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Effects of fucosylated milk of goat and mouse on *Helicobacter pylori* binding to Lewis b antigen

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Abstract

AIM: To evaluate the effects of animal milk containing fucosylated antigens on *Helicobacter pylori* (*H pylori*) binding to Lewis b antigen.

METHODS: A mammary gland expression vector containing human α1-3/4-fucosyltransferase cDNA sequences was constructed. Transient expression of human α1-3/4-fucosyltransferase cDNA in goat mammary cell and establishment of transgenic mice were performed. The adhesion inhibitory properties of milk samples were analyzed by using *H pylori*.

RESULTS: Goat milk samples were found to inhibit bacterial binding to Lewis b antigen. The highest inhibition was observed 42 h after injection of the plasmid. The binding activity of *H pylori* to Lewis b antigen reduced mostly, by 83%, however milk samples from transgenic mice did not inhibit *H pylori* binding to Lewis b antigen.

CONCLUSION: The use of "humanized" animal milk produced by the transgenic introduction of fucosylated antigen can perhaps provide an alternative therapy and preventive measure for *H pylori* infection.

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INTRODUCTION

Helicobacter pylori (*H pylori*), a human specific gastric pathogen, was first isolated in 1983[1]. Twenty years of research has found that *H pylori* infection is one of the major causes of upper gastrointestinal tract diseases, such as chronic active gastritis and peptic ulcer disease[2-6]. In chronic active gastritis, gastric ulcer and gastroduodenal ulcer, the incidences of *H pylori* infection are 71-94%, 72-100% and 73-100% respectively. In addition, *H pylori* infection has been linked with the development of gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT)[7-13]. It has been defined as a Class I carcinogen by WHO[14,15].

H pylori colonize human gastric mucosa by adhering both to the mucous epithelial cells and to the mucus layer[16]. Specific receptor structures in combination with the unique tissue-specific distribution of receptors can restrict microbial colonization to a limited number of hosts, tissues and cell lineages[17]. *H pylori* can bind tightly to epithelial cells using various bacterial surface components[18-20]. The best characterized adhesin, BabA, is a 78-kD outer-membrane protein that binds to the fucosylated Lewis b (Le^b) blood group antigen[21]. Accumulating evidence in animal models suggests that BabA is relevant to *H pylori*-associated diseases[22]. Le^b antigen is one of the most important receptors, governing adhesion of *H pylori* to gastric mucosa. Boren *et al.* found that the fucosylated Lewis blood group antigens Le^b and H-1 were the carbohydrate structures that specifically mediated the adherence of *H pylori* to human gastric epithelial cells *in situ*.

Le^b antigen is a human blood group antigen. In human cells, the synthesis of Lewis antigens is regulated by a series of glycosyltransferases that act sequentially upon a precursor molecule. The fucosyltransferases are responsible for the final step in this process. Their function is to add a fucose residue to precursor molecules to form human blood antigens, such as Le^a, Le^b and H1 antigen (Figure1). Addition of fucose to the terminal galactose residue of the lacto series core chain oligosaccharide results in the H1 antigen. Le^b antigen is formed by the addition of a "branched" fucose residue to H1-antigen, catalyzed by α1-3/4-fucosyltransferase. The fucosylated blood group antigens, typically found on erythrocytes, are also expressed on the gastro-intestinal epithelium. Le^b is the dominant fucosylated blood group antigen expressed on the gastric surface mucous cells in the gastric epithelial lining. The fucosylated blood group antigens are also present in the mucins of the gastric mucus layer and, in addition, as natural "scavengers" or clearance factors in secretions such as saliva, tears, and human milk.

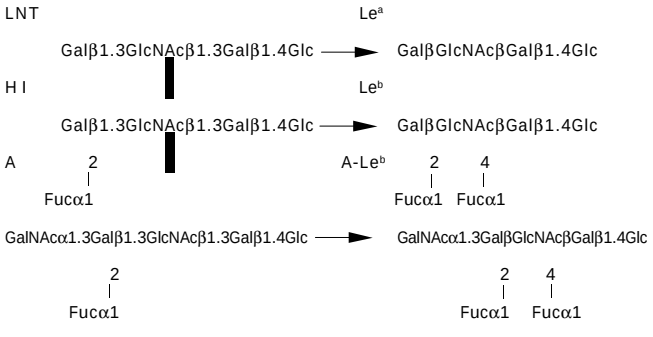

Figure 1 Formation of fucosylated blood group antigens.

