MICROBIAL CHARACTERISATION

Inhibition of *Helicobacter pylori* infection by bovine milk glycoconjugates in a BALB/cA mouse model

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The attachment of *Helicobacter pylori* to the human gastric mucosa is a complex process involving several specific structures recognised by the cell surface receptors. Sialylated multivalent high mol. wt glycoproteins have been shown to inhibit *H. pylori* sialic acid-specific haemagglutination. This study explored whether sialylated glycoconjugates from bovine milk could inhibit an experimental *H. pylori* infection in a mouse model. BALB/cA mice (6–8 weeks old) were inoculated with a mouse-passaged *H. pylori* strain 317p. Four weeks after infection the mice were given lactoferrin (iron-free LF or 20% iron-saturated LF) or bovine milk fat globule membrane fractions (MFGM or defatted MFGM) orally (400 mg/kg body weight) once daily for 10 days and then killed to examine for bacterial colonisation and gastritis. Mice treated with iron-free LF, 20% iron-saturated LF, MFGM or defatted MFGM showed 30%, 10%, 20% or 20% healing rates, respectively, when compared with the *H. pylori*-infected control. Gastric colonisation by *H. pylori* was remarkably decreased in all mice treated with bovine milk glycoconjugates and the inflammation score was also significantly lower in treated mice than in infected control animals. The fact that there was no significant difference between iron-free LF and iron-saturated LF or MFGM and defatted MFGM suggested that iron is not crucial for inhibition of *H. pylori* by lactoferrin and that the lipid part of MFGM is not important for anti-*H. pylori* activity. In conclusion, bovine milk glycoconjugates showed potencies to inhibit *H. pylori* infection in this mouse model and, therefore, could be considered as candidates for non-antibiotic strategies against *H. pylori* infection in man.

Introduction

Helicobacter pylori is the causative agent of chronic type B gastritis and a key factor in peptic ulcer disease, stomach cancer and MALT lymphoma [1–3]. The attachment of *H. pylori* to the human gastric mucosa is a complex process involving several specific structures recognised by gastric mucin and cell surface receptors [4]. *H. pylori* sialic acid-binding haemagglutinin(s) represent a group of such putative cell surface adhesins, proposed to be crucial for the bacterial colonisation of the stomach [5]. Sialylated glycoconjugates in gastrointestinal mucins, as well as sialylated bovine milk glycoproteins, inhibit *H. pylori* sialic acid-specific haemagglutination [6]. Lactoferrin (LF) is an iron-binding glycoprotein that is found in various

secretions, including tears, milk, and semen, and also in the secretory granules of neutrophils. It has been reported to have various biological functions [7] and antibiotic properties against various gram-negative bacteria [8]. The components of bovine milk fat globule membrane are high mol. wt mucin and the roles of mucin-like components from milk in the prevention of bacterial adhesion have been reported for several pathogens [9–11].

The rapid increase of antibiotic resistance of various pathogenic microbes and the development of new resistance mechanisms are threats for the treatment of several infective diseases [12]. Of the potential novel non-antibiotic treatment strategies, anti-adhesive compounds to inhibit tissue invasion by bacterial pathogens are promising candidates [13, 14]. This group of investigators has previously standardised a mouse model for *H. pylori* infection [15–17] and recently reported that antioxidants such as astaxanthin and vitamin C inhibit *H. pylori* infection in this model

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[18]. The present study investigated whether bovine milk glycoproteins could inhibit an experimental *H. pylori* infection in a BALB/cA mouse model, to develop a possible anti-adhesion strategy in the future.

Materials and methods

Glycoconjugates

Milk fat globule membrane fractions (MFGM and defatted MFGM) were prepared from bovine buttermilk obtained from a butter-making plant of Snow Brand Milk Products (Tokyo, Japan) as described previously [6]. Lactoferrin (iron-free LF and 20% iron-saturated LF) from bovine milk were also obtained from Snow Brand Milk Products. These milk compounds were dissolved in sterilised water just before administration.

Bacterial strains

A *H. pylori* mouse-passaged strain EU317p (originally isolated from a patient with gastritis), which is a CagA⁺, VacA⁺ and sialic acid-binding strain, was used in this study. *H. pylori* was cultured on GAB-Camp agar (Gc agar base; Becton Dickinson, Franklin Lakes, USA) supplemented with horse serum 10% and incubated for 48 h at 37°C under micro-aerobic conditions [19]. The cells were harvested into 0.01 M sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl (PBS), centrifuged at 3000 rpm for 10 min and resuspended in the same buffer to a final concentration of 10⁹ cfu/ml.

