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Inhibition of Human Immunodeficiency Virus Type 1 by Lactic Acid Bacteria from Human Breastmilk

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Abstract

Background: Human breastmilk provides a rich source of commensal lactic acid bacteria (LAB) to the infant during breastfeeding and stimulates abundant growth and colonization of these bacteria at mucosal surfaces in the infant gastrointestinal tract. While conferring critical nutritional and immunologic support to the developing newborn, breastmilk also serves as a vehicle for human immunodeficiency virus type 1 (HIV-1) transmission from mother to child during breastfeeding. Whether breastmilk LAB confer protection against mucosal exposure to HIV-1 in breastfeeding infants is unknown.

Study Design: In the present study, we sought to evaluate LAB isolated from the breastmilk of healthy women for the ability to inhibit HIV-1 infection in vitro. A total of 38 strains of breastmilk bacteria were evaluated in this study. Both heat-killed bacteria and cell-free conditioned supernatants from bacterial cultures were tested for the ability to inhibit infection with HIV-1 using viral isolates with tropism for CCR5 (R5), CXCR4 (X4), or R5/X4 dual-tropism.

Results: Significant inhibition of R5-tropic HIV-1 was demonstrated using heat-killed bacteria, most notably among breastmilk strains of *Lactobacillus* and *Pediococcus*. Selected strains of breastmilk LAB also demonstrated significant inhibition of HIV-1 infection against virus with tropism for X4 and R5/X4.

Conclusion: These results demonstrate for the first time that commensal LAB from human breastmilk inhibit HIV-1 infection in vitro and suggest a possible role for these bacteria in mucosal protection against HIV-1 in the breastfeeding infant.

Introduction

Transmission of human immunodeficiency virus (HIV) type 1 (HIV-1) from mother to child during breastfeeding is a significant source of new pediatric HIV-1 infections worldwide.[1] Yet, most breastfed infants remain uninfected, despite repeated exposure of their oral and gastrointestinal mucosal surfaces to both cell-free HIV-1 and cell-associated virus in maternal milk. Recognition of the protective benefits of breastfeeding has sparked considerable interest in determining factors in human breastmilk that may augment natural defenses in the infant and confer protection against vertical transmission of HIV-1 from mother to child in the setting of lactation.

Breastmilk is widely appreciated to be a complex biological fluid containing a variety of soluble factors and maternal cells with antimicrobial and immunomodulatory properties.[2] Among them are a number of nonimmune, preimmune, and immune factors that may modulate (positively or negatively) the risk of HIV-1 transmission via breastfeeding. Recently, we have shown that breastmilk contains innate factors that potently block infection of $CD4^+$ cells with cell-free HIV but do not affect infection with cell-associated virus, suggesting distinct inhibitory mechanisms exist, possibly involving multiple constituents in the milk.[3]

Comparatively little is known of the protective effects of naturally occurring microbiota in breastmilk, specifically strains of lactic acid bacteria (LAB) that are present in human milk and are known to rapidly colonize the infant oral and gastrointestinal mucosa soon after birth. Human milk provides a continuous source of bacteria to the infant for several weeks after birth, and these bacteria constitute a major factor in the initiation and development of the neonatal intestinal microbiota.[4–11] Bacteria present in breastmilk vary based on different geographic and environmental conditions and may include opportunistic pathogens. However, some strains of breastmilk bacteria have been found to display probiotic properties, including antimicrobial and immunomodulatory activities.[12–14]

In this context, we sought to assess in exploratory studies the ability of 38 naturally occurring bacterial strains,

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previously isolated from the breastmilk of healthy women, to inhibit HIV-1 infectivity in vitro.

Materials and Methods

Viruses

HIV-1 isolates used in these studies include R5-tropic HIV-1_{BaL} and two primary isolates, X4-tropic HIV-1_{HC4} and R5/X4 HIV-$1_{C7/86}$ (kindly provided to our laboratory by Dr. John Moore, Weill Medical College of Cornell University, New York, NY). All HIV-1 isolates were propagated in phytohemagglutinin-activated peripheral blood mononuclear cells and titered on TZM-bl cells (NIH AIDS Research and Reference Reagent Program [Germantown, MD], contributed by Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme Inc. [Durham, NC]).

