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Direct Activity of Recombinant Human Lactoferrin against *Helicobacter pylori*

STEPHAN MIEHLKE, RITA REDDY, MICHAEL S. OSATO, PAULINE P. WARD, ORLA M. CONNEELY, AND DAVID Y. GRAHAM*

Department of Medicine, Veterans Affairs Medical Center, and the Division of Molecular Virology and the Department of Cell Biology, Baylor College of Medicine, Houston, Texas

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We report the activity of recombinant human lactoferrin against *Helicobacter pylori*. Lactoferrin exerted a time- and dose-dependent action against 8 of the 13 clinical isolates of *H. pylori* tested in vitro. These results highlight a potential therapeutic use for lactoferrin against *H. pylori* infection.

Lactoferrin is a multifunctional iron-binding glycoprotein which is found in high concentrations in milk and in several mucosal secretions, i.e., saliva, tears, and plasma at concentrations of up to 14 mg/ml (6, 8) and also at high concentrations within specific granules of polymorphonuclear leukocytes (7). Lactoferrin is an important factor in the host defense against a wide range of gram-negative and gram-positive bacteria (1, 2). Two different mechanisms appear to be involved in the antibacterial action of lactoferrin at the mucosal surface. First, the high iron-binding affinity of the protein causes a bacteriostatic effect by depriving bacteria that require iron as an essential growth nutrient (3). However, this effect may often be temporary, since certain gram-negative bacteria are capable of adapting to iron-restrictive conditions by synthesizing siderophores that can remove iron from lactoferrin. The second antibacterial property of lactoferrin is due to an antimicrobial domain consisting mainly of a loop of 18 amino acid residues located in a region distinct from its iron-binding sites (2). At physiological concentrations, apolactoferrin directly damages the outer membrane of gram-negative bacteria by causing release of lipopolysaccharides (4). We have previously reported the production of recombinant human lactoferrin (rhLF) in *Aspergillus awamori* (9). The recombinant protein was shown to be indistinguishable from native breast milk lactoferrin in terms of size, immunoreactivity, iron-binding, receptor-binding, and antimicrobial properties (9). In the present study, we evaluated the in vitro effect of rhLF on the growth of *Helicobacter pylori*, the major pathogen in gastritis and peptic ulcer disease (5).

Thirteen clinical isolates of *H. pylori* from patients with duodenal ulcer disease were inoculated on brain heart infusion agar plates supplemented with 7% fresh horse blood and incubated at 37°C, 12% CO_2, and 100% humidity. Bacteria were harvested, resuspended, and diluted in saline to obtain an optical density at 625 nm of 0.2 (McFarland standard 1).

rhLF (95% iron free) at a stock concentration of 21 mg/ml in 5 mM sodium phosphate (pH 7.5) was used. A 2-ml assay in 24-well plates, each containing 1.9 ml of brain heart infusion agar was prepared. The tested concentrations of rhLF were 0.375, 0.75, 1.5, and 3.0 mg/ml. One hundred microliters of each bacterial suspension was inoculated into each well. A negative control was prepared with phosphate buffer. The plates were incubated at 37°C, 12% CO_2, and relative humidity of 100%. At 24, 48, and 72 h, 100 μl of each well was inoculated on blood agar plates in a 1:100 dilution with saline and incubated. Colony counting was performed after 5 days of incubation.

rhLF exhibited potent activity against *H. pylori* in a dose-dependent manner. Table 1 shows the number of cultures inhibited of the 13 *H. pylori* strains tested after incubation with increasing concentrations of rhLF. To determine whether the antibacterial effect was related to the iron concentration of the growth medium, two *H. pylori* strains that were susceptible to 3.0 mg of rhLF per ml were incubated on iron-rich blood agar plates containing 0.75 or 3.0 mg of rhLF per ml. Neither strain grew at an rhLF concentration of 3.0 mg/ml, while both grew poorly at a concentration of 0.75 mg/ml, suggesting that the iron concentration of the culture media does not influence the bactericidal effect of rhLF on *H. pylori* (data not shown).

H. pylori infection is now established as the major pathogenic factor in chronic gastritis and peptic ulcer disease. In addition, there is accumulating evidence that *H. pylori* plays an important role in the process of gastric carcinogenesis (5). The results presented in this study demonstrate an in vitro dose-dependent activity of rhLF against *H. pylori*. This activity was independent of the iron concentration of the growth medium. We found a total loss of viable CFU at a recombinant lactoferrin concentration of 1.5 mg/ml after 72 h in 8 of 13 *H. pylori* strains (61.5%) tested.

The in vitro activity of lactoferrin against a wide range of bacteria has previously been reported (3, 4). In addition, an isolated peptide containing a highly cationic domain from the N terminus of the protein was shown to exert a more potent antimicrobial activity than the native lactoferrin (2). The isolated peptide, as well as its synthetic analog, has a direct activity against a wide range of gram-negative and gram-positive bacteria. Intriguingly, in the present study, the effect of recom-

TABLE 1. Number of the 13 *H. pylori* strains showing inhibition of growth after incubation with increasing concentrations of rhLF

Time (h)	No. of strains inhibited at rhLF concn (mg/ml) of[a]:				
	0.18	0.375	0.75	1.5	3.0
24	0	0	0	0	0
48	0	0	4	7	6
72	0	0	5	8	8

[a] All values represent number of strains inhibited out of 13 strains tested. MICs ranged from 9.25 to 18.5 μM.

* Corresponding author. Mailing address: Veterans Affairs Medical Center (111D), 2002 Holcombe Blvd., Houston, TX 77030. Phone: (713) 795-0232. Fax: (713) 790-1040.

binant lactoferrin against *H. pylori* mimics the action of the isolated peptide more than the action of the native protein, although the exact mechanism of action is currently unknown.

In summary, we have demonstrated a potent activity of rhLF against clinical isolates of *H. pylori*. This activity was bactericidal in nature and was independent of the iron concentration of the growth medium. A potential role for rhLF in the treatment of human *H. pylori* infection is currently being evaluated.

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