Animals

Sixty conventional BALB/cA mice (6–8 weeks old) were used in this study (B&K Universal Company, Stockholm, Sweden). Mice were housed with a 12-h light-dark schedule, fed with rat and mouse standard diet no. 2 (B&K Universal Company) and allowed water *ad libitum* [15]. The experiment was done with the permission of a Swedish animal ethical committee in Lund.

Experimental design

Fifty mice were inoculated orally through a feeding tube three times at 2-day intervals with 0.1 ml of a *H. pylori* suspension containing 10⁹ cfu/ml, and as a negative control group, 10 mice, were given PBS only [17]. Inoculated mice were then randomly divided into five groups. Four groups of mice were given iron-free LF, 20% iron-saturated LF, MFGM or defatted MFGM at a dose of 400 mg/kg body weight orally once daily for 10 days at 4 weeks after inoculation. Infected control and normal control mice were given distilled water through the feeding tube. Mice were killed after the final treatment and their stomachs were collected. Half of each stomach, including all types of mucosa, was fixed in 10% buffered formalin for histology

studies [16]. The remaining part of the stomach was used to culture *H. pylori*.

Culture

Stomach biopsies were rinsed in phosphate-buffered saline (PBS), smeared directly on GAB-Camp agar and incubated at 37°C for 5–10 days under micro-aerobic conditions [17]. The presence of *H. pylori* on the culture plates was confirmed by urease, catalase and oxidase tests, Gram's staining and PCR [15]. The absence of *H. pylori* colonies on the plate was classed as culture negative. The number of colonies per plate was counted as cfu/culture unit.

Histopathology

Murine stomach tissues were fixed in 10% buffered formalin, embedded in paraffin, then 4-μm sections were prepared and stained with haematoxylin and eosin by standard procedures. The degree of inflammation was scored in a blind manner on a scale of 0–3 for body and antrum as follows: 0, normal; 1, few inflammatory cells; 2, moderate inflammatory cells in several layers; 3, high level of inflammation with nests containing >50 inflammatory cells, often in more than three cell layers [17]. The inflammation score of each mouse was calculated as the average of body and antrum.

Growth inhibition assay

Bacteria (2 × 10⁶ cfu) were grown in triplicate tubes containing 2 ml of trypticase soy broth (TSB) supplemented with horse serum 10% and Dent-supplement (Oxoid) with or without iron-free LF or 20% iron-saturated LF at 0.5–4 mg/ml at 37°C. Samples (20 μl) were taken 2 and 5 days after the start of incubation for counting viable cells. After incubation for 5 days, the bacterial cells were harvested by centrifugation and replaced with fresh medium without lactoferrin for a further 5 days growth. Turbidity was then measured at an optical density (OD) of 600 nm. The control growth was set as 100% and inhibition of growth was given a value between 0 and 100%.

Haemagglutination inhibition assay

Bacterial suspensions were diluted with PBS to give 4 haemagglutinating units (HAU) [6]. One HAU was defined as the amount of bacteria that caused complete agglutination. Inhibition tests were performed in the wells of U-shaped microtitration plates by mixing 15 μl of bacterial sample with 15 μl of a serial dilution of inhibitor for 1 h at room temperature, after which 15 μl of human erythrocyte 0.75% suspension was added to each well and allowed to settle for 1–2 h.

Adherence assay

The adherence assay was done by the method described previously [20, 21]. Briefly, bacteria were pre-incubated with serially diluted inhibitors in wells of U-shaped microtitration plates for 15 min at room temperature. Then 25 μ l of the mixture from each well was added to HeLa S3 cell monolayers (derived from a cervix carcinoma) and incubated with gentle agitation for 20 min at room temperature. Monolayers were washed three times with 100 μ l of PBS to remove unbound bacteria. Remaining bacteria were quantified by the urea-phenol red assay. The inhibitory concentration (IC) was calculated as a percentage from the following formula: Inhibition (%) = $[(OD_{\text{experimental}} - OD_{\text{negative}})/(OD_{\text{positive}} - OD_{\text{negative}})] \times 100$, where negative wells contained only epithelial monolayers without bacteria and positive wells contained bacteria and monolayer without inhibitor.