Breastmilk bacteria and growth conditions

A total of 38 bacterial strains belonging to 15 different species were included in this work (Table 1). They were isolated from the breastmilk of healthy women in previous studies, and the methods for isolation and characterization of these bacteria have been described in detail elsewhere.[4,8] Strains of LAB used in the current study were grown in Mann Rogosa Sharpe broth (Oxoid, Basingstoke, UK), while the other bacterial strains were grown in brain-heart infusion (Oxoid) broth.

Preparation of heat-killed bacteria and cell-free bacterial supernatants

Flasks with 14 mL of Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (10%) and

Table 1. Inhibition of R5 HIV-1_{BaL} by Breastmilk Bacteria

Bacterial strain	*Inhibition of HIV infectivity (mean ± SD %)*[a]	*Bacterial growth (log cfu)*[b]	*Relative inhibition (% HIV inhibition/log cfu)*[c]
Lactobacillus curvatus/graminis VM25	55.50 ± 2.12	6.62	8.38
Lactobacillus fermentum VM8	23.00 (—)	6.11	3.76
L. fermentum VM25	41.50 ± 0.71	6.08	6.83
L. fermentum VM21	42.50 ± 0.71	6.08	6.99
L. fermentum VMA	52.50 ± 0.71	3.48	15.10
Lactobacillus gasseri VM21	14.00 ± 1.41	3.85	3.64
L. gasseri VM29	17.50 ± 3.54	4.60	3.80
L. gasseri VM23	28.50 ± 0.71	5.70	5.00
L. gasseri VM22	42.67 ± 0.58	6.30	6.77
Lactobacillus plantarum VM31	42.50 ± 20.51	7.30	5.82
Lactobacillus salivarius VM25	27.00 ± 9.64	4.90	5.51
L. salivarius VM5	31.57 ± 9.18	6.71	4.71
Lactobacillus vaginalis VM69	35.50 ± 7.78	7.67	4.63
L. vaginalis VM76	36.50 ± 4.95	7.49	4.87
Weissella cibaria VM6	35.50 ± 7.78	6.15	5.78
Weissella salpiscis/confusa VM22	24.50 ± 3.54	6.51	3.77
W. salpiscis/confusa VM8	33.00 ± 4.24	6.81	4.85
W. salpiscis/confusa VM11	37.50 ± 3.54	6.78	5.53
Lactococcus lactis VM17	22.00 ± 2.83	5.08	4.33
L. lactis VM265	29.00 ± 5.66	6.74	4.30
Pediococcus pentosaceus VM3	41.50 ± 17.68	6.41	6.47
P. pentosaceus VM17	43.00 ± 0.00	6.20	6.93
P. pentosaceus VM21	45.50 ± 4.95	6.85	6.65
P. pentosaceus VM95	49.00 (—)	7.70	6.36
Staphylococcus epidermidis VM10	27.00 ± 10.15	7.18	3.76
S. epidermidis VM38	28.67 ± 5.86	6.54	4.38
S. epidermidis VM69	29.00 ± 7.81	5.08	5.71
S. epidermidis VM4	32.00 ± 6.56	6.41	4.99
Staphylococcus hominis VM49	6.67 ± 3.79	5.85	1.14
S. hominis VM12	27.00 ± 10.15	5.18	5.22
S. hominis VM16	28.25 ± 4.86	6.18	4.57
S. hominis VM14	38.25 ± 6.13	6.20	6.17
Streptococcus salivarius VM18	22.33 ± 6.66	7.91	2.82
S. salivarius VM28	39.00 ± 9.90	5.41	7.20
Enterobacter sp. VM4	24.33 ± 2.52	6.85	3.55
Enterobacter sp. VM5	36.00 ± 8.72	6.15	5.86
Klebsiella pneumoniae VM1	6.67 ± 3.79	7.48	0.89
K. pneumoniae VM2	41.00 ± 7.96	5.36	7.65

[a]Percentage inhibition of R5-tropic HIV-1_{BaL} in TZM-bl target cells.
[b]Bacterial concentration expressed as the log cfu.
[c]Relative inhibition of infectivity expressed as the ratio between the values of inhibition of HIV infectivity (percentage) and the bacterial concentration (log cfu).

