from the cell as an initial event, ultimately leading to cell death. The data do not, however, suggest that the inhibition is reversible by washing, but that the killing event can be halted before completion by removal of exogenous lactoferrin.

The length of the lag period is dose dependent. As the concentration of lactoferrin is decreased relative to the number of target cells, the length of time required for the initiation of killing increases. Total killing of 10^7 CFU of *S. mutans* per ml within 1 h at 37°C requires a concentration of apolactoferrin of at least 1.2 μM. The optimal number of molecules per target coccus can thus be calculated to approach 10^6. However, greater than 99% loss in viability within the 1-h incubation period can be observed with 10-fold less lactoferrin (Table 1; 2). Killing can be detected with 1,000-fold less lactoferrin over longer incubation periods (Arnold et al., unpublished data).

This multiplicity requirement for lactoferrin might indicate multiple target sites (either all essential or a few essential sites in the presence of a greater number of nonessential sites). The influence of the metabolic state on susceptibility to lactoferrin killing suggests a quantitative rather than qualitative difference between exponential- and early-stationary-phase cells. Cells harvested during stationary phase are much more resistant to lactoferrin killing; however, with sufficiently high concentrations of lactoferrin, cell death is observed. During exponential growth the cells are committed to division, with the bulk of nutrients and energy going to reproduction. As cells approach stationary phase, there is an increased buildup of storage reserves. Perhaps lactoferrin acts either directly or indirectly to deplete essential reserves (possibly iron, carbohydrate, or energy) below the minimal levels required to maintain viability. The early inhibition of glucose utilization by lactoferrin might suggest the involvement of carbohydrate (3). The fact that this inhibition occurs in the absence of cell death and that such inhibition is reversible suggest that inhibition of carbohydrate metabolism is an early effect preceding cell death. If carbohydrate transport is blocked, (for example, the phosphotransferase system) and the cell is stimulated to deplete internal stores, cell death could be envisaged. The data (kinetics, multiplicity, and temperature requirements) are not contradictory to such a mechanism. If this hypothesis is true, then depletion of the energy reserves of stationary-phase cells should increase their sensitivity to lactoferrin killing. Preliminary studies suggest this to be true; however, these experiments are not straightforward because of the ability of *S. mutans* to conserve energy stores under nutrient limitation. Similar studies are being pursued with mutants lacking the ability to synthesize iodophilic polysaccharides and with wild-type strains in unbalanced growth (12).

The temperature requirements suggest an energy dependence for lactoferrin killing that might reflect a requirement for active bacterial metabolism during the cidal event or might indicate a temperature dependence for activity of the lactoferrin molecule itself. Active depletion of carbohydrate stores would require metabolic activity.

The enhanced bactericidal activity observed with slightly acid pH's would be expected, considering the secretory environment in which lactoferrin is normally operating. The fact that there is a rapid drop in intracellular pH that precedes bacterial killing in neutrophils (10) might also reflect lactoferrin involvement in the cidal activity of these cells. The reduction of activity at neutral pH and the loss of killing under slightly alkaline conditions might be due to the significant conformational changes of lactoferrin that occur with increasing pH (4). If lactoferrin activity requires direct access to the cell membrane, then an unfolded molecule might be essential either for proper exposure of active sites or for penetration of cell structures exterior to the membrane. Likewise, the loss of activity with iron saturation of lactoferrin (1–3) might be the result of conformational changes (11) rather

than direct blocking of active sites. If iron were the target site involved in the killing event, then a competitive inhibition would be expected in the presence of excess iron either as ferric, ferrous, or iron-ferritin. The lack of a reduction in lactoferrin activity in these experiments would suggest either that iron is not a target site or that the affinity of lactoferrin for iron associated with bacteria is greater than any of the three forms of iron tested.

There is no overt lysis of lactoferrin-treated cells as detected microscopically. It is possible that lactoferrin action results in disruption of cytoplasmic membrane, possibly due to its chelating capacity. The membrane stabilization afforded by 1 mM Mg^{2+} does not protect the cell against the cidal activity of lactoferrin. Present studies are investigating the leakiness of lactoferrin-treated cells as well as the effects of lactoferrin on cell-bound iron, adenosine triphosphate levels, and carbohydrate storage polymers.

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