J. Pharm. Pharmacol. 2000, 52: 1541–1546
Received May 11, 2000
Accepted August 4, 2000

Recombinant Human Lactoferrin is Effective in the Treatment of *Helicobacter felis*-infected Mice

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Abstract

Recombinant human lactoferrin possesses in-vitro antibiotic and anti-inflammatory activity similar to the native form. It was tested for in-vivo activity in mice infected with the gastritis-inducing bacterium *Helicobacter felis*.

A two-week course of treatment with lactoferrin was sufficient to partially reverse both infection-induced gastritis and the infection rate, and fully reverse gastric surface hydrophobicity changes. A comparison of lactoferrin with amoxicillin and standard triple therapy revealed no differences in infection rate.

These results show that recombinant human lactoferrin is effective in a mouse model of *Helicobacter* infection, and support further testing of this promising agent for this application.

Lactoferrin is a multi-functional glycoprotein with antibiotic, anti-inflammatory and immune-modulatory properties (Baveye et al 1999; Vorland 1999). It is found in neutrophils and in human exocrine secretions such as breast milk, tears, saliva, bile and pancreatic juice, where it is associated with mucosal defense. Lactoferrin derived from human and bovine milk has been tested for in-vitro activity in a number of systems, but its in-vivo efficacy has not been as fully investigated. The reasons for this are related to the high cost and limited availability of a sufficiently purified and characterized product. Consequently, the potential use of lactoferrin as a pharmaceutical agent has led to the development of a recombinant human form which was shown to possess many identical properties to the native form (Ward et al 1992, 1995).

We have tested recombinant human lactoferrin for efficacy in an animal model of bacterial-induced gastritis, the *Helicobacter felis*-infected mouse (Lee et al 1990; Dick-Hegedus & Lee 1991; Fox et al 1993). This is a model for *Helicobacter pylori*-induced gastritis in man, a known precursor condition to either peptic ulcer or gastric cancer (NIH 1994; Honda et al 1998; Watanabe et al 1998). We (Dial et al 1998) and others (Wada et al 1999) previously showed that bovine lactoferrin was capable of suppressing *Helicobacter* infection in mice. Miehlke et al (1996) also showed that recombinant human lactoferrin inhibited the growth of *H. pylori* in an in-vitro culture system. Therefore, this study was designed to investigate the effect of recombinant human lactoferrin on *H. felis*-infected mice, and then compare it with the activity of another antibiotic (amoxicillin) and with the currently accepted triple therapy (metronidazole, tetracycline and bismuth subsalicylate) for *Helicobacter* infection. Our results suggest that recombinant human lactoferrin holds promise for the treatment of this common, chronic infection.

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Materials and Methods

Materials Recombinant human lactoferrin was supplied by Agennix Inc. (Houston, TX). It was dissolved immediately before use each day in sterile saline. The antibiotics amoxicillin, metronidazole and tetracycline were purchased from Sigma Chemical Co. (St Louis, MO). Bismuth subsalicylate was a gift from Procter and Gamble Co. (Cincinnati, OH). These latter four agents were administered as suspensions in saline.

Animals
C57BL/6 female mice were obtained from Taconic Farms, Germantown, NY. At 4–6 weeks of age, *Helicobacter* infection of mice was accomplished by oral inoculation of 10^9 colony-forming units per mouse with *H. felis* (ATCC 49179) a total of 3 times at 2-day intervals. Mice were then used at 4–9 weeks post infection. Age-matched, uninfected control mice were maintained during the same time. All protocols were approved by the Animal Welfare Committee of The University of Texas Health Science Center at Houston.

Time-course of lactoferrin effects on H. felis *infection*
At 2 and 4 weeks post inoculation, infected and uninfected mice were killed with carbon dioxide and gastric tissue was collected for analysis of gastritis (stomach/body weight ratio), surface hydrophobicity as a measure of gastric barrier integrity (contact angle) and bacterial presence (urease colour test). These parameters have been previously validated and shown to reflect disease processes (Lichtenberger et al 1999). Tissue was fixed in 10% formalin, embedded in paraffin and processed for haematoxylin and eosin staining. Histological analysis of gastritis was performed in a blinded manner using a score of 0–6, with 0 denoting no inflammatory cells (polymorphonuclear cells or mononuclear cells) in the submucosa or lamina propria and 6 denoting marked numbers of inflammatory cells in both areas, similar to Ferrero et al (1994). From 4–6 weeks post infection, the remaining infected mice were randomly distributed into treatment groups ($n = 8$ per group) and administered, daily by oral gavage in 0·1-mL volume, either saline (control), recombinant human lactoferrin $100\,mg\,kg^{-1}$, or triple therapy (metronidazole 0·675 mg, tetracycline 1·5 mg and bismuth subsalicylate 0·185 mg). These doses were chosen for their similarity to (bovine lactoferrin: Dial et al (1998)), or effectiveness in (triple therapy: Dick-Hegedus & Lee (1991)), previous studies. Dosing was always performed in the early afternoon. After 14 days of treatment, and after an additional 14 days without treatment, mice were killed with carbon dioxide and tissue was collected.