nonessential amino acids (1%) were inoculated with 140 μL of the respective overnight bacterial cultures ($\sim$9 $\log_{10}$ colony-forming units [cfu]/mL). The flasks were incubated for 24 hours at 37°C in aerobic conditions. After incubation, the subcultures were centrifuged at 30,000×g for 20 minutes to separate the bacterial cell pellet from the cell-free conditioned supernatant (CF-CS). The pellets were washed once with phosphate-buffered saline and centrifuged at 6,000×g for 20 minutes. The resulting bacterial pellets were resuspended in 14 mL of DMEM, and the suspension was separated into aliquots (1 mL). To inactivate the bacteria, the aliquots were heated at 65°C for 30 minutes prior to storage at −80°C. The CF-CS preparations were adjusted to neutral pH 7.0, sterilized by filtering (pore size, 0.2 μm), and stored at −80°C.

TZM-bl reporter assay for infectious HIV-1

TZM-bl cells were used to assess the effect of heat-killed bacteria and CF-CS on HIV-1 infectivity. TZM-bl cells are derived from HeLa cells and engineered to stably express the HIV-1 receptor (CD4) and co-receptors (CCR5 and CXCR4) required for infection along with reporter genes for luciferase and β-galactosidase under control of the HIV-1 promoter. For all experiments, HIV-1 infection of TZM-bl cells was quantified by measuring induction of luciferase expression. TZM-bl cells were first seeded at 1×10^4 cells per well in 96-well plates in a final volume of 100 μL of DMEM containing 1% fetal bovine serum and allowed adhere overnight. Heat-killed bacteria (100 μL of the bacterial cell suspension) or CF-CS (100 μL) was added to triplicate wells, followed by addition of 100 median tissue culture infective doses ($TCID_{50}$) of R5-tropic HIV-1_{BaL}. The plates were incubated for 48 hours at 37°C in 5% CO_2, after which HIV-1 infectivity was quantified in cell lysates by measuring the levels of luciferase activity using the Steady-Glo® luciferase assay system (Promega, Madison, WI). Cell viability was quantified in the cultures after 48 hours using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt-based reagent (CellTiter 96® AQ_{ueous} One, Promega).

Inhibition of HIV-1 infection was calculated as a percentage of the luminescence values in the treated wells compared to untreated control cultures. An additional parameter, representing the ratio between the values for HIV inhibition (percentage) and the bacterial concentration (log cfu), was also calculated for each strain and reported in Table 1 as the relative inhibition of infectivity.

Statistical analysis

Association between percentage HIV-1 inhibition using the heat-killed bacteria and the amount of bacteria added to the assay was evaluated using Pearson's correlation coefficient. Microbiological data, recorded as cfu, were transformed to logarithmic values. Differences in the relative inhibition of HIV-1 infectivity among the main bacterial genuses were evaluated using the Kruskal-Wallis test.

After the normality and variance homogeneity of the data were confirmed, Student's t test was used to assess the significance of differences in HIV-1 inhibition mediated by heat-killed bacteria and by CF-CS, with comparisons made using one-way analysis of variance (ANOVA) followed by Fisher's least significant difference procedure. Data were analyzed using the statistical software package StatGraphics Centurion XV (version 15.0.04) (StatPoint Technologies, Inc., Herndon, VA).

Results

The bacterial strains used in this study were previously isolated from the breastmilk of healthy lactating HIV-negative women and represent 38 different strains among the four main genuses of *Lactobacillus*, *Weissella*, *Pediococcus*, and *Staphylococcus* (Table 1). The characteristics of these bacterial isolates have been described elsewhere.[4,8]

We first sought to evaluate the effect of killed bacteria on HIV-1 infectivity using an R5-tropic isolate of HIV-1 (HIV-1_{BaL}). The results of these experiments demonstrate a reduction in viral infectivity ranging from 6.7% to 55.5% depending on the strain of bacteria tested (Table 1). The greatest inhibition of HIV-1_{BaL} was achieved using bacterial cells from strains of *L. curvatus* VM25 (55.5%), *L. fermentum* VMA (52.5%), *P. pentosaceous* VM95 (49.0%), and *P. pentosaceous* VM21 (45.5%). Of note is that all the pediococcal strains inhibited R5 HIV-1_{BaL} infection between 41.5% and 49.0% as compared to untreated controls. By comparison, the lowest levels of HIV-1_{BaL} inhibition were observed using heat-killed bacteria from *S. hominis* VM49 (6.7%) and *K. pneumoniae* VM1 (6.7%).

