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Induction of Release of Tumor Necrosis Factor from Human Monocytes by Staphylococci and Staphylococcal Peptidoglycans

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The role of cytokines in gram-positive infections is still relatively poorly defined. The purpose of this study was to establish whether or not intact staphylococci and purified peptidoglycans and peptidoglycan components derived from staphylococci are capable of stimulating the release of tumor necrosis factor (TNF) by human monocytes. We show here that intact staphylococci and purified peptidoglycans, isolated from three *Staphylococcus epidermidis* and three *S. aureus* strains, were indeed able to induce secretion of TNF by human monocytes in a concentration-dependent fashion. TNF release was detected by both enzyme immunoassay and the L929 fibroblast bioassay. In the enzyme immunoassay, a minimal concentration of peptidoglycan of 1 μg/ml was required to detect TNF release by monocytes, whereas in the bioassay a peptidoglycan concentration of 10 μg/ml was needed to detect a similar amount of TNF release. Peptidoglycan components such as the stem peptide, tetra- and pentaglycine, and muramyl dipeptide were unable to induce TNF release from human monocytes.

Coagulase-negative staphylococci have emerged as important pathogens in foreign-body-related infections (29), particularly in immunocompromised hosts and in relation to central venous catheters (37). Clinical presentation of coagulase-negative staphylococcal infections may range from mild signs of local inflammation at the site of insertion of the intravescular catheter to a fulminant septicemia resembling the clinical syndrome of gram-negative shock (10, 21). It has been shown that in the pathophysiology of gram-negative infections tumor necrosis factor (TNF) and interleukin 1 (IL-1) are responsible for the majority of the clinical and hematologic manifestations of endotoxic shock (4, 8); most of the biological effects of endotoxin appear to be triggered primarily by the lipid A component of the lipopolysaccharide (LPS) molecule (12).

In the pathogenesis of staphylococcal infections, various cell wall components of these bacteria have been shown to be capable of inducing septic shock-like symptoms. In an animal model, evidence has been presented that peptidoglycan (PG), the major cell wall component of these bacteria (22), may cause a variety of signs associated with septic shock, such as hypotension, leukopenia, thrombocytopenia (28), arthritis (30), and allergic skin reactions (35).

It has been suggested that, analogous to LPS in gram-negative infections, cell wall components of staphylococci, notably, PG, act by triggering the release of TNF and IL-1 (32). This is supported by the fact that killed staphylococcal whole cells are capable of inducing the release of TNF and IL-1 from human mononuclear cells (38). Moreover, staphylococcal PG has been shown to be able to induce TNF release from rabbit macrophages (17). Interestingly, it has been shown that pneumococcal cell surface components induce IL-1 but not TNF (23).

The purpose of the present study was to assess whether PG, the major cell wall component of staphylococci, was able to directly induce the release of TNF from human monocytes. We report here that whole cells of *Staphylococcus epidermidis* and *S. aureus* strains, as well as purified PGs from these strains, are indeed capable of triggering the production of TNF by human monocytes.

MATERIALS AND METHODS

Bacterial strains. Three clinical isolates of coagulase-negative staphylococci (*S. epidermidis* 291, 354, and 412) derived from cases of catheter-related bacteremias and three *S. aureus* strains (*S. aureus* Wood, *S. aureus* 52A5, and *S. aureus* EMS) were included in the study. As a control, *Escherichia coli* O111:B4 was also included in the study. To stimulate human monocytes, colonies were freshly inoculated into Mueller-Hinton broth (Difco, Detroit, Mich.) and incubated for 18 h at 37°C. Prior to the TNF induction assay, bacteria were washed three times with phosphate-buffered saline (PBS; pH 7.4), counted spectrophotometrically, subsequently heat inactivated for 30 min at 60°C, and resuspended in PBS (pH 7.4) to a final concentration of 5×10^{10} CFU/ml. *E. coli* O111:B4 was grown in Mueller-Hinton broth at 37°C for 18 h, washed three times with PBS (pH 7.4), and counted spectrophotometrically. The bacteria were subsequently killed by incubation in 0.1% formaldehyde in saline at 37°C for 1 h and washed three times in medium 199 enriched with Earle's salt solution without serum and antibiotics. Sterility was confirmed by incubation of the killed bacteria on a blood agar plate overnight. Spectrophotometric counts were correlated to CFU by constructing standard

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curves relating optical density to colony counts by direct plating for each bacterial strain.