H pylori infection is one of the most common infections in humans. Epidemiological data show that it affects about half of the human population. Without specific therapy, *H pylori* infection can persist for decades or even the host's lifetime. But only about 15% of *H pylori*-infected individuals actually have *H pylori*-associated diseases. It is likely to be associated with other additional factors such as genetic predisposition, age of infection, and the genotype of infective strain. The prevalence varies greatly among countries and among population groups within the same country. The overall prevalence of *H pylori* infection is strongly correlated with socioeconomic conditions. Eighty-five percent of *H pylori* can be eradicated by combination therapy in clinic, however, using antibiotics for several weeks may bring about other problems such as bacterial resistance. So many researchers are looking for other methods to prevent *H pylori* infection, such as preventing *H pylori* binding to or colonizing the gastric mucosa.

If we can add some *H pylori* specific receptor, for example, Le^b blood antigen or its analog to food, then *H pylori* binding to the human gastric mucosa may be prevented or reduced, and the bacteria will be excreted by the alimentary tract or destroyed by human antibody. Flak *et al.* reported that the α1-3/4-fucosyltransferase was expressed in the gastric mucosa of mice and that *H pylori* could bind to the mice gastric mucosa. At present, there are no reports of α1-3/4-fucosyltransferase being expressed in animal galactophore. Therefore, our aim was first to introduce human α1-3/4-fucosyltransferase into animals and get them expressed in the animal mammary gland and thus produce Le^b antigen in milk. This kind of milk does not only have nutritional value, it is also a natural source of lectin, a molecule that can block *H pylori* binding to the human gastric mucosa, and therefore prevent *H pylori* infection and reduce the severity of the infectious process. In this way, people can prevent *H pylori* infection by drinking this kind of milk daily.

This paper describes the transient expression of human α1-3/4-fucosyltransferase gene in goat mammary gland and the establishment of transgenic mouse model. A new test to prevent and cure *H pylori* infection and gastrointestinal diseases associated with *H pylori* infection is put forward in our study.

MATERIALS AND METHODS

Experimental animals

Kunming white mice were purchased from Beijing Laboratory Animal Research Center. Laoshan goats were provided by Beijing Sangao Corporation.

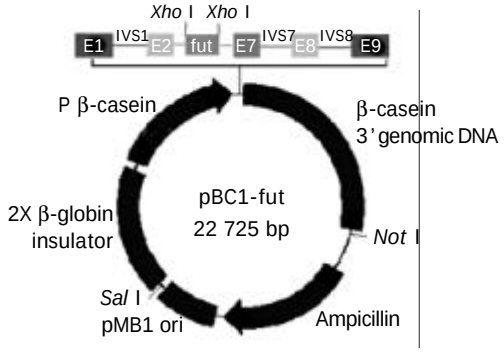

Figure 2 Map of expression vector: pBC1-fut.

Construction of expression vectors

A 1 115-bp α1-3/4-fucosyltransferase cDNA encompassing the entire 1 086-nt coding region specifying the 361-AA transmembrane glycoprotein, containing an upstream Kozak consensus sequence and *Xho*I site, a downstream *Xho*I site, was generated by PCR from a Fut/pCDM8 plasmid. The PCR product was then purified and digested by *Xho*I, and the digestion product was subcloned into pBC1 vector, which had been treated previously with *Xho*I, and transformed into *E. coli* DH5α. pBC1 is a specific milk expression vector which contains the goat β-casein promoter and other proprietary DNA sequences. The positive transfected clone containing the properly oriented α1-3/4-fucosyltransferase cDNA was screened by colony PCR. The expression vector, named pBC1-fut, allowed the α1-3/4-fucosyltransferase cDNA to be placed in its downstream of the β-casein promoter (Figure 2).

