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Lipoteichoic Acid and Interleukin 1 Stimulate Synergistically Production of Hepatocyte Growth Factor (Scatter Factor) in Human Gingival Fibroblasts in Culture

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Lipoteichoic acids (LTA) from various gram-positive bacteria, including oral streptococci such as *Streptococcus sanguis*, enhanced the production of hepatocyte growth factor (HGF) (scatter factor) by human gingival fibroblasts in culture, whereas lipopolysaccharides (LPS) from various gram-negative bacteria did not. In contrast, LPS induced interleukin 1 activity in human gingival epithelial cells in culture, while LTA had little effect. LTA and recombinant human interleukin 1α enhanced synergistically the production of HGF/SF in human gingival fibroblast cultures. Recombinant human HGF, in turn, enhanced the proliferation of human gingival epithelial cells in culture.

Human hepatocyte growth factor (HuHGF) was first purified from the plasma of patients with fulminant hepatic failure as a hepatocyte-specific growth factor (16), then cDNA for HuHGF was cloned from a cDNA library of human placenta (29), and recombinant HuHGF showed the same biological activity as the native HuHGF on rat and human hepatocytes in culture (39). On the other hand, HuHGF has been found to be the same protein as the scatter factor (SF) (15, 46), which enhanced the motility of epithelial cells. HuHGF/SF exhibits various biological activities, such as mitogenic (20, 23, 27), motogenic (15, 27, 38), and morphogenic (30, 31), on epithelial and endothelial cells and tumor cytotoxic effects (37) (for reviews, see reference 17). On the other hand, several cell lines of mesenchymal origin have been shown to produce HuHGF/SF (17). We have previously reported that human gingival fibroblasts also produce HGF/SF, and the production is enhanced by inflammatory cytokines, such as interleukin 1α (IL-1α), IL-1β, and tumor necrosis factor alpha (42). Periodontal tissues are markedly colonized by oral bacteria. Lipoteichoic acid (LTA) and lipopolysaccharide (LPS) are widely distributed and ubiquitous cell surface amphiphiles of gram-positive and -negative bacteria, respectively, and these components share various biological activities (47). Streptococci and lactobacilli are important constituents of normal flora of the oral cavity; *Streptococcus sanguis* is a dominant species in dental plaques (18). Most of these gram-positive bacteria possess LTA (9). By contrast, in the inflamed gingiva, the number of gram-negative bacteria is increased (33). Therefore, gingival cells may be exposed to LTA and LPS as well as inflammatory cytokines in inflamed conditions.

In this study, we demonstrated that LTAs from various bacteria, including oral streptococci, enhanced the production of HGF/SF in human gingival fibroblasts and that human gingival epithelial cells produced IL-1 upon stimulation with LPS. We also found that IL-1α and LTA induced synergistically HGF/SF production in human gingival fibroblasts in culture. We further examined the effects of HGF/SF on gingival epithelial cells and found that recombinant HuHGF (rHuHGF) enhanced DNA synthesis by human gingival epithelial cells.

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MATERIALS AND METHODS

LTA, LPS, and cytokines. LTA specimens prepared from *S. sanguis*, *Streptococcus mutans*, *Streptococcus pyogenes*, *Streptococcus faecalis*, *Staphylococcus aureus*, and *Bacillus subtilis* were purchased from Sigma Chemical Co. (St. Louis, Mo.). LTA prepared from *Lactobacillus plantarum* ATCC 155 was a gift from S. Kokeguchi (Okayama University Dental School, Okayama, Japan). To examine the contamination of these LTA preparations with extraneous LPS and peptidoglycan/β-glucan, a colorimetric *Limulus* test (Endospecy test; Seikagaku Co. Ltd., Tokyo, Japan) (34) and a recently developed SLP test (Wako Pure Chemicals, Osaka, Japan) utilizing a prophenol-oxidase cascade in silkworm larva plasma, which is activated specifically with bacterial peptidoglycan and fungal β(1-3)-D-glucan (2), were carried out (Table 1). Among the LTAs tested, *S. faecalis* LTA exhibited marked activity in the *Limulus* and SLP tests. To obtain a purified LTA without LPS and peptidoglycan/β-glucan, the commercial LTA of *S. sanguis* was subjected to hydrophobic interaction chromatography on an Octyl-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) column according to the method of Fischer et al. (10). A single peak fraction thus obtained (Fig. 1) was lyophilized and used as a purified LTA in some experiments. The purified LTA had little activity in both the *Limulus* and SLP tests (Table 1). An ultrapurified LPS prepared from *Salmonella abortusequi* (Novo-Pyrexal) (14) was a gift from C. Galanos (Max Planck Institut für Immunbiologie, Freiburg, Germany). Various chemotypes of LPS prepared by the PCP method (13) from *Salmonella minnesota* R60 (Ra mutant), *S. minnesota* R345 (Rb mutant), *S. minnesota* R5 (Rc mutant), *S. minnesota* R7 (Rd1 mutant), *S. minnesota* R3 (Rd2 mutant), and *S. minnesota* R595 (Re mutant) were also supplied by C. Galanos. Synthetic *Escherichia coli*-type lipid A, LA-15-PP (compound 506), was purchased from Daiichi Chemical Co. (Tokyo, Japan). rHuIL-1α (lymphocyte activating activity, 2.3×10^7 U/mg of protein) (12) was supplied by Dainippon Pharmaceutical Co. (Osaka, Japan). rHuHGF (48) was provided by Takehisa Ishii, Mitsubishi Chemical Co. (Yokohama, Japan).