Statistical analysis

The Mann-Whitney U test and χ^2 test were used for defining colonisation and inflammation distribution. The level of significance was selected as $p < 0.05$.

Results

All 10 mice in the infected control group were *H. pylori* culture positive at 5.5 weeks after inoculation. In the groups treated with iron-free LF or 20% iron-saturated LF, seven or nine mice within each group of 10 mice were *H. pylori* culture positive. Of the mice treated with MFGM or defatted MFGM, eight of 10 animals were *H. pylori* culture positive (Table 1). There was no statistical difference between any of the treated groups of mice and infected control animals. However, only 30%, 10%, 20% and 20% healing rates, respectively, were obtained from *H. pylori*-infected mice after treatment with iron-free LF, 20% iron-saturated LF, MFGM and defatted MFGM.

The numbers of *H. pylori* colonies cultured from gastric biopsies were significantly decreased in mice treated with iron-free LF or 20% iron-saturated LF compared with *H. pylori*-infected control animals

($p < 0.01$). MFGM or defatted MFGM also decreased *H. pylori* colonisation in the mouse stomachs remarkably when compared with the *H. pylori*-infected control animals ($p < 0.05$) (Fig. 1). There was no significant difference between the bovine milk glycoconjugates tested in this model.

The infected control mice showed severe inflammation in the stomach when compared with normal control mice 5.5 weeks after inoculation ($p < 0.001$). The LF (iron-free or 20% iron-saturated)- and MFGM (fat or defatted)-treated mice showed a significant decrease in inflammation score compared with infected control animals ($p < 0.05$) (Fig. 2), but still had a significantly higher inflammation score than non-*H. pylori*-inoculated animals ($p < 0.001$). The histopathological analysis of these different groups of mice is illustrated in Fig. 3.

The effect of bovine lactoferrin on the growth of *H. pylori* strain 317 was examined *in vitro* in broth culture. The mean IC₅₀ was 1 mg/ml for iron-free LF and 4 mg/ml for iron-saturated LF (Table 2). Incubation for 5 days with various concentrations of LF showed a greater inhibitory effect on growth of *H. pylori* than incubation for 2 days (data not shown). Both bovine MFGM and its defatted fraction inhibited haemagglutination of *H. pylori* at similar concentrations (Table 2). Iron-saturated LF inhibited haemagglutination of *H. pylori*, whereas iron-free LF did not inhibit haemagglutination at a concentration of 33 mg/ml (Table 2). The cell adherence assay demonstrated that all the milk glycoconjugates tested inhibited the binding of *H. pylori* to HeLa S3 cell monolayers (Table 2).

Discussion

This study aimed to investigate whether sialylated glycoconjugates from bovine milk could inhibit an experimental *H. pylori* infection in BALB/cA mice. It was found that all tested bovine milk glycoproteins decreased the gastric colonisation of *H. pylori* and the gastritis.

Iron-free and iron-saturated LF showed 30% and 10% healing rates of *H. pylori* colonisation in this mouse model. Lactoferrin can act as an antimicrobial agent like transferrin through iron deprivation or can damage the outer membrane of bacteria directly by the binding of lactoferrin [22, 23]. Recently, the iron uptake of various *Helicobacter* species was compared [24]. It was found that gastric *Helicobacter* spp., except *H. pylori*, were non-siderophore-producing organisms and were able to obtain iron only from haem and haemoglobin. However, non-gastric *Helicobacter* spp. produced siderophores and were able to use a wide range of iron sources for growth, such as bovine and human lactoferrin and transferrin, haem and haemo-

Table 1. *H. pylori* culture from mouse gastric biopsies

Groups of mice	<i>H. pylori</i> -positive/ total mice	Healing rate (%)
Normal	0/10	100
<i>H. pylori</i>	10/10*	0
<i>H. pylori</i> + lactoferrin (iron-free)	7/10	30
<i>H. pylori</i> + lactoferrin (20% iron-saturated)	9/10	10
<i>H. pylori</i> + MFGM	8/10	20
<i>H. pylori</i> + defatted MFGM	8/10	20

* $p < 0.01$ versus normal mice by χ^2 test. There was no statistical difference between any treated group and the infected control group.

Fig. 1. Inhibition of *H. pylori* colonisation in mice by bovine milk glycoconjugates. All treated mice showed significantly lower gastric colonisation than the infected control mice (* $p < 0.01$ versus LF and $p < 0.05$ versus MFGM). Data are mean values with SEM.