Preparation of staphylococcal cell wall components. Cell wall components were prepared by the method of Wilkinson et al. (22, 39) with minor modifications. Bacteria were grown in peptone yeast extract (PYK) medium for 2 to 4 h at 37°C. The log-phase bacteria were inoculated into 25 liters of PYK medium and incubated for 18 h at 37°C with shaking. Bacteria were harvested by centrifugation (5,000 × *g*, 10 min, 4°C) and subsequently washed three times in sterile bidistilled water. The pellet from the last washing step was added to the chamber of a Bead Beater (Biospec Products, Bartlesville, Okla.), which was cooled with ice, and mixed with sterile glass beads (0.1-mm diameter). The Bead Beater was operated for five cycles of 90 s each, with a pause of 90 s between each cycle. After sedimentation of the beads, the supernatant was collected and the beads were washed three times. The pooled supernatants from these washing procedures were centrifuged for 10 min at 14,000 × *g*. Cell walls were harvested by removing the white top layer of the pellet. The isolation of crude and purified cell walls and the purification of PG were performed essentially as described before (22, 39). All PG preparations were tested for contamination with endotoxin by the *Limulus* amoebocyte lysate test (Chromogenix, Molndal, Sweden) at a sensitivity of 2 pg/ml. In all of the preparations, tested in a concentration of 10 μg/ml, the endotoxin content was <2 pg/ml. The presence of toxic shock syndrome toxin 1, staphylococcal enterotoxins A through E, and the exfoliative toxins A and B in the bacterial strains was determined by probing for the presence of the respective genes by polymerase chain reaction. None of the strains was found to be positive for any of the genes (34a). Tetraglycine (G 3882; Sigma, St. Louis, Mo.), pentaglycine (G 5755; Sigma), muramyl dipeptide (*N*-acetylmuramyl-L-alanyl-D-isoglutamine, A 9519; Sigma), and PG stem peptide (L-alanyl-D-isoglutaminyl-L-lysyl-D-alanyl-D-alanine acetate, A 1035; Sigma) were obtained commercially.

Chemical characterization of PG. PGs were hydrolyzed in sealed tubes with 6 M HCl for 18 h at 110°C. Acid was removed under vacuum in the presence of KOH. Amino acids were determined in an amino acid analyzer (Alpha Plus; LKB, Uppsala, Sweden). The purity of the PGs as determined by amino acid analysis showed the amino acids serine, glutamine, glycine, alanine, and lysine, present in the following ratios: PG 291, 1:1:4:3:1; PG 354, 1:1:5:3:1; PG 412, 1:1:2:2:1; PG WOOD, 1:2:10:5:1; PG EMS, 1:1:4:2:1; PG 52A5, 0:1:5:2:1. Non-PG amino acids, notably, leucine and isoleucine, were not detected. Teichoic and lipoteichoic acid extractions were successful, as shown by the low phosphorus content of the PG preparations (1.5 to 6.2 μg/mg). Phosphorus was determined as described by Bartlett (2).

Sonication of PG. PG resuspended in bidistilled water (2 mg/ml) was sonicated at 40,000 Hz (Branson Sonifier B12) on ice. Prior to use, PGs were sonicated three times for 10 s each time. In some experiments, PG preparations were sonicated for longer periods.

Enzyme treatment of PG. In some experiments, PG preparations were treated with the enzymes lysostaphin (L7386; Sigma), lysozyme (Fluka, Buchs, Switzerland), mutanolysin (M9901; Sigma), SALE (*S. aureus* lytic enzyme) (16), and M1 (15) (both SALE and M1 were kindly provided by S. Kotani, Osaka College of Medical Technology, Osaka, Japan). PG (2 mg/ml) was incubated with M1 (10 μg/ml) in sodium acetate buffer (pH 5.5). Incubation with the other enzymes (lysostaphin, lysozyme, and mutanolysin) was in PBS (final concentration, 10 μg/ml) for 4 h at 37°C. Subsequently, the enzymes were inactivated by heat treatment (10 min at 100°C). LPS from *E. coli* O111:B4 was treated with M1 as mentioned above for PG.