Cells and culture conditions. Specimens of healthy human gingival tissue were obtained from a patient (female, 8 years old) during a fenestration procedure. The specimen was cut into pieces, and fibroblasts were prepared as described previously (41). Briefly, the explants were cultured in alpha minimal essential medium (α-MEM) (ICN, Amsterdam, The Netherlands) supplemented with 10% fetal bovine serum (FBS; GIBCO Laboratories, Grand Island, N.Y.), 200 μg of kanamycin (Meiji, Tokyo, Japan) per ml, and 0.25 μg of amphotericin B (Fungizone; GIBCO) per ml at 37°C in a humidified atmosphere of 5% CO_2 in air with a medium change every 3 to 5 days until confluent cell monolayers were formed. After three to four subcultures, homogeneous, slim, spindle-shaped cells growing in characteristic swirls were obtained. These fibroblasts were used throughout the experiments. Healthy human gingival tissue was also obtained from a patient (female, 10 years old) during enucleation of wisdom teeth, cut into pieces, and cultured in keratinocyte–serum-free medium (GIBCO) containing bovine pituitary extract (0.05%, vol/wt) and human epidermal growth factor 1-51 (recombinant from *E. coli*, 820 μM) supplemented with 200 μg of kanamycin per ml and 0.25 μg of amphotericin B per ml with a medium change every 3 to 5 days

TABLE 1. Contamination of test LTA specimens with endotoxin and peptidoglycan/β-glucan

LTA source	*Limulus* activity[a] (ng/mg)	SLP activity[b] (μg/mg)
S. sanguis	277	29.4
S. mutans	28.1	56.3
S. pyogenes	460	129.0
S. faecalis	17,500	166.0
S. aureus	86.3	129.0
L. plantarum	12.3	10.0
B. subtilis	15.2	28.9
S. sanguis[c]	0.1	0.6

[a] *Limulus* activity was determined by a colorimetric method, using the Endospecy test (Seikagaku Co.). Activity was expressed as an equivalent weight to the reference LPS of *E. coli* O111:B4 (Difco Laboratories, Detroit, Mich.).

[b] Activity was expressed as an equivalent to the reference peptidoglycan of "*Micrococcus lysodeikticus*" included in the SLP test kit.

[c] Purified LTA prepared from the above-listed *S. sanguis* LTA.

until confluent cell monolayers were formed. After three passages, the cells were identified by immunostaining with anti-cytokeratin type I and II antibodies (Funakoshi, Tokyo, Japan). Human gingival epithelial cells obtained in this manner (Fig. 2) were used in this study. The human melanoma A375S2 cell line, which is highly sensitive to the cell growth inhibitory activity of IL-1 (32), was supplied by T. Koga (Kyusyu University Faculty of Dentistry, Fukuoka, Japan).

Determination of HGF/SF in fibroblast culture supernatants. Fibroblasts (2×10^4) were seeded into each well of a 96-well culture plate in 100 μl of α-MEM supplemented with 10% FBS and 200 μg of kanamycin per ml. After overnight cultivation, the cells were washed and the medium was changed to α-MEM supplemented with 1% FBS and 200 μg of kanamycin per ml. Then, the cells were cultured in triplicate with test materials for 24 h. The triplicate supernatants were collected and stored at −20°C until use. The concentrations of immunoreactive HGF/SF in culture supernatants were determined in duplicate by a commercial enzyme-linked immunosorbent assay (ELISA) kit (Otsuka Pharmaceutical Co., Tokushima, Japan) originally described by Tsubouchi et al. (45). The assays were performed according to the manufacturer's instructions, and the data were calculated from a standard curve prepared for each assay.

Measuring IL-1 activity. Human gingival epithelial cells were cultured in a 96-well culture plate with or without stimulants, and the supernatants were collected. IL-1 activity in culture supernatants was measured in terms of the inhibitory effect on proliferation of A375S2 cells in vitro as described by Nakai et al. (32), with slight modification. Briefly, A375S2 cells were cultured for 24 h at a density of 10^4 cells per 100 μl per well in α-MEM containing 10% FBS and 200 μg of kanamycin per ml. Then, diluted test supernatants (25 μl) were added to the wells in triplicate. After a further 24 h of incubation, the medium was discarded, and the remaining viable cells were fixed and stained with 2% formaldehyde containing 0.2% crystal violet. The intensity of staining was measured with a microplate reader (Well Reader SK 601; Seikagaku Co.) at a wavelength of 595 nm. The dilution of test specimens that caused a 50% inhibitory proliferation of cells was determined from a titration curve. IL-1 activity in test specimens was expressed in nanograms per milliliter and was estimated from the ratio of a 50% inhibitory proliferation dose of the test specimen to that of standard rHuIL-1α (Dainippon Pharmaceutical Co.).

Northern (RNA) blot analysis. Northern blot analysis was performed as described previously (42). Briefly, cDNA for HuHGF was excised with *Hin*dIII and *Bam*HI, and cDNA for glyceraldehyde-3-phosphate dehydrogenase, which was provided by S. Sakiyama (Chiba Cancer Center Research Institute and Hospital, Chiba, Japan) (44), was excised with *Eco*RI. The cDNAs were purified from vector sequences by agarose gel electrophoresis and then labeled by using random primers, fragments from Klenow enzyme (labeling grade; Boehringer Biochemicals, Mannheim, Germany), and [α-^{32}P]dCTP according to the method described by Feinberg and Vogelstein (8). The specific radioactivity of labeled cDNA was 1×10^8 to 5×10^8 cpm/μg of DNA. After experimental treatments with test materials, total cellular RNA was extracted from about 6×10^6 cells (one 150-mm-diameter plate) by the acid guanidinium thiocyanate-phenol-chloroform method as described by Chomczynski et al. (5). Total RNA (20 μg per lane) was electrophoresed in 1.2% agarose gel in 0.66 M formaldehyde and transferred electrophoretically to a nylon membrane (Zeta-Probe; Bio-Rad, Hercules, Calif.) as previously described by Thomas (43). The membrane was hybridized with ^{32}P-labeled HuHGF cDNA as a probe and washed according to the protocols for reduction of background as recommended by Bio-Rad. The blots were exposed to an imaging plate for 1 h for analysis with an imaging analyzer or exposed to X-ray film at −80°C for several days for autoradiography. Equal loading in lanes was checked by successive hybridization with ^{32}P-labeled glyceraldehyde-3-phosphate dehydrogenase cDNA by the method of Sambrook et al. (36). Photographs of the imaging plates were analyzed by a Bioimaging analyzer (model BAS-1000 Mac; Fujifilm Inc., Tokyo, Japan).