Transient expression

pBC1-fut was purified using Qiagen Plasmid Maxi kit. About 1 mg pBC1-fut was injected into a lactating goat's right and left mammary glands from the goat glandular duct. Milk samples at different times were then collected over a 100-h (4 d) period from both right (R) and left (L) udders. Then, milk samples were analyzed in a dilution series (25-, 50-, 100- and 200-fold) for adhesion inhibition properties.

Transgenic mice production

The 16-kb DNA fragment inserted was isolated by agarose gel electrophoresis and recovered by electro-elution. To remove any contamination, products were spot dialyzed against 40 mL TE (10 mmol/L Tris, 0.1 mmol/L EDTA, pH7.4) for 30 min (VSWP02 500 membrane, Millipore). Purified DNAs were diluted to 2-3 ng/μL in TE buffer and microinjected into the pronuclei of fertilized eggs of Kunming white mice.

Genomic DNAs were isolated from the tails of transgenic mice using a standard method. A pair of primers was designed to screen for transgenic mice: upper primer: 5' - GATTGACAA GTAATACGCTGTTTCCTC-3' and downstream primer: 5' - CATCAGAAGTTAAACAGCACAGTTAG-3' . PCR reactions using genomic DNAs as template were performed under the following condition: 30 cycles of 94 ℃ for 1 min, 58 ℃ for 1 min, and 74 ℃ for 1 min. After PCR screening, transgenic mice were confirmed by Southern hybridization. The probe was created by ^{32}P labeling α1-3/4-fucosyltransferase cDNA. Genomic DNA from transgenic mice and negative mouse as well as expression vector DNA were digested by *Bam*HI. Copies of the transgene were estimated by comparing the band density of the vector control with that in transgenic mice. Hybridizations were at 65 ℃ in Church (10 g/L BSA, 70 g/L SDS, 1 mmol/L EDTA, 0.5 mol/L sodium phosphate, pH 7.2). Final washes were in 2×SSC, 0. 5×SDS at 65 ℃. Signal from the membrane was detected using a Phosphor Screen (Molecular Dynamics, US).

Analysis of adhesion inhibitory properties of the milk

Goat milk was collected at different time points for 100 h from both right (R) and left (L) udders. The transgenic milk was collected at the 7 th day of lactation. The milk was centrifuged at 18 000 r/min for 1 h at 4 ℃. The fat on the surface was removed and the clear part of the supernatant was put in a new tube and used as the sample. Le^b antigen was labeled with ^{125}I by the chloramine T method. Milk samples were analyzed in dilution series (25-, 50-, 100- and 200-fold). The samples were mixed with an *H pylori* strain (CCUG17875), which bound bind Le^b antigen efficiently, on a cradle for 17 h at room temperature. After this period, ^{125}I radioactivity in bacterial pellet was measured with a gamma counter.

Western blotting

After electrophoresis on 80 g/L SDS-PAGE, proteins were transferred to a nitrocellulose extra blotting membrane (Sartorius, Germany). Le^b monoclonal antibodies (Immucor, GA) and HRP-conjugated goat anti-rabbit-IgG (Cappel Laboratories, US) were used to detect Le^b antigen.

Table 1 Blocking effect on *H pylori* binding to Lewis b antigen by goat milk

Time	Bind / Free Le^{b*} (%)				Sample	Bind / Free Le^{b*} (%)			
	25×	50×	100×	200×		25×	50×	100×	200×
R 6 h	32.5	43.4	49.6	53.9	L 6 h	36.9	47.1	52.1	55.1
R 12h	28.2	41.7	49.4	53.0	L 12 h	34.7	45.3	51.3	53.4
R 18 h	25.5	40.5	49.4	53.1	L 18 h	29.4	42.3	49.2	53.2
R 23 h	16.8	32.3	43.9	50.6	L 23 h	22.1	37.3	46.5	51.7
R 30 h	15.0	31.0	43.1	49.4	L 30 h	25.6	37.1	46.3	51.9
R 36 h	11.9	28.7	41.2	48.3	L 36 h	20.0	35.4	45.1	50.8
R 42 h	11.2	27.0	40.1	48.0	L 42 h	17.2	33.3	43.6	49.7
R 54 h	16.6	32.3	43.4	49.5	L 54 h	20.3	35.1	44.9	50.4
R 60 h	20.2	35.9	45.1	50.5	L 60 h	25.9	38.7	47.6	51.7
R 66 h	24.6	38.3	45.8	51.0	L 66 h	22.6	36.2	44.9	50.6
R 78 h	35.2	45.6	51.6	54.2	L 78 h	31.9	43.1	50.2	53.3
R 84 h	37.8	46.2	51.7	56.0	L 84 h	36.3	45.9	51.1	54.0
R 90 h	37.1	46.5	51.9	54.8	L 90 h	38.5	46.3	51.3	54.8
R 102 h	42.6	49.4	54.1	57.1	L 102 h	38.8	46.3	51.3	55.0
Control 1	61.2	61.2	61.5	61.5	Control 2	61.0	60.8	61.1	61.2