We found variable rates of growth among the different bacterial strains in culture, but there was no statistically significant relationship between bacterial growth achieved in culture and the subsequent antiviral activity of the killed bacteria (Pearson's correlation coefficient = −0.097). Additionally, there were no significant differences among the main bacterial groups tested as to the relative inhibition of HIV-1 infectivity as defined by the inhibition of HIV infectivity/log cfu ($p = 0.090$ by Kruskal-Wallis test).

In further experiments, we tested the CF-CS from the bacterial cultures for HIV-inhibitory activity. Among the 38 strains tested, inhibition of R5 HIV-1_{BaL} was observed with CF-CS from just six strains (Table 2) with the highest levels of inhibition associated with *L. salivarius* VM5 (41.8%), *L. gasseri* VM22 (39.2%), and *L. lactis* VM17 (30.0%). Two of these strains (*L. salivarius* VM5 and *L. lactis* VM17) displayed greater anti-HIV activity associated with the CF-CS (Table 2) compared to the corresponding killed bacteria (Table 1), suggesting distinct antiviral effects mediated by bacterial cellular components versus soluble bacterial products.

Table 2. Inhibition of R5 HIV-1_{BaL} by CF-CS from Breastmilk Bacteria

Bacterial strain	*% inhibition of HIV-1*
L. salivarius VM5	41.8 ± 4.92
L. gasseri VM22	39.2 ± 3.49
L. lactis VM17	30.0 ± 1.41
S. salivarius VM18	17.0 ± 2.83
S. epidermidis VM4	11.5 ± 3.53
S. epidermidis VM69	10.0 ± 6.55

Data are mean ± SD values for percentage inhibition of R5 HIV-1_{BaL} infection of TZM-bl target cells treated with CF-CS from bacterial cultures compared to untreated control cells.

Table 3. Inhibition of X4 and R5/X4-Tropic HIV-1 by LAB from Human Breastmilk

Viral isolate (tropism), bacterial strain	*Inhibition of infectivity (%)*	
	Killed bacteria	*CF-CS*
HIV-1HC4 (X4)		
L. salivarius VM5	49.50 ± 30.41	20.50 ± 3.54
L. gasseri VM22	81.50 ± 2.12	11.50 ± 7.78
S. salivarius VM18	81.00 ± 1.41	4.00 ± 1.41
L. lactis VM17	76.50 ± 3.54	11.00 ± 0.00
HIV-1C7/86 (R5/X4)		
L. salivarius VM5	36.67 ± 12.42	12.50 ± 3.54
L. gasseri VM22	37.50 ± 19.09	8.00 ± 5.66
S. salivarius VM18	64.00 (—)	12.00 ± 1.41
L. lactis VM17	62.00 ± 4.24	1.00 (—)

Data are mean ± SD values for percentage inhibition of HIV-1 in TZM-bl target cells.

Four strains of breastmilk bacteria (*L. salivarius* VM5, *L. gasseri* VM22, *L. lactis* VM17, and *S. salivarius* VM18) with demonstrated biological activity against R5 HIV-1_{BaL} (Table 1) were selected for further evaluation against two additional primary HIV-1 isolates with tropism for CXCR4 (HIV-1_{HC4}) or R5/X4 (HIV-$1_{C7/86}$). The inhibitory activity of both the heat-killed bacteria and the corresponding CF-CS from these strains was evaluated (Table 3). Results demonstrate that the heat-killed bacteria from all four strains reduced HIV-1 infection with both X4 HIV-1_{HC4} and R5/X4 HIV-$1_{C7/86}$, suggesting that the inhibitory effect mediated by the bacterial cells is not restricted by the co-receptor tropism of the virus.