LPS. LPS from *E. coli* O111:B4 (Sigma) was dissolved in pyrogen-free water (2 mg/ml) and sonicated on ice as described for PG. The solution was stored at −20°C. Prior to use, the solution was sonicated in a water bath for 5 min.

Isolation, culture, and stimulation of human monocytes. Human peripheral blood mononuclear cells were isolated from a buffy coat of healthy donors, by the method of Böyum (5), slightly modified as described previously (22). Peripheral blood mononuclear cells were washed once in Hanks' balanced salt solution with gelatin (0.1%, wt/vol); subsequently, the cells were washed in medium 199 (GIBCO, Breda, The Netherlands) supplemented with bicarbonate (1.2 g/liter), HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; 2 g/liter), vancomycin (25 μg/ml), and gentamicin (10 μg/ml). After being washed, the peripheral blood mononuclear cells were adjusted to a concentration of 3×10^6 cells per ml in medium 199 without antibiotics and sera. One-third of the peripheral blood mononuclear cells were monocytes, as determined by May-Grunwald-Giemsa staining. The cells were transferred to flat-bottom tissue culture plates with 96 wells (Nunc, Kamstrup, Denmark), 100 μl (3×10^5 cells per well), and then incubated in a 5% CO_2 atmosphere at 37°C for 1 h in order to adhere. Nonadherent cells were removed by aspiration, and the monolayer of monocytes (examined by May-Grunwald-Giemsa and trypan blue staining) was then stimulated with 100 μl of whole bacteria, PG, or LPS at the concentrations indicated. After incubation at 37°C (5% CO_2) for 18 h, the contents of the wells were collected and centrifuged for 15 min at 7,000 × *g*; the supernatants were stored at −70°C prior to determination of TNF concentrations. Control monocytes were incubated with medium 199 without additions. Quantitation of TNF in monocyte supernatants was performed both by measuring cytotoxicity for L-929 murine fibroblasts and by an enzyme immunoassay (EIA; Medgenix Diagnostics, Fleurus, Belgium). Experiments were carried out in duplicate (EIA) or in triplicate (L-cell assay) and repeated at least three times on separate days. For the EIA, a standard curve was prepared with six TNF concentrations (0, 50, 150, 500, 1,000, and 1,500 pg/ml). Results of the EIA were expressed in picograms of TNF per milliliter. The L-cell bioassay was performed as described previously (34).

RESULTS

Induction of TNF release by intact staphylococci and PGs. Figure 1 shows that whole bacterial cells of *S. epidermidis* 354 and *S. aureus* Wood stimulated TNF production by human monocytes in a concentration-dependent fashion. A minimal bacterium-to-monocyte ratio of 10:1 was required to induce TNF release. *S. epidermidis* 354 induced slightly higher TNF levels than *S. aureus* Wood and the control, *E. coli* O111:B4, at concentrations of 10^7 and 10^8 CFU/ml.

Figure 2 shows that purified PG (100 μg/ml) isolated from *S. epidermidis* 354 and *S. aureus* Wood similarly induced the release of TNF by human monocytes. Supernatants were collected after 2, 4, 16, 24, and 48 h of incubation. LPS (100 ng/ml) from *E. coli* O111:B4 was used as a control. After 2 h of incubation, TNF release induced by PG Wood and 354 reached 53 and 60%, respectively, of the final maximum TNF value. For LPS, the corresponding value was 4%. The peak of TNF was reached after 16 h of incubation for both

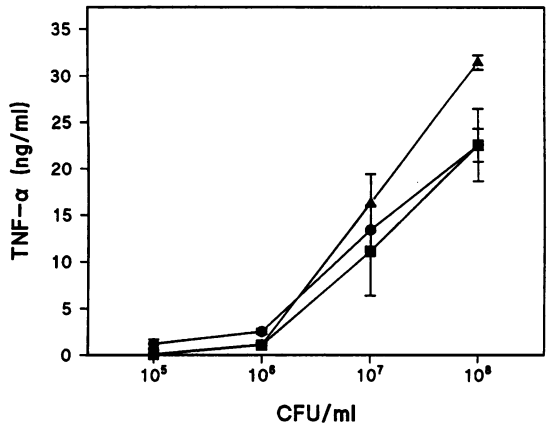

FIG. 1. TNF levels measured by EIA in the supernatant of human monocytes (10^6 per ml) stimulated with heat-killed *S. epidermidis* 354 (▲) and *S. aureus* Wood (■) and formalin-killed *E. coli* O111:B4 (●) at the concentrations indicated. Means ± standard errors of three experiments with different donors are shown.