Measurement of DNA synthesis by human gingival epithelial cells. The cells were placed at a density of 5×10^3 cells per 100 μl per well in a 96-well culture plate. After overnight cultivation, the medium was changed to a physiological concentration (1.8 mM) of Ca^{2+} without bovine pituitary extract and epidermal growth factor, and then the cells were further cultured with various concentrations of rHuHGF for 30 h in triplicate. During the final 6 h of the culture, the cells were pulse-labeled with [^{3}H]thymidine (18.5 kBq [0.5 μCi]/5.6 to ~8.4 pmol per well; Moravek Biochemicals, Brea, Calif.). The cells were then trypsinized and collected on a glass fiber filter mat (LABO MASH, Tokyo, Japan) with an auto cell-harvester (LABO MASH). The radioactivity incorporated by cells was measured by the conventional scintillation method.

Statistical analyses. In most assays, the mean ± standard error of the mean was obtained, and the statistical significance of the difference between each test and its respective control was examined by Student's *t* test. In some experiments, analyses of variance on the interaction between LTA and IL-1α were carried out to examine the synergistic effect of the agents.

RESULTS

HGF/SF production by human gingival fibroblasts stimulated with LTA. We first examined the effect of LTA and LPS specimens on the production of immunoreactive HGF/SF by human gingival fibroblasts. As shown in Fig. 3, various LTA specimens stimulated the secretion of immunoreactive HGF/SF by the fibroblasts. In particular, *S. sanguis* LTA greatly enhanced HGF/SF production by human gingival fibroblasts; the level was higher than that induced by 10 ng of rHuIL-1α per ml. Other LTAs had activities comparable to or slightly less than that of rHuIL-1α. LPS prepared from *S. abortusequi* had no effect on human gingival fibroblasts. Various chemotypes of LPS of *S. minnesota* and the synthetic *E. coli*-type lipid A also had little activity (data not shown).

IL-1 production by human epithelial cells stimulated with LPS. IL-1 is present in the inflammatory periodontal tissue (4, 22, 26). Human gingival fibroblasts are capable of producing IL-1 upon stimulation with cytokines such as IL-1α, IL-1β, and tumor necrosis factor alpha (24, 41), whereas they did not produce IL-1 when they were treated with the LTAs and LPSs noted above (data not shown). Thus, we examined the IL-1-inducing activity of LTA and LPS in human gingival epithelial cell cultures. LTAs, except those of *S. sanguis* and *S. faecalis*, did not induce IL-1 activity in human gingival epithelial cell cultures, whereas LPS of *S. abortusequi* induced marked IL-1 activity (Table 2). LTA from *S. faecalis* was suspected to be

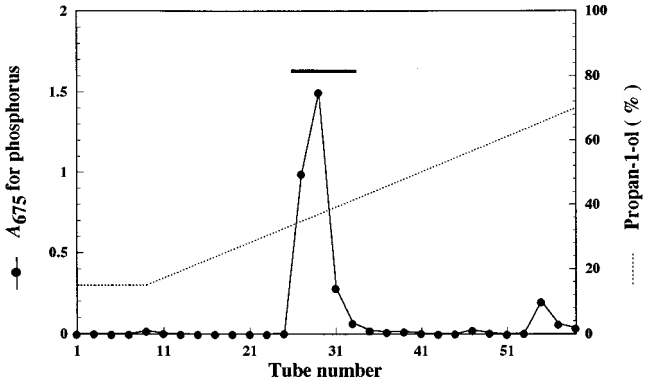

FIG. 1. Hydrophobic interaction chromatography of a commercial LTA from *S. sanguis* on an Octyl-Sepharose column. The LTA specimen was subjected to hydrophobic interaction chromatography on an Octyl-Sepharose CL-4B column (1.8 by 12 cm) equilibrated with 0.1 M sodium acetate buffer (pH 4.5) containing 15% propan-1-ol. The column was successively eluted with the buffer described above and a linear gradient of 15 to 70% propan-1-ol in the same buffer. Fractions (2 ml each) were collected and monitored by their phosphorus content according to the method of Bartlett (3) with a slight modification. The fraction indicated by the bar was pooled and used as a purified LTA.