Although inhibition of HIV-1 was not determined by the virus tropism, differences were apparent in the levels of inhibition among the four strains tested. In all cases, the killed bacteria yielded greater inhibition of X4 HIV-1_{HC4} compared to R5 HIV-1_{BaL} (mean percentage inhibition of 72% vs. 30%, $p = 0.036$ by one-way ANOVA), while an intermediate inhibitory effect was observed using R5/X4 HIV-$1_{C7/86}$ (mean percentage inhibition of 50%). The observed differences in inhibition of R5, X4, and R5/X4 strains of HIV-1 by killed bacteria suggest that inhibition is influenced by interaction of the bacteria with sites on the HIV-1 envelope glycoproteins, which contain the determinants of viral tropism. Interestingly, CF-CS from these same four strains showed greater inhibition of R5 HIV-1 (mean percentage inhibition of 32%) compared to either X4 (12%) or R5/X4 (8%) HIV-1 ($p = 0.006$ by one-way ANOVA) (Tables 2 and 3).

Taken together, these results indicate that distinct mechanisms act to block HIV infection depending on whether the virus is exposed to bacterial cells or to soluble microbial products. The majority of strains tested in this study demonstrated HIV-1 inhibition only with bacterial cells, but several strains were able to reduce viral infection as a result of soluble microbial products in the conditioned supernatants.

Discussion

Human colostrum and breastmilk play key roles in the initiation, development, and composition of the infant intestinal microbiota. These complex biological fluids contain a variety of factors, such as inmunoglobulin, inmunocompetent cells, fatty acids, polyamines, oligosaccharides, lysozyme, lactoferrin, and antimicrobial peptides that modulate microbial growth in the intestinal ecosystem, and they are important and continuous sources of commensal bacteria to the infant intestine.[4–6,10,11] In the present study, we demonstrate for the first time that strains of LAB isolated from human breastmilk have the ability to inhibit infection with HIV-1 in vitro.

Our results show that HIV-inhibitory activity is largely associated with the bacterial cells and, in a few cases, with the CF-CS from the bacterial cultures, suggesting that more than one mechanism may be used by endogenous bacteria to block viral infectivity. HIV-inhibitory activity was demonstrated against R5-, X4-, and dual-tropic strains HIV-1 and was not strictly associated with the tropism of the virus. However, differences in the potency of inhibition among R5, X4, and R5/X4 viruses using either killed bacteria or CF-CS suggest that HIV-1 tropism, which is governed by determinants in the viral envelope glycoproteins, may influence HIV-1 interaction with both bacterial cells and products.

The mechanisms by which LAB from human breastmilk inhibit HIV-1 in vivo are not known. Previous studies have shown that oral lactobacilli isolated from the saliva of healthy human subjects can bind and capture HIV-1 in vitro through lectin-like interactions between mannose residues on the viral envelope glycoprotein gp120 and the bacteria.[16,17] This physical interaction has been likened to an "HIV-1 trap"[16,17] and may serve to sequester virus and prevent it from attaching to cellular receptors on mucosal tissues. Strains of LAB present in human breastmilk may similarly act to capture HIV-1 and prevent access of the virus to cells lining the infant's gastrointestinal tract. Presumably these physical interactions are mediated by constituents of the bacterial surface structures, such as peptidoglycans or exopolysaccharide moieties, and may depend on the strain of bacteria, the stage of bacterial growth, and the growth conditions of the bacteria within the localized environment.

Although HIV-inhibitory activity was consistently associated with strains of *Lactobacillus* and *Pediococcus* (from 17% to 55%), we observed differences in antiviral activity even among different isolates of the same strain (Table 1, e.g., *L. gasseri*). These differences were independent of bacterial growth in culture, suggesting subtle variation in the composition of the bacterial cell surface may affect the ability of a given strain to interact with HIV-1. Indeed in other studies, only seven of 107 strains of oral *Lactobacillus* isolated from the saliva of 100 volunteers were found to co-aggregate with yeast through a mannose-glucose-dependent interaction analogous to the interaction with HIV-1.[16,17]

Overall, these findings suggest that strains of bacteria in human breastmilk have the capacity to inactivate HIV-1 by interaction with bacterial cells; however, the HIV-inhibitory activity is highly strain-specific and may be affected by subtle differences in the composition of bacterial surface components. The degree to which human breastmilk LAB help prevent HIV-1 infection in vivo may therefore depend not only on exposure of the infant to maternal bacteria during breastfeeding, but also on exposure to those strains with the greatest ability to interact with and bind HIV.