PG and LPS; subsequently, a slight drop in the amount of detectable TNF occurred. Similar to the TNF release triggered by intact staphylococci, the release of TNF induced by purified PG was found to be concentration dependent (Table 1). A PG concentration of 1 μg/ml or more was required to detect TNF release from human monocytes in the EIA, while in parallel experiments employing the L-cell assay, a 10-fold-higher concentration of PG was required to induce a detectable TNF release.

Table 2 shows that polymyxin B (10 μg/ml) did not inhibit monocyte TNF release induced by PG (100 μg/ml), whereas the induction of TNF release in response to LPS (100 ng/ml) from *E. coli* O111:B4 was completely abolished by polymyxin B.

Incubation of monocytes with muramyl dipeptide, pentaglycine, tetraglycine, and the PG stem peptide did not induce any release of TNF in the concentration range of 50 to 250 μg/ml. At a concentration of 500 μg each of muramyl

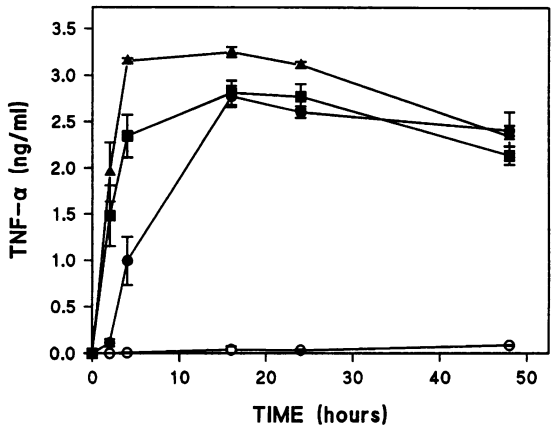

FIG. 2. Time curves of TNF responses by human monocytes (10^6 per ml) after stimulation with PG (100 μg/ml) from *S. epidermidis* 354 (▲) and *S. aureus* Wood (■) and with LPS (100 ng/ml) from *E. coli* O111:B4 (●). Control monocytes (○) were incubated in medium 199 alone. Supernatants were collected after 2, 4, 16, 24, and 48 h and stored at −70°C prior to TNF determination by EIA. Means ± standard errors of three experiments with different donors are shown.

TABLE 1. Release of TNF by human monocytes induced by staphylococcal PG as determined by EIA and L929 fibroblast bioassay

PG concn (μg/ml)	TNF (pg/ml)[a]	
	EIA	Bioassay
0 (medium 199)	44 ± 15	0
0.1	92 ± 42	0
1	257 ± 69	0
10	1,482 ± 156	613 ± 297
100	2,905 ± 88	1,871 ± 566

[a] Means ± standard deviations of three separate experiments (three different monocyte donors).

dipeptide and the PG stem peptide per ml, minimal TNF production of 10 and 28 pg/ml, respectively, was detected.

Prior to use, preparations were sonicated three times for 10 s each time. Such preparations showed maximal induction of TNF release. PGs sonicated for shorter periods gave irreproducible results. Prolonged sonication of PGs resulted in a decrease in the capacity to induce TNF release. The reduction in TNF release amounted to 40 to 60% and 80 to 90% after sonication of the PGs for 30 and 60 min, respectively. Digestion of PG by the enzymes lysozyme, lysostaphin, mutanolysin, SALE, and M1 resulted in either a strong reduction or a complete loss of the capacity of PG to induce TNF release. Interestingly, this reduced capacity of PG to induce TNF release was parallelled by a decrease in the optical density of the PG solution. An example of this association is presented in Fig. 3, showing the effect of treatment of PG with M1 on its TNF-inducing capacity and on optical density of the PG solution. LPS O111, treated with M1 in the same way as PG, showed a decrease in TNF-inducing capacity (30 to 40% depending on the donor) after 5 min of treatment, but this inhibitory effect of M1 on LPS-inducing capacity did not increase any further beyond this time point (data not shown).