Dose–response of lactoferrin on H. felis *infection*
Mice were infected with *H. felis* as described above and at 7 weeks post infection, the mice were distributed into groups ($n = 8$ per group) treated with either saline (control) or recombinant human lactoferrin 100, 200, or $400\,mg\,kg^{-1}$. After 14 days of treatment, the mice were killed and gastric tissue was collected for analysis of gastritis, surface hydrophobicity and bacterial presence.

Comparison of lactoferrin and other antibiotics on H. felis *infection*
Mice were infected with *H. felis* as described above and distributed into groups ($n = 7–12$ per group) treated with saline control, recombinant human lactoferrin $200\,mg\,kg^{-1}$, amoxicillin $19\,mg\,kg^{-1}$ (approx. 50% the recommended dose), recombinant human lactoferrin 10 or $100\,mg\,kg^{-1}$ plus amoxicillin at $19\,mg\,kg^{-1}$, or triple therapy. Daily dosing continued for 14 days, after which the mice were killed and gastric tissue was collected for estimation of gastritis, surface hydrophobicity, and bacterial presence.

Measurements
All measurements were performed under sterile conditions on tissue collected immediately following death. Gastritis was estimated by determination of the ratio of the wet weight of the entire stomach (mg) to the body weight of the mouse (g). Surface hydrophobicity of the gastric mucosa as measured by contact angle analysis was determined as previously described (Hills et al 1983). We have shown these two parameters to be generally sensitive indicators of infection in this mouse model (Lichtenberger et al 1999). Bacterial presence was determined by placing a biopsy containing both antral and oxyntic tissue into a solution to test for the presence of urease (Hazell et al 1987). If a colour change was noted within 24 h, it was considered positive for the bacteria.

Statistical analysis
The results depicted in the figures are expressed as the mean ± standard error of the mean. Statistical significance was determined by Student's *t*-test. The results in the tables are expressed as a proportion and were tested for differences by Fisher's exact test. The level of significance was set at $P < 0·05$.

Results

Time-course of lactoferrin effects on H. felis *infection*
At least 4 weeks of infection were required to elicit consistent differences in gastritis (Figure 1) and

contact angle (Figure 2) in *H. felis*-infected mice. Subsequently, the 2-week course of treatment with a single daily dose of recombinant human lactoferrin (100 mg kg^{-1}) was able to prevent some, but not all, of the changes that occurred in saline-treated infected mice, and gave a response that was between, and not significantly different from, that of the uninfected control and the *H. felis* + saline treatment. A 2-week wash-out period in which no treatment was given, gave results similar to those found immediately upon treatment completion. Therefore, subsequent studies were always terminated at the end of the dosing period. For comparison, the triple-therapy group at the end of the study had a stomach/body weight ratio of $8{\cdot}9 \pm 0{\cdot}1$ and a contact angle of $28 \pm 3°$, both of which were significantly different from the *H. felis* + saline group and not different from the uninfected control. Urease tests for the presence of the bacteria showed no improvement from recombinant human lactoferrin treatment (100% infection rate immediately after treatment and 62% two weeks later), but a clear benefit from triple therapy (13% infection rate two weeks after treatment). Histological analysis of gastritis in uninfected controls and infected mice (with or without lactoferrin treatment) revealed a positive correlation with the stomach/body weight ratio ($r = 0{\cdot}88$, $P < 0{\cdot}0005$). This gastritis (inflammation) score was significantly higher in saline-treated infected mice than in uninfected controls, and was significantly reduced by triple therapy (Figure 3). The dose of lactoferrin used in this study produced an inflammation score that was between, and not statistically different from, that of uninfected controls and saline-treated infected mice.