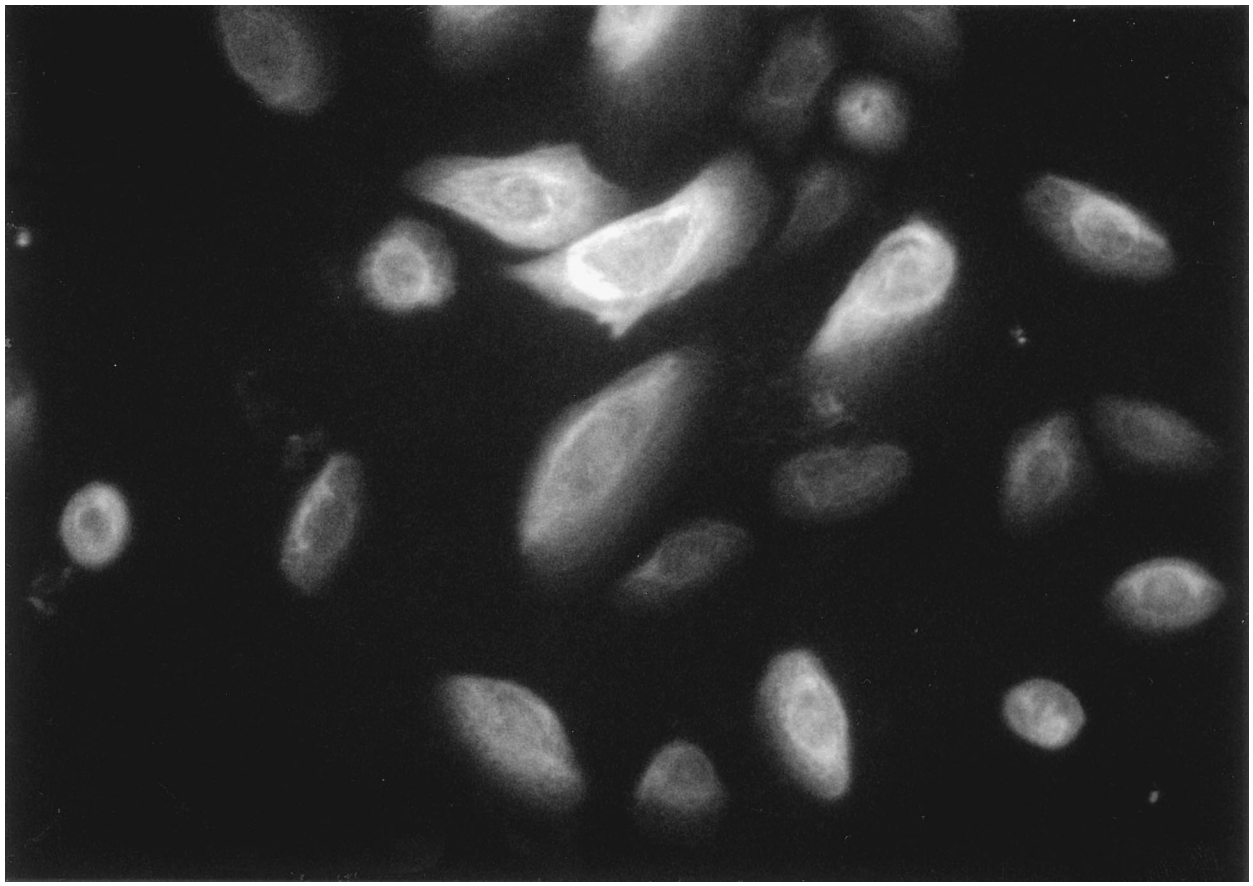

FIG. 2. Detection of cytokeratin in human gingival epithelial cells. Human gingival epithelial cells were cultured in a collagen-coated chamber slide. Then, double antibody immunohistochemistry was performed. The cells were incubated with mouse anti-cytokeratin type I and II antibodies and then with fluorescence-labeled $F(ab')_2$ goat anti-mouse immunoglobulin G heavy and light chains. The cells were observed with a fluorescence microscope.

considerably contaminated with LPS on the basis of the data from the *Limulus* test (Table 1).

Synergistic effect of LTA and IL-1 on human gingival fibroblasts to produce HGF/SF. The human gingival fibroblasts stimulated with both *S. sanguis* LTA and rHuIL-1α secreted a several-times-larger quantity of HGF/SF than those stimulated with the LTA or with rHuIL-1α alone (Fig. 4). A significant result for the synergistic effect of LTA and IL-1α on HGF/SF production was obtained by analysis of variance including an interaction term. Similar results were obtained with LTAs from *S. faecalis* and *L. plantarum* (data not shown). By contrast, the synthetic *E. coli*-type lipid A did not augment the activity of rHuIL-1α (data not shown). We further examined the HGF/SF production by the fibroblasts stimulated with the purified LTA of *S. sanguis* in the presence or in the absence of IL-1α. As shown in Fig. 5, the purified LTA significantly enhanced HGF/SF production and exhibited synergistic effects with IL-1α. Next, we extracted total RNA from human gingival fibroblasts stimulated with the crude *S. sanguis* LTA and/or rHuIL-1α and carried out Northern blot analysis to determine the levels of HuHGF mRNA expression. As shown in Fig. 6B, in the cells stimulated with LTA alone, the levels of HuHGF mRNA expression increased dose dependently. In cells treated with *S. sanguis* LTA and rHuIL-1α, the levels of HuHGF mRNA expression were more than additive.

Effect of rHuHGF on the DNA synthesis by human gingival epithelial cells. We next examined the effect of rHuHGF on DNA synthesis by human gingival epithelial cells. [^{3}H]thymidine incorporation in the epithelial cells increased dose dependently in cells treated with rHuHGF (Fig. 7). DNA synthesis in the cells reached a peak at a concentration of 10 ng/ml of rHuHGF.

DISCUSSION

Previously, we demonstrated that human fibroblasts stimulated with inflammatory cytokines such as IL-1α, IL-1β, and tumor necrosis factor alpha exhibited enhanced production of HGF/SF. In this study, we found that bacterial LTA enhanced the production of HGF/SF by human gingival fibroblasts. Furthermore, LTA and IL-1α enhanced synergistically the production of HGF/SF. The physicochemical properties and distribution of LTA in gram-positive bacterial cells are similar to those of endotoxic LPS of gram-negative bacteria. Therefore, several investigators suggested that LTA has LPS-like bioactivities (47). However, bacterial endotoxin did not enhance the production of HGF/SF in our assay system. The most active LTA in this study was that prepared from *S. sanguis*, which is the dominant bacterium in the oral cavity (18), especially in the early stage of dental plaque formation, and was suggested to be associated with gingival health (7, 33). HGF/SF is a mitogen and motogen on epithelial cells (20, 23, 27, 38). We found that gingival epithelial cells produce IL-1 in response to LPS. This inflammatory cytokine stimulated fibroblasts to produce HGF. Therefore, gingival fibroblasts stimulated by LTA released from oral streptococci represented by *S. sanguis* in the pres-

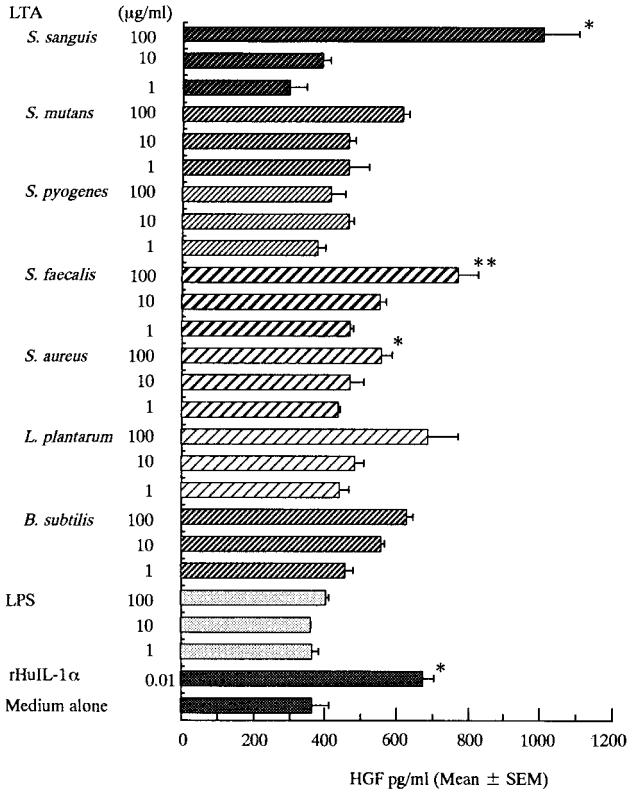

FIG. 3. HGF/SF secretion from human gingival fibroblasts stimulated with various LTAs and *S. abortusequi* LPS. Fibroblasts were cultured at a density of 2.0×10^4 cells per 100 μl per well in a 96-well plastic culture plate. Stimulants were added and incubated for 24 h. Triplicate culture supernatants were collected, and the concentration of HGF/SF in the pooled specimens was determined by an ELISA in duplicate assay. Values are means ± standard errors of the mean. * and **, statistically different from the control (medium alone), as determined by Student's *t* test (*, $P < 0.05$; **, $P < 0.01$).