R: Right udders; L: Left udders; Control 1: Milk from right udders of unimmunized goats; Control 2: Milk from left udders of unimmunized goats.

RESULTS

Transient expression

As shown in Table 1 and Figure 3, milk samples from experimental goats inhibited *H pylori* binding to Le^b antigen, and the time of the highest inhibition efficacy was at 42 h after DNA immunization. Furthermore, milk collected from both right (R) and left (L) udders had inhibitory effect on *H pylori* binding to Le^b. Unimmunized goat (the negative controls) did not inhibit bacterial binding. The binding activity of *H pylori* to Le^b antigen reduced mostly, 83 % in the 25-fold diluted milk samples.

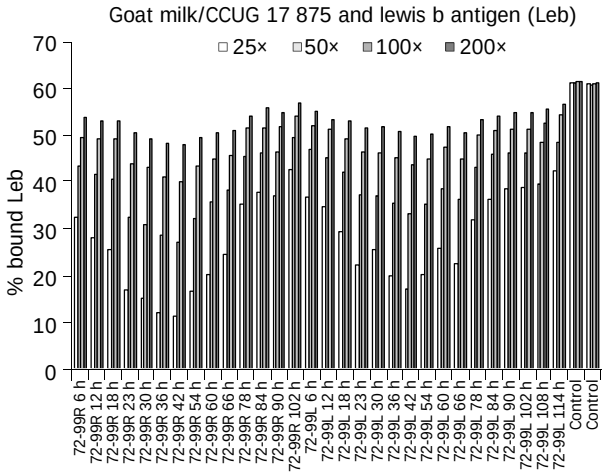

Figure 3 Blocking effect on *H pylori* binding to Lewis b antigen by goat milk. Abscissa: Goat milk collected at different time points and control milk. Ordinate: Rate of *H pylori* binding to Lewis b antigen.

Production of transgenic mice

Five of 84 mice including 2 males and 3 females were identified as being transgenic mice by PCR (Figure 4). The serial numbers of the positive mice were 15, 42, 47, 63 and 71. Efficiency of microinjection was about 6%, within the usual range of 5-20%. These transgenic mice were confirmed by using human α1-3/4-fucosyltransferase cDNA as a probe in Southern blotting (Figure 5). Transgene copy numbers were also determined by Southern blotting. We analyzed three female's milk for the blocking effect on binding of *H pylori* to Le^b antigen, but no inhibitory activity was detected by our experimental system.

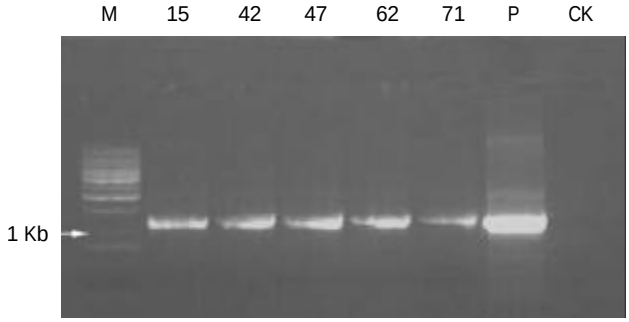

Figure 4 PCR results of transgenic mice. M: DNA ladder; P: pBC1-fut plasmid; CK: Non-transgenic mouse. 15, 42, 47, 62 and 71: Serial numbers of gene positive mice.