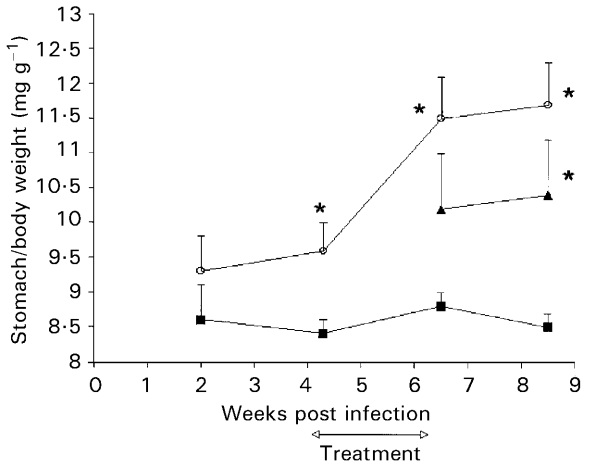

Figure 1. Time-course of changes in stomach/body weight ratio in control (■) and *H. felis*-infected mice . Infected mice were treated for two weeks (weeks 4–6) with 100 mg kg^{-1} of recombinant human lactoferrin (▲) or with saline (○). Results are expressed as the ratio of stomach to body weight (mg g^{-1}). $*P < 0{\cdot}05$ vs uninfected control.

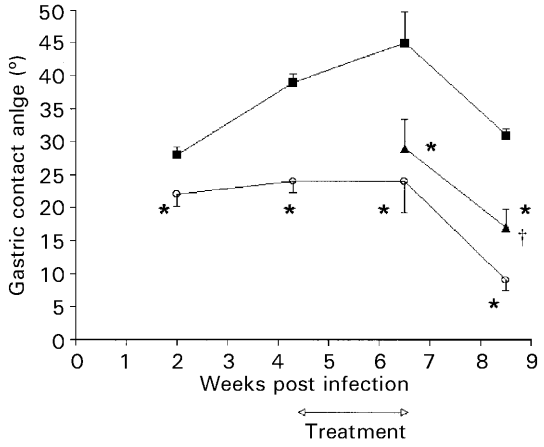

Figure 2. Time-course of changes in gastric contact angle in control (■) and *H. felis*-infected mice. Infected mice were treated for two weeks (weeks 4–6) with 100 mg kg^{-1} of recombinant human lactoferrin (▲) or saline (○). Results are expressed in degrees. $*P < 0{\cdot}05$ vs uninfected control; $\dagger P < 0{\cdot}05$ vs *H. felis* + saline.

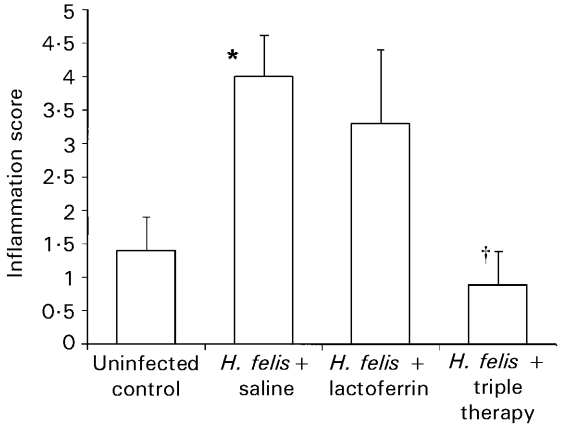

Figure 3. Histological gastritis in control and *H. felis*-infected mice. Infected mice were treated for two weeks with either saline, 100 mg kg^{-1} recombinant human lactoferrin or triple therapy (0·675 mg metronidazole, 1·5 mg tetracycline, 0·185 mg bismuth subsalicylate). After another two weeks, gastric tissue was assessed for inflammation. $*P < 0{\cdot}05$ vs uninfected control; $\dagger P < 0{\cdot}05$ vs *H. felis* + saline.

Dose–response of lactoferrin on H. felis *infection*

At a sufficient concentration, recombinant human lactoferrin was capable of fully or partially reversing all three indicators of *H. felis* infection. Figure 4 illustrates the final stomach/body weight ratio, and shows that the bacteria induced a gastritis that manifested as a significant increase in this ratio. Recombinant human lactoferrin treatment reduced the ratio at the two higher doses, to a value between, and not different from, that of the saline-treated infected mice and the uninfected mice.