ence of inflammatory cytokines may produce a larger amount of HGF/SF, which in turn stimulates gingival epithelial cells to proliferate and migrate to protect periodontal tissues against bacterial invasion. Furthermore, HGF/SF was suggested to activate osteoblastic cells (21) and stimulate the bone-resorptive activity of osteoclasts in the presence of osteoblastic cells

TABLE 2. IL-1-inducing activity of various LTA and reference LPS specimens in human gingival epithelial cell cultures[a]

Test material and origin	IL-1 concn (pg/ml) at indicated stimulant dose (μg/ml)		
	1	10	100
LTA			
S. sanguis	<10	<10	700
S. mutans	<10	<10	<10
S. pyogenes	<10	<10	<10
S. faecalis	<10	760	2,600
S. aureus	<10	<10	<10
B. subtilis	<10	<10	<10
LPS[b]	10,800	87,000	>100,000

[a] Human gingival epithelial cells were cultured at a density of 2.0×10^4 cells per 100 μl per well in a 96-well culture plate. Stimulants were added and incubated for 24 h. The culture supernatants were collected, and the IL-1 activity in the pooled supernatants was measured as described in the text.

[b] An ultrapurified LPS prepared from *S. abortusequi* (Novo-Pyrexal).

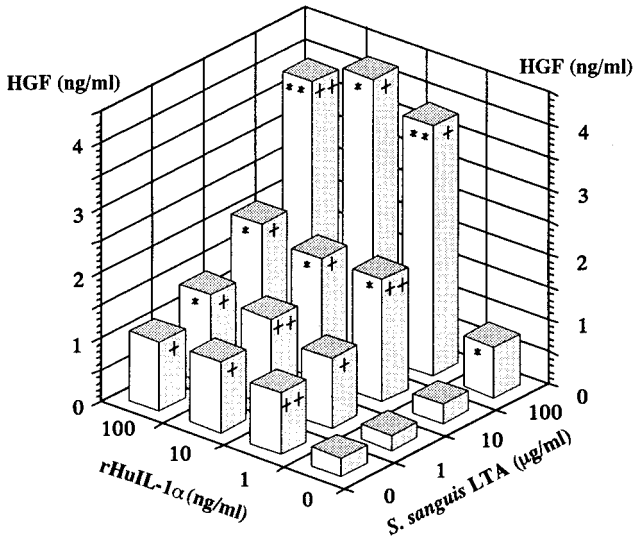

FIG. 4. Synergistic effect of LTA and rHuIL-1α on secretion of HuHGF by human gingival fibroblasts in culture. Experimental conditions were the same as described in the legend to Fig. 2, except for stimulants and their concentrations, which are indicated. Triplicate culture supernatants were collected, and the concentration of HGF/SF was determined by an ELISA in duplicate assay. Standard errors of the mean were less than 15% of the respective mean value. *, **, +, and ++, significantly different from the respective control (* and **, rHuIL-1α-alone control in the case of synergistic experiments or medium-alone control in the case of LTA-alone experiments; + and ++, LTA-alone control in the case of synergistic experiments or medium-alone control in the case of rHuIL-1α-alone experiments) as determined by Student's *t* test (* and +, $P < 0.05$; ** and ++, $P < 0.01$). A significant result ($P < 0.01$) for the synergistic effect of LTA and IL-1α on HGF/SF production was obtained by analysis of variance including an interaction term.

(11). Therefore, HGF/SF produced by gingival fibroblasts may be involved in the pathogenesis of periodontal diseases. In this context, an immunomodulator extracted from periodontal disease-related *Prevotella intermedia* with hot phenol-water, which is different from endotoxin, also enhanced the production of HGF/SF by human gingival fibroblasts (40). Thus, HGF/SF, like other cytokines, may be involved in both destructive and

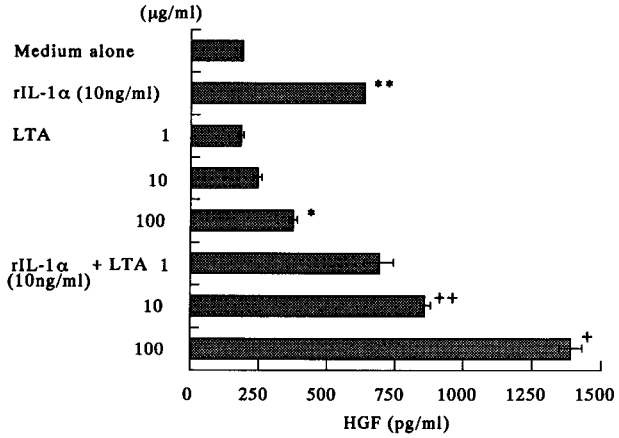

FIG. 5. HGF/SF secretion from human gingival fibroblasts stimulated with a purified LTA from *S. sanguis* and/or rHuIL-1α. Experimental conditions were the same as described in the legend to Fig. 2. *, **, +, and ++, significantly different from the respective control (* and ** medium-alone control; + and ++ LTA-alone control) as determined by Student's *t* test (* and +, $P < 0.05$; ** and ++, $P < 0.01$). A significant result ($P < 0.01$) for the synergistic effect of LTA and IL-1α on HGF/SF production was obtained by analysis of variance including an interaction term.