DISCUSSION

The results of the present study show that intact cells of *S. epidermidis* and *S. aureus* as well as purified PGs of these species directly stimulate the release of TNF by human monocytes in vitro. The stimulatory effect was time and concentration dependent.

With respect to the induction of TNF by staphylococcal whole cells, our results confirm those reported previously by others (11, 38) and also show that whole cells provide a much

TABLE 2. Release of TNF by human monocytes induced by staphylococcal PG and endotoxin: effect of polymyxin B

Monocyte stimulus	TNF (pg/ml)[a]		
	Control	+ Polymyxin B	% Inhibition
LPS (100 ng/ml), *E. coli* O111	2,114 ± 340	0	100
PG (100 μg/ml), *S. epidermidis*[b]	2,827 ± 958	2,340 ± 552	17
PG (100 μg/ml), *S. aureus*[b]	2,540 ± 536	2,646 ± 263	−3

[a] As determined by EIA. Results shown represent means ± standard deviations of three separate experiments (three different monocyte donors).

[b] PGs from *S. epidermidis* 291, 354, and 412 and *S. aureus* EMS, Wood, and 52A5 were used, and data were pooled.

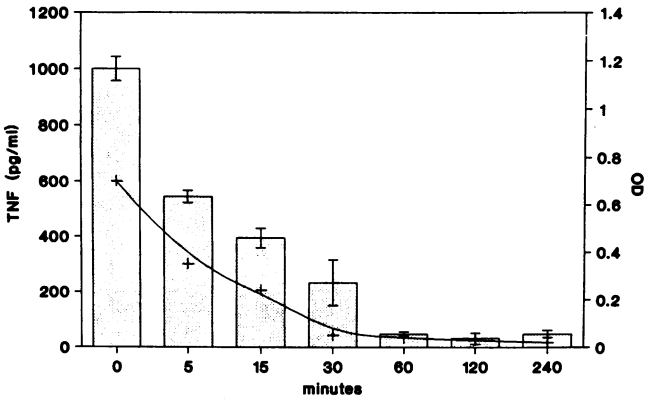

FIG. 3. Effect of M1 (10 μg/ml) on the capacity of *S. epidermidis* 354 PG (100 μg/ml) to induce TNF production by human monocytes. The influence of M1 treatment on the optical density (OD) of the PG solution was determined spectrophotometrically at 660 nm as represented by the solid lines. Bars show means ± standard errors of TNF released in three separate experiments. TNF was determined by EIA.

more effective stimulus for TNF induction than the purified PGs. This is evident from the fact that 10^7 staphylococcal cells corresponding to 0.1 μg of PG yielded a much more effective stimulus than the purified PG, from which at least 1 to 10 μg/ml was needed to induce TNF release. This suggests that whole staphylococcal cells contain constituents other than PG, which either together or acting synergistically with PG are responsible for the much higher TNF-inducing capacity of the whole bacterial cells. This aspect clearly needs further study.

Detectable TNF production was triggered by purified PG in a concentration of 1 to 10 μg/ml depending on the assay used (EIA or L929 fibroblast bioassay). TNF secretion was maximal 16 h after induction, with a slight subsequent decline upon longer incubation. This is in accordance with the kinetics of LPS found by us and Schindler et al. (26). However, much higher TNF levels were noticed after 2 and 4 h of incubation when the monocytes were stimulated with PG compared with stimulation with LPS. Since freeze-thawing of monocytes did not yield higher amounts of TNF (data not shown), it may be concluded that virtually all TNF produced by monocytes upon stimulation by PG is released into the extracellular environment.

Variations in TNF secretion were found with different donor monocytes; high and low responders with regard to TNF production have been observed in monocytes from healthy volunteers (33). In addition, to detect TNF release by human monocytes induced by PG, the EIA was found to be more sensitive than the bioassay.

Purified PG is an insoluble material, which yielded irreproducible results with respect to the induction of TNF release unless it was well dispersed prior to use. Sonication of PG for three 10-s periods resulted in a macroscopically homogeneous suspension which showed a maximal capacity for induction of TNF release. Prolonged sonication, however, resulted in a decrease in this capacity, probably due to either disruption of the tertiary structure or size reduction of the macromolecule or both.