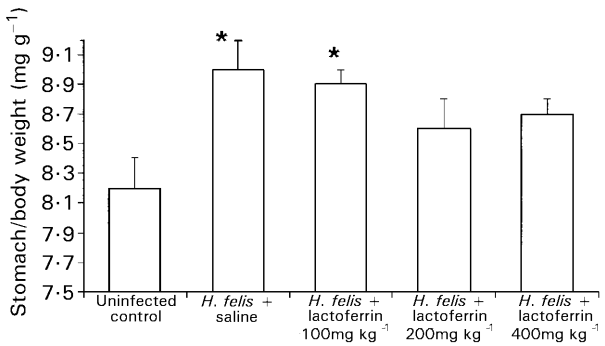

Figure 4. Dose–response effects of recombinant human lactoferrin on stomach/body weight ratio in control and *H. felis*-infected mice. Infected mice were treated for two weeks with recombinant human lactoferrin (100, 200 or 400 mg kg^{-1}), after which the stomach and body weights were obtained. *$P < 0{\cdot}05$ vs uninfected control.

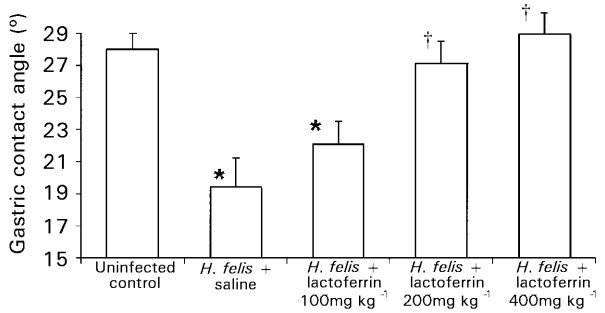

Figure 5. Dose-response effects of recombinant human lactoferrin on gastric contact angle in control and *H. felis*-infected mice. Infected mice were treated for two weeks with recombinant human lactoferrin (100, 200 or 400 mg kg^{-1}), followed by contact angle analysis of the stomach. *$P < 0{\cdot}05$ vs uninfected control; †$P < 0{\cdot}05$ vs *H. felis* + saline.

Figure 5 and shows that infection induced a significant reduction in the gastric contact angle. Recombinant human lactoferrin treatment significantly reversed the decrease in contact angle in a dose-dependent manner, and returned the value to control levels at the higher doses. Finally, the results of the urease test for the presence of the bacteria (Table 1) show that recombinant human lactoferrin treatment caused a significant reduction in bacterial presence at two of the three doses.

Table 1. *H. felis* infection rate in mice after recombinant human lactoferrin treatment.

Treatment	No. positive/group (%)
Uninfected control	0/8 (0%)
H. felis + saline	6/8 (75%)*
H. felis + lactoferrin 100 mg kg^{-1}	1/8 (12·5%)†
H. felis + lactoferrin 200 mg kg^{-1}	2/8 (25%)
H. felis + lactoferrin 400 mg kg^{-1}	1/8 (12·5%)†

Control or *H. felis*-infected mice were treated for two weeks with recombinant human lactoferrin. Gastric biopsies were tested for the presence of *H. felis* with the urease colour test. *$P < 0{\cdot}05$ vs uninfected control; †$P < 0{\cdot}05$ vs *H. felis* + saline.

Comparison of lactoferrin and other antibiotics on H. felis *infection*

This experiment showed a favourable response when the in-vivo activity of recombinant human lactoferrin was compared with that of known antibiotic treatments for *Helicobacter* infection. The high rate of infection in saline-treated infected mice could be significantly reduced by recombinant human lactoferrin, amoxicillin, lactoferrin plus amoxicillin, and triple therapy (Table 2). When recombinant human lactoferrin and amoxicillin were combined, complete clearance of the bacteria was obtained, although this effect was not statistically different from the agents alone. The gastritis and contact-angle changes of infected mice compared with controls were altered only 13 and 19%, respectively, in this study (data not shown), which suggests a rather mild infection. In general, it is difficult to perceive improvements when such small differences are found.

Discussion

This study has shown that recombinant human lactoferrin possesses antibiotic activity in-vivo in a mouse model of *Helicobacter* infection. This is noteworthy because the human form of lactoferrin has not been tested previously in an animal model of this infection, and it now can be seen to be effective. This study also shows that recombinant human lactoferrin has activity when administered orally.