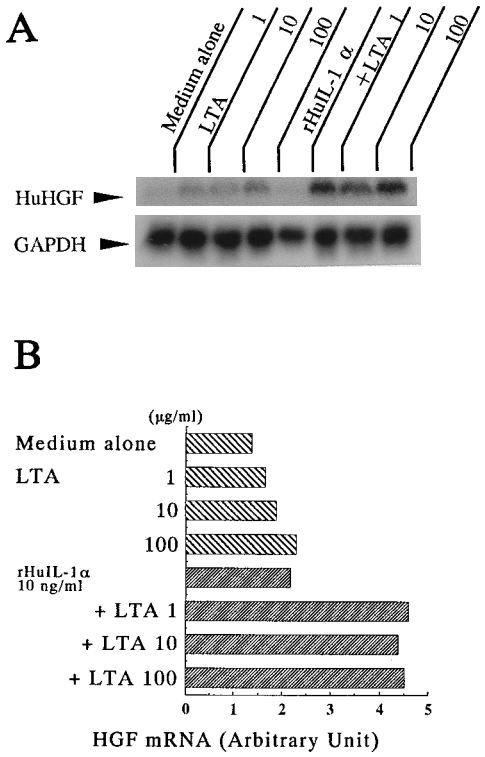

FIG. 6. HuHGF mRNA expression in human gingival fibroblasts stimulated with *S. sanguis* LTA in the presence or in the absence of rHuIL-1α. (A) Confluent human gingival fibroblasts were incubated with *S. sanguis* LTA in the presence or in the absence of rHuIL-1α (10 ng/ml) for 6 h. Total cellular RNA (20 μg per lane) extracted from the cells was electrophoresed and blotted onto a nylon membrane, and then HuHGF mRNA was determined by Northern blotting by using ^{32}P-labeled cDNA for HuHGF as a probe. Equal loading in lanes was checked by rehybridization with ^{32}P-labeled cDNA for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (B) Relative densities of the bands of HuHGF mRNA shown in panel A were analyzed by a BAS-1000 Mac. The results are representative of two different experiments.

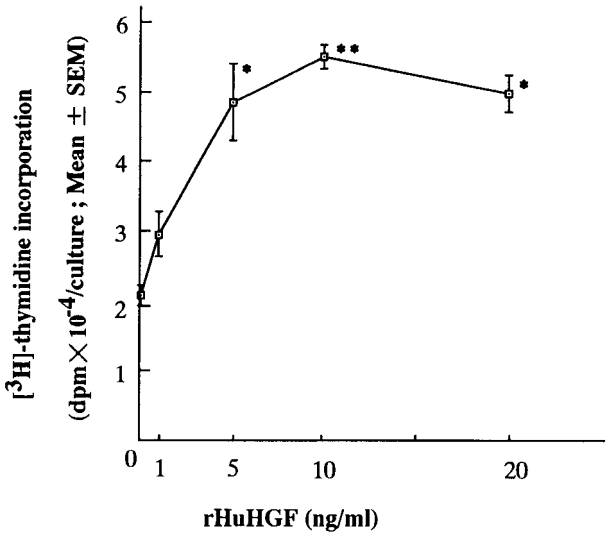

FIG. 7. Effect of rHuHGF on DNA synthesis by human gingival epithelial cells. Cells (5×10^3) were seeded into each well of a 96-well culture plate in 100 μl of keratinocyte–serum-free medium supplemented with 200 μg of kanamycin per ml. On the next day, the cells were washed and the medium was changed to a physiological concentration (1.8 mM) of Ca^{2+} without epidermal growth factor and bovine pituitary extract. Then, the cells were incubated with various concentrations of rHuHGF for 30 h in triplicate. During the final 6 h of culture, the cells were pulse-labeled with [^{3}H]thymidine, and then the radioactivity incorporated by the cells was measured. Values are means ± standard errors of the mean. * and **, significantly different from the control (medium alone), as determined by Student's *t* test (*, $P < 0.05$; **, $P < 0.01$).

reparative phases in periodontal diseases under different physiological and pathological conditions.

Most LTAs used in this study were commercial ones and were contaminated with endotoxin. The activity of *S. faecalis* LTA to induce IL-1 in gingival epithelial cell cultures may be derived from the endotoxin in the preparation. In the fibroblast cultures, the contamination of the preparation with endotoxin was not considered to have influenced our results, because endotoxin was inactive in this assay. However, the LTAs were also contaminated with proteins (data not shown), peptidoglycans, and/or β-glucan. Recently, De Kimpe et al. (6) reported that peptidoglycan and LTA acted in synergy to induce cytokines and nitric oxide synthases and caused shock in rats. Therefore, we prepared purified LTA from *S. sanguis* according to the method of Fischer et al. (10), in which proteins, endotoxin, and peptidoglycans/β-glucan were detected negligibly. The purified LTA thus obtained enhanced the production of HGF/SF in gingival fibroblasts and exhibited a synergistic effect with IL-1α to produce HGF/SF in the cells (Fig. 5).

At present, the mechanism of the synergistic effect of LTA and rHuIL-1α on the stimulation of gene expression of HuHGF is not clear. Miyazawa et al. (28) reported three consensus sequence elements in the HuHGF promoter region, namely, nuclear factor for IL-6 expression binding site (NF-IL6) (1), IL-6 response element (IL-6RE) (19), and nuclear factor κB (NF-κB) binding site (25), which are located at positions −151 to −143, −304 to −299, and −1019 to −1010 from the major transcription initiation site, respectively, and Tamura et al. (42) have suggested the involvement of these sequence elements in HuHGF induction by IL-1α, IL-1β, and tumor necrosis factor alpha in fibroblasts. In addition, Plaschke-Schlütter et al. (35) recently identified two regulatory sequences in the mouse HGF promoter region by deletion mapping followed by chloramphenicol acetyltransferase assays and footprint analysis, namely, a negative element at positions −258 to −239 from the major transcription start site and a positive element near the major transcription start site (−66 to +34). Because the promoter sequences of mouse and human HGF genes are highly homologous up to position −453 from the major transcription start site (35), these elements may be responsible for regulation of HuHGF gene expression by LTA or IL-1 in the fibroblasts used in this study, but further studies are needed to clarify the regulatory mechanism of HuHGF gene expression.