In our hands, the anti-HIV activity of LAB from human breastmilk was predominantly associated with the bacterial

cells; however, we did observe HIV inhibition associated with the cell-free supernatants from six strains of breastmilk bacteria, notably strains of *Lactobacillus* and *Lactococcus* (Table 2). It is well known that LAB produce an array of soluble metabolites and signaling molecules, including factors such as bacteriocins, which act as natural antimicrobial agents by preventing the outgrowth of competing strains of bacteria and other organisms in the local milieu.[18–20] Several classes of bacteriocins from LAB have been identified, and the range of their activity against pathogenic bacteria has been explored most often in the context of the food industry.[21,22]

The activity of microbial bacteriocins against viruses is less well defined but may still have a significant role in host defense. A small cationic peptide (Enterocin CRL35) produced by the lactic acid bacterium *Enterococcus faecium* CRL35 has been shown to inhibit the late stages of herpes simplex virus types 1 and 2 replication in vitro,[23] providing clear evidence that a naturally produced bacterial peptide can exert antiviral activity. Interestingly, Enterocin CRL35 blocked herpes simplex virus late protein synthesis in Vero cells without causing cellular cytotoxicity, suggesting distinct mechanisms of action that target bacteria versus cell-associated viruses. In the present study, strains of breastmilk LAB with the highest levels of anti-HIV activity in the CF-CS were found to be positive for bacteriocins (V.M., unpublished data), suggesting a possible role for these factors in HIV inhibition.

In vivo application of selected strains of breastmilk LAB in the form of probiotics may be considered a valid approach to augment host immune responses and strengthen barrier function in the gastrointestinal tract of both adults and infants.[7,12,14] *L. gasseri* CECT5714, a strain isolated from breastmilk, is metabolically active in the human intestine, increasing the production of functional metabolites such as butyrate 38, which is the main energy source for enterocytes and plays a key role in maintaining the mucosal barrier in the intestine of healthy individuals.[24] Oral administration of *L. salivarius* CECT5713 and *L. fermentum* CECT5716, other strains isolated from human milk, has been shown to facilitate the recovery of inflamed tissue in a murine colitis model, an effect associated with amelioration of inflammatory response mediators, such as tumor necrosis factor-α and nitric oxide.[25] HIV transmission is exacerbated by underlying conditions and co-infections that stimulate inflammatory responses in tissues.[26–28] Strains of probiotic breastmilk bacteria with both antiviral activity and proven anti-inflammatory effects may therefore be considered advantageous for application in breastfeeding infants exposed to HIV to reduce the risk of virus transmission.[29]

While promising in concept, further studies are needed to fully elucidate the mechanism(s) by which breastmilk LAB inhibit the infectivity of HIV-1, by both bacterial cells and soluble microbial products, and to identify those LAB strains with the most significant potential for development as probiotics.

Conclusions

Our results demonstrate for the first time that strains of LAB present in human breastmilk have the capacity to significantly reduce HIV-1 infection in vitro. These findings suggest that commensal bacteria present in human breastmilk, in addition to modulating the maturation of the immune system and development of associated microbiota in the infant gastrointestinal tract, may also play a role in protection against viral pathogens such as HIV-1. Transmission of HIV-1 in breastmilk occurs across mucosal surfaces in the infant oral, pharyngeal, and gastrointestinal tissues, sites that are abundantly colonized with commensal bacteria within days of birth. As many of the strains of colonizing bacteria have their origins in the mother's breastmilk, this suggests a unique mechanism for conferring protection to the infant against viral pathogens. Further work is now needed to identify those strains of bacteria with the most potent antiviral activity and to elucidate the mechanisms by which they exert this effect. Application of breastmilk LAB in the form of probiotics may provide a promising avenue by which to strengthen the natural mucosal defenses in the gastrointestinal tract of breastfeeding infants born to HIV-infected mothers.

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Disclosure Statement

No competing financial interests exist.

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