We chose to examine three different parameters of *H. felis* infection in mice which explored separate aspects of the infection. The gastritis mea-

Table 2. *H. felis* infection rate in mice after treatment with recombinant human lactoferrin and antibiotics.

Treatment	No. positive/group (%)
Uninfected control	0/12 (0%)
H. felis + saline	10/11 (91%)*
H. felis + lactoferrin 200 mg kg^{-1}	2/8 (25%)†
H. felis + amoxicillin 19 mg kg^{-1}	1/8 (12·5%)†
H. felis + amoxicillin + lactoferrin 10 mg kg^{-1}	0/8 (0%)†
H. felis + amoxicillin + lactoferrin 100 mg kg^{-1}	0/8 (0%)†
H. felis + triple therapy	3/7 (43%)†

Control or *H. felis*-infected mice were treated for two weeks with the indicated agents. Gastric biopsies were tested for the presence of *H. felis* with the urease colour test. *$P < 0{\cdot}05$ vs uninfected control; †$P < 0{\cdot}05$ vs *H. felis* + saline.

surement (stomach/body weight ratio) was a reflection of the hypertrophy of the gastric mucosa due in part to inflammation and oedema. Indeed, this simple ratio correlated well with histological assessments of inflammation. The surface hydrophobicity measurement (contact angle) estimated the degree of gastric barrier integrity disruption due to phenomena at the luminal surface. And the urease colour test was a direct measure of the infection rate. Collectively, these parameters could give a broad view of the extent of the infection. In a previous study (Lichtenberger et al 1999), as with this one, we found that the gastritis and contact-angle measurements deviated from control values early in the infection and were maintained at the altered levels until the infection was reduced or eradicated. The use of these parameters was further supported by their reversal following triple therapy.

Study of the time-course of *H. felis* infection showed a robust infection with an approximately 35% increase in stomach size (gastritis) and a 50% or greater drop in contact angle. The initial 2-week course of treatment at a single dosage of recombinant human lactoferrin ($100\,mg\,kg^{-1}$) appeared to be effective at partially reversing gastritis and surface hydrophobicity changes, with no effect on infection rate. A subsequent dose–response experiment revealed that higher doses of recombinant human lactoferrin could be more effective on contact angle and infection rate if the infection were less severe ($\sim$20% increase in gastritis, $\sim$35% reduction in contact angle). The variability of infection between studies cannot be ascribed to any single event, as care was taken to utilize the same animal supplier and only use freshly cultured bacteria from a common source. However, taken together, these data support the finding that recombinant human lactoferrin alone has considerable efficacy at combating *Helicobacter* infection in-vivo. The variability between these experiments did not change the basic outcome that shows a beneficial effect of recombinant human lactoferrin on *H. felis*-infected mice.

The combination of recombinant human lactoferrin with amoxicillin was chosen to test lactoferrin with another antibiotic commonly used against *Helicobacter* infection. The study had been designed to utilize sub-optimal doses of each agent, but both agents were quite effective alone on the infection rate. However, the combination of amoxicillin and recombinant human lactoferrin did produce an infection level of zero. While the general mildness of the infection in this study makes interpretation of the gastritis and contact-angle data difficult, the infection-rate data support the further testing of lactoferrin at other doses and with other antibiotics to produce a combination that maximizes antibiotic activity, minimizes dosage and potentially lessens side effects of other commonly used antibiotics. The mechanism by which recombinant human lactoferrin exerts its effect in this mouse model is likely to be a combination of properties. Primary among these is that its antibiotic activity could be suppressing growth or eradicating the organism. The protein's anti-inflammatory activity could also contribute to the reduction in gastritis. Lactoferrin's reported ability to block or displace bacterial attachment (Wada et al 1999) could reduce bacterial numbers and associated inflammation. We have reported a healing effect of recombinant human lactoferrin on rat gastric mucosa (Dial et al 1999) that might augment its other protective actions.

There is a need for improved therapies to treat *H. pylori* infections, as current treatments are plagued by growing antibiotic resistance, uncomfortable side effects and poor patient compliance (Graham 2000). The use of recombinant human lactoferrin may bypass these disadvantages. In that regard, a recent Phase I trial showed recombinant human lactoferrin to be safe, although not efficacious at reducing the bacterial load, after 5 doses given during a 24-h period (Opekun et al 1999). Further long-term studies with this promising agent are warranted.

Acknowledgements

Funded by Agennix Inc., Houston, TX, and NIH R41 DK55374.

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