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REFERENCES

1. **Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto.** 1990. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. EMBO J. **9:**1897–1906.
2. **Ashida, M., and H. I. Yamazaki.** 1990. Biochemistry of the phenoloxidase

system in insects: with special reference to its activation, p. 239–265. *In* E. Ohnishi and H. Ishizaki (ed.), Molting and metamorphosis. Japan Scientific Societies Press, Tokyo, Japan.
3. **Bartlett, G. R.** 1959. Phosphorus assay in column chromatography. J. Biol. Chem. **234:**466–468.
4. **Charon, J. A., T. A. Luger, S. E. Mergenhagen, and J. J. Oppenheim.** 1982. Increased thymocyte-activating factor in human gingival fluid during gingival inflammation. Infect. Immun. **38:**1190–1195.
5. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
6. **De Kimpe, S. J., M. Kengatharan, C. Thiemermann, and J. R. Vane.** 1995. The cell wall components peptidoglycan and lipoteichoic acid from *Staphylococcus aureus* act in synergy to cause shock and multiple organ failure. Proc. Natl. Acad. Sci. USA **92:**10359–10363.
7. **Dzink, J. L., S. S. Socransky, and A. D. Haffajee.** 1988. The predominant cultivable microbiota of active and inactive lesions of destructive periodontal diseases. J. Clin. Periodontol. **15:**316–323.
8. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
9. **Fischer, W.** 1990. Bacterial phosphoglycolipids and lipoteichoic acids. Handb. Lipid Res. **6:**123–234.
10. **Fischer, W., H. U. Koch, and R. Haas.** 1983. Improved preparation of lipoteichoic acids. Eur. J. Biochem. **133:**523–530.
11. **Fuller, K., J. Owens, and T. J. Chambers.** 1995. The effect of hepatocyte growth factor on the behaviour of osteoclasts. Biochem. Biophys. Res. Commun. **212:**334–340.
12. **Furutani, Y., M. Notake, M. Yamayoshi, J. Yamagishi, H. Nomura, M. Ohue, R. Furuta, T. Fukui, M. Yamada, and S. Nakamura.** 1985. Cloning and characterization of the cDNAs for human and rabbit interleukin-1 precursor. Nucleic Acids Res. **13:**5869–5882.
13. **Galanos, C., O. Lüderitz, and O. Westphal.** 1969. A new method for the extraction of R lipopolysaccharides. Eur. J. Biochem. **9:**245–249.
14. **Galanos, C., O. Lüderitz, and O. Westphal.** 1979. Preparation and properties of a standardized lipopolysaccharide from *Salmonella abortus equi* (Novo-Pyrexal). Zentralbl. Bakteriol. Mikrobiol. Hyg. Abt. 1 Orig. Reihe A **243:**226–244.
15. **Gherardi, E., and M. Stoker.** 1990. Hepatocytes and scatter factor. Nature (London) **346:**228.
16. **Gohda, E., H. Tsubouchi, H. Nakayama, S. Hirono, O. Sakiyama, K. Takahashi, H. Miyazaki, S. Hashimoto, and Y. Daikuhara.** 1988. Purification and partial characterization of hepatocyte growth factor from plasma of patient with fulminant hepatic failure. J. Clin. Invest. **81:**414–419.
17. **Goldberg, I. D., and E. M. Rosen.** 1993. Hepatocyte growth factor-scatter factor (HGF-SF) and the c-*met* receptor. Birkhäuser Verlag, Basel.
18. **Hardie, J. M., and P. D. Marsh.** 1978. Streptococci and the human oral flora, p. 157–206. *In* F. A. Skinner and L. B. Quesnel (ed.), Streptococci. Academic Press, London.
19. **Hattori, M., L. J. Abraham, W. Northemann, and G. H. Fey.** 1990. Acute-phase reaction induces a specific complex between hepatic nuclear proteins and the interleukin 6 response element of the rat α_2-macroglobulin gene. Proc. Natl. Acad. Sci. USA **87:**2364–2368.
20. **Igawa, T., S. Kanda, H. Kanetake, Y. Saitoh, A. Ichihara, Y. Tomita, and T. Nakamura.** 1991. Hepatocyte growth factor is a potent mitogen for cultured rabbit renal tubular epithelial cells. Biochem. Biophys. Res. Commun. **174:**831–838.
21. **Inaba, M., H. Koyama, M. Hino, S. Okuno, M. Terada, Y. Nishizawa, T. Nishino, and H. Morii.** 1993. Regulation of release of hepatocyte growth factor from human promyelocytic leukemia cells, HL-60, by 1,25-dihydroxyvitamin D_3, 12-*O*-tetradecanoylphorbol 13-acetate, and dibutyryl cyclic adenosine monophosphate. Blood **82:**53–59.
22. **Kabashima, H., K. Maeda, Y. Iwamoto, T. Hirofuji, M. Yoneda, K. Yamashita, and M. Aono.** 1990. Partial characterization of an interleukin-1-like factor in human gingival crevicular fluid from patients with chronic inflammatory periodontal disease. Infect. Immun. **58:**2621–2627.
23. **Kan, M., G. Zhang, R. Zarnegar, G. Michalopoulos, Y. Myoken, W. L. McKeehan, and J. I. Stevens.** 1991. Hepatocyte growth factor/hepatopoietin A stimulates the growth of rat kidney proximal tubule epithelial cells (RPTE), rat nonparenchymal liver cells, human melanoma cells, mouse keratinocytes and stimulates anchorage-independent growth of SV-40 transformed RPTE. Biochem. Biophys. Res. Commun. **174:**331–337.
24. **Le, J., and J. Vilček.** 1987. Tumor necrosis factor and interleukin 1: cytokines with multiple overlapping biological activities. Lab. Invest. **56:**234–248.
25. **Lenardo, M. J., and D. Baltimore.** 1989. NF-κB: a pleiotropic mediator of inducible and tissue-specific gene control. Cell **58:**227–229.
26. **Masada, M. P., R. Persson, J. S. Kenney, S. W. Lee, R. C. Page, and A. C. Allison.** 1990. Measurement of interleukin-1α and -1β in gingival crevicular fluid: implications for the pathogenesis of periodontal disease. J. Periodontal Res. **25:**156–163.