The results of enzymatic treatment of PG also indicate that particle size as well as tertiary structure are critically important for the induction of TNF release. Structural requirements for the induction of cytokines have also been found by Takahashi et al. (31) for endotoxic LPS and for the induction of IL-1 by cell walls. Treatment of PG with M1, SALE, lysostaphin, lysozyme, and mutanolysin resulted in a decrease in the capacity to induce TNF release. Although these enzymes act at different sites of the PG macromolecule, they all reduce its size and disrupt its tertiary structure.

In order to determine the component of the PG macromolecule responsible for the induction of TNF secretion, several PG fractions were tested. Neither muramyl dipeptide nor tetra- and pentaglycine or the PG stem peptide was able to induce TNF secretion. Only at very high, nonphysiological concentrations of muramyl dipeptide and the PG stem peptide were low levels of TNF release found.

These results may offer an explanation for the apparent discrepancy between our findings and those of Riesenfeld-Orn et al. (23) with respect to the capacity of PG to induce TNF release by human monocytes in vitro. The latter investigators found that soluble PG fragments composed of disaccharide peptide mono-, di-, and trimers from pneumococci were unable to stimulate monocyte TNF production but were capable of inducing IL-1 release by these cells. This is in agreement with our finding that the TNF-inducing capacity of staphylococcal PG was rapidly lost when the macromolecule was degraded into smaller fragments by either enzyme treatment or sonification. In addition, the lack of TNF-inducing activity of muramyl dipeptide, tetra- and pentaglycine, and the PG stem peptide found in the present study lends further support to the notion that a certain size and/or tertiary configuration of the PG macromolecule is required to induce TNF release by human monocytes.

Previous studies have demonstrated that other components from gram-positive bacteria such as staphylococcal lipoteichoic acid (38), PG precursors from *Enterococcus faecalis* (13), a polysaccharide from *Streptococcus mutans* (3), and staphylococcal toxins, notably, toxic shock syndrome toxin 1 (14, 20), are capable of inducing TNF release by monocytes. Since the PG preparations were free of teichoic and lipoteichoic acids and the bacterial strains lacked the genes for toxic shock syndrome toxin 1 and the known staphylococcal exotoxins, it is unlikely that the TNF production induced by PG as observed in the present study is due to contamination by any of these products.

Nonetheless, in a staphylococcal infection any of these constituents and toxins may act together, perhaps synergistically, to induce TNF and other cytokines, notably, IL-1. Such a process may possibly have a protective and homeostatic effect in a local infection, but in a systemic staphylococcal infection this sequence may have the disastrous result of the induction of septic shock (38). Elevated TNF levels have been observed in patients with gram-positive septic shock (6, 7). However, the exact role of TNF in gram-positive septic shock remains to be determined.

The locally protective effect of TNF induction against infection is strongly supported by the results of a recent study by Vaudaux et al. (36). They found that in a guinea pig model of staphylococcal foreign body infection both locally induced (by staphylococcal PG) and locally injected TNF prevented the subsequent development of infection by inoculation of *S. aureus*. This local effect of TNF in preventing infection may explain observations in older studies that animals could be protected against a variety of infections after prior treatment with intact cells or cell walls of gram-positive organisms, such as *Corynebacterium parvum* (1), *Lactobacillus casei* (25), *Listeria monocytogenes* (19), and group A streptococci (24). Prior intravenous treatment of mice with *S. epidermidis* strains resulted in an enhanced

protection of mice against subsequent infection with *Listeria monocytogenes* (9). A recent study documented the importance of endogenous TNF in host resistance to *Listeria monocytogenes* infections (18). In mice, it was found that nonspecific resistance induced by streptococcal cell walls was dependent on the PG moiety (19). In conclusion, induction of TNF locally and possibly also systemically may, in part, explain the protective effect of prior treatment with staphylococcal cells, cell walls, and PG against a variety of infections.

Finally, our study has shown the ability of intact staphylococci and staphylococcal PGs to induce TNF secretion by human monocytes. Future studies are needed to further elucidate the basic structure(s) or component(s) of the PG macromolecule that is involved in the induction of TNF release both in vitro and in vivo.

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