Fig. 2. Inflammation score of gastritis in BALB/cA mice treated with milk compounds. * $p < 0.001$ versus all *H. pylori*-infected mice; † $p < 0.05$ versus all treated groups. Data are mean values with SEM.

globin. Antibiotic-like properties of bovine lactoferrin against *H. pylori* growth through iron deprivation have been reported and the treatment of *H. felis*-infected mice with lactoferrin partially reversed mucosal disease manifestations [25]. In the experimental *H. pylori* mouse model used in the present study, lactoferrin inhibition of *H. pylori* infection was independent of iron-scavenging properties, as both iron-free and 20% iron-saturated LF gave similar effects *in vivo* and in the adhesion assay. In a germ-free mouse model, bovine lactoferrin (30% iron-saturated) decreased the number of *H. pylori* colonies significantly and inhibited the attachment of *H. pylori* to the stomach mucosa [26]. Iron-free LF inhibited *H. pylori* growth *in vitro* at a lower concentration (1 mg/ml) compared with iron-saturated LF (4 mg/ml). The involvement of the

ingested lactoferrin in the stimulation of carbohydrate absorption, which is independent of its iron-chelating capacity as *in vitro*, has been proposed as the mechanism of bovine lactoferrin-induced bacteriostasis *in vivo* [27].

Sialylated bovine milk glycoproteins inhibit *H. pylori* sialic acid-specific haemagglutination effectively [6]. In the mouse model studied, both MFGM and defatted MFGM decreased gastric *H. pylori* colonisation and its associated gastritis. The lipid part of the MFGM fraction does not seem to be involved in this mechanism, as similar results were obtained with a delipidated preparation in both *in-vivo* and *in-vitro* experiments. The importance of sialylated oligosaccharides on human gastric epithelial cells as binding

Fig. 3. Histopathological analysis of mouse stomachs. (A) Normal control animal: only a few lymphocytes, inflammation score 0.5; (B) *H. pylori*-infected control animal: heavy infiltrate of granulocytes, lymphocytes and plasma cells close to muscular mucosa (arrows) and also many inflammation cells between crypts, inflammation score 2.5; (C, D) *H. pylori*-infected mice treated with iron-free LF or iron-saturated LF: only a few cells around the lamina muscularis mucosa and scattered inflammatory cells in-between glands, inflammation scores 1 and 1.5, respectively; (E, F) *H. pylori*-infected mice treated with MFGM or defatted MFGM: very few cells around the lamina muscularis mucosa and scattered inflammatory cells in-between glands, score 1. Magnification $\times 20$ (bar = 100 μm).

sites for *H. pylori* has been described [21] and 3'-sialyllactose (3'SL) suppressed *H. pylori* infection in a rhesus monkey model [13]. Monkeys treated with 3'SL at 100 mg or 500 mg/kg body weight/day for 28 and 56 days showed a 33% curing rate (two of six animals). The dose of MFGM used to treat the infected mice in the present study was 400 mg/kg body weight/day,

which is similar to that used in the monkeys. Prolonging the therapeutic period may increase the healing rate for *H. pylori* infection.

A Mongolian gerbil model was recently optimised to study the gastric inflammation response to *H. pylori* infection with a strong induction of pro- and anti-

Table 2. Inhibition of *H. pylori* growth, haemagglutination and binding to HeLa S3 monolayers

Inhibitor	Concentration that caused 50% inhibition of (IC50 mg/ml)		
	growth	haemagglutination	adhesion
Lactoferrin (iron-free)	1	>33	2.81
Lactoferrin (20% iron-saturated)	4	0.2	2.5
MFGM	NT	0.065	5.63
Defatted MFGM	NT	0.031	5.31

NT, not tested.

oxidants [28]. Also, a guinea-pig model has been developed for experimental *H. pylori* infection yielding a strong gastric inflammation response [29]. These animal models may now be used to evaluate various candidates for a non-antibiotic treatment strategy to combat *H. pylori* infections.

In conclusion, inhibition of *H. pylori* infection in this mouse model with selected milk glycoconjugates looks promising. However, longer duration of therapy or a combination of several glycoconjugates could possibly yield better results. The anti-adhesion concept should be investigated further for possible use in *H. pylori* infections in man.

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