27. **Matsumoto, K., K. Hashimoto, K. Yoshikawa, and T. Nakamura.** 1991. Marked stimulation of growth and motility of human keratinocytes by hepatocyte growth factor. Exp. Cell Res. **196:**114–120.
28. **Miyazawa, K., A. Kitamura, and N. Kitamura.** 1991. Structural organization and the transcription initiation site of the human hepatocyte growth factor gene. Biochemistry **30:**9170–9176.
29. **Miyazawa, K., H. Tsubouchi, D. Naka, K. Takahashi, M. Okigaki, N. Arakaki, H. Nakayama, S. Hirono, O. Sakiyama, K. Takahashi, E. Gohda, Y. Daikuhara, and N. Kitamura.** 1989. Molecular cloning and sequence analysis of cDNA for human hepatocyte growth factor. Biochem. Biophys. Res. Commun. **163:**967–973.
30. **Montesano, R., K. Matsumoto, T. Nakamura, and L. Orci.** 1991. Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. Cell **67:**901–908.
31. **Montesano, R., G. Schaller, and L. Orci.** 1991. Induction of epithelial tubular morphogenesis in vitro by fibroblast-derived soluble factors. Cell **66:**697–711.
32. **Nakai, S., K. Mizuno, M. Kaneta, and Y. Hirai.** 1988. A simple, sensitive bioassay for the detection of interleukin-1 using human melanoma A375 cell line. Biochem. Biophys. Res. Commun. **154:**1189–1196.
33. **Nisengard, R. J., M. G. Newman, and J. J. Zambon.** 1994. Periodontal disease, p. 360–384. *In* R. J. Nisengard and M. G. Newman (ed.), Oral microbiology and immunology, 2nd ed. The W. B. Saunders Co., Philadelphia.
34. **Obayashi, T., H. Tamura, S. Tanaka, M. Ohki, S. Takahashi, M. Arai, M. Masuda, and T. Kawai.** 1985. A new chromogenic endotoxin-specific assay using recombined limulus coagulation enzymes and its clinical applications. Clin. Chim. Acta **149:**55–65.
35. **Plaschke-Schlütter, A., J. Behrens, E. Gherardi, and W. Birchmeier.** 1995. Characterization of the scatter factor/hepatocyte growth factor gene promoter. J. Biol. Chem. **270:**830–836.
36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., p. 7.37–7.52. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
37. **Shima, N., M. Nagao, F. Ogaki, E. Tsuda, A. Murakami, and K. Higashio.** 1991. Tumor cytotoxic factor/hepatocyte growth factor from human fibroblasts: cloning of its cDNA, purification and characterization of recombinant protein. Biochem. Biophys. Res. Commun. **180:**1151–1158.
38. **Stoker, M., E. Gherardi, M. Perryman, and J. Gray.** 1987. Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. Nature (London) **327:**239–242.
39. **Strain, A. J., T. Ismail, H. Tsubouchi, N. Arakaki, T. Hishida, N. Kitamura, Y. Daikuhara, and P. McMaster.** 1991. Native and recombinant human hepatocyte growth factors are highly potent promoters of DNA synthesis in both human and rat hepatocytes. J. Clin. Invest. **87:**1853–1857.
40. **Takada, H., K. Iki, T. Sakuta, A. Sugiyama, S. Sawamura, and S. Hamada.** 1995. Lipopolysaccharides of oral black pigmented bacteria and periodontal diseases, p. 59–68. *In* J. Levin, C. R. Alving, R. S. Munford, and H. Redl (ed.), Bacterial endotoxins: lipopolysaccharides from genes to therapy. John Wiley & Sons, New York.
41. **Takada, H., J. Mihara, I. Morisaki, and S. Hamada.** 1991. Induction of interleukin-1 and -6 in human gingival fibroblast cultures stimulated with *Bacteroides* lipopolysaccharides. Infect. Immun. **59:**295–301.
42. **Tamura, M., N. Arakaki, H. Tsubouchi, H. Takada, and Y. Daikuhara.** 1993. Enhancement of human hepatocyte growth factor production by interleukin-1α and -1β and tumor necrosis factor-α by fibroblasts in culture. J. Biol. Chem. **268:**8140–8145.
43. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77:**5201–5205.
44. **Tokunaga, K., Y. Nakamura, K. Sakata, K. Fujimori, M. Ohkubo, K. Sawada, and S. Sakiyama.** 1987. Enhanced expression of a glyceraldehyde-3-phosphate dehydrogenase gene in human lung cancers. Cancer Res. **47:**5616–5619.
45. **Tsubouchi, H., Y. Niitani, S. Hirono, H. Nakayama, E. Gohda, N. Arakaki, O. Sakiyama, K. Takahashi, M. Kimoto, S. Kawakami, M. Setoguchi, T. Tachikawa, S. Shin, T. Arima, and Y. Daikuhara.** 1991. Levels of the human hepatocyte growth factor in serum of patients with various liver diseases determined by an enzyme-linked immunosorbent assay. Hepatology **13:**1–5.
46. **Weidner, K. M., N. Arakaki, G. Hartmann, J. Vandekerckhove, S. Weingart, H. Rieder, C. Fonatsch, H. Tsubouchi, T. Hishida, Y. Daikuhara, and W. Birchmeier.** 1991. Evidence for the identity of human scatter factor and human hepatocyte growth factor. Proc. Natl. Acad. Sci. USA **88:**7001–7005.
47. **Wicken, A. J., and K. W. Knox.** 1977. Biological properties of lipoteichoic acids, p. 360–365. *In* D. Schlessinger (ed.), Microbiology—1977. American Society for Microbiology, Washington, D.C.
48. **Yoshiyama, Y., N. Arakaki, D. Naka, K. Takahashi, S. Hirono, J. Kondo, H. Nakayama, E. Gohda, N. Kitamura, H. Tsubouchi, T. Ishii, T. Hishida, and Y. Daikuhara.** 1991. Identification of the N-terminal residue of the heavy chain of both native and recombinant human hepatocyte growth factor. Biochem. Biophys. Res. Commun. **175:**660–667.

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