A high molecular mass constituent of cranberry juice inhibits *Helicobacter pylori* adhesion to human gastric mucus

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Abstract

Because previous studies have shown that a high molecular mass constituent of cranberry juice inhibited adhesion of *Escherichia coli* to epithelial cells and coaggregation of oral bacteria, we have examined its effect on the adhesion of *Helicobacter pylori* to immobilized human mucus and to erythrocytes. We employed three strains of *H. pylori* all of which bound to the mucus and agglutinated human erythrocytes via a sialic acid-specific adhesin. The results showed that a high molecular mass constituent derived from cranberry juice inhibits the sialic acid-specific adhesion of *H. pylori* to human gastric mucus and to human erythrocytes. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Since the discovery in the early 80s implicating *Helicobacter pylori* as the major cause for a variety of gastrointestinal diseases including gastric, duodenal and peptic ulcers, as well as gastric cancer, numerous studies have focused on the virulence factors of this pathogen [1–4]. Asymptomatic carriage of *H. pylori* by about half of the population is the major source of infection in humans. It has been suggested that the best way to prevent *H. pylori* infections is to eliminate the pathogen from its most common habitat, the gastric mucus layer [5]. To cause ulcer and other gastric diseases, the organism must leave the mucus layer and adhere to the underlying epithelium