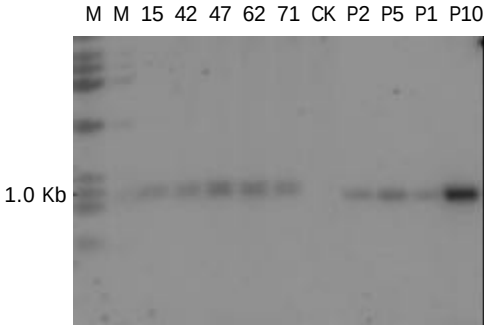

Figure 5 Southern blotting of transgenic mice. M: DNA ladder; CK: Non-transgenic mouse; P1, P2, P5 and P10: pBC1-fut plasmid equivalent to 1, 2, 5 and 10 gene copies, respectively. 15, 42, 47, 62 and 71: Serial numbers of gene positive mice.

Western blotting

We performed Western blotting and stained the membranes of the goat and transgenic mice milk protein with Le^b monoclonal antibody. The goat milk sample blot showed a beautiful, time-dependent induction of the blood group antigens secreted into milk (Figure 6). The band densities at different time points were consistent with the results of the transient expression described above. The band density was strongest at the 42 h after DNA immunization. But the transgenic mice milk did not give a positive signal, suggesting that this milk did not contain Le^b antigen or its analog.

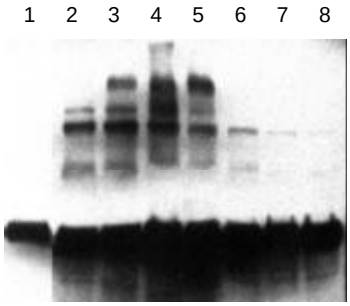

Figure 6 Western blotting of the transient expression milk of goat. Lanes 1-8: Milk collected at different time points of 6, 18, 30, 42, 60, 78, 90, 100 h postinjection, respectively.

DISCUSSION

In our experiment, goat milk could block *H pylori* binding to Le^b antigen, which is one of the most important receptors governing adhesion of *H pylori* to gastric mucosa. The activity of *H pylori* binding to Le^b antigen reduced as much as 83% in some samples. The result showed that some milk proteins might be fucosylated and structurally similar to human Le^b blood group antigen, so they were able to bind the bacteria. Goat milk fucosylated protein can bind *H pylori in vitro*. So "humanized" goat milk, in which human α1-3/4-fucosyltransferase has been introduced into goat mammary gland, may be an alternative therapy and a prevention method for *H pylori* infection.

Unfortunately, the transgenic mice milk collected did not block *H pylori* binding to Le^b antigen. The reasons might be as follows: (1) Level of α1-3/4-fucosyltransferase gene expression in mammary gland of mice was very low. Thus the quantity of Le^b antigen in the milk was so low that the milk could not effectively block *H pylori* binding to Le^b antigen. One way to increase the expression of α1-3/4-fucosyltransferase would be to introduce the complete genomic sequence of the α1-3/4-fucosyltransferase gene to mammary gland of mice. (2) There is no precursor of Le^b antigen in mice milk, so, even if α1-3/4-fucosyltransferase was expressed in mouse galactophore, the transgenic glycosylation patterns that were generated by the activity of α1-3/4-fucosyltransferase did not form epitopes that were recognized by the *H pylori* Le^b-binding adhesions. Therefore the milk could not block *H pylori* binding to Le^b antigen. Since other results have shown that α1-3/4-fucosyltransferase expression in the gastric mucosa of mice can block the binding of *H pylori* to mouse gastric mucosa, it is possible that there might be differences between the carbohydrate core chains of the Le^b antigen in milk glands and gastric mucosa in mice.

Although the results for the transgenic mice were not positive, the successful introduction of α1-3/4-fucosyltransferase cDNA into goat mammary cell and expression of Le^b antigen analog were an important finding. The "humanized" milk by the transgenic introduction of fucosylated antigens can be an alternative therapy and a prevention method for *H pylori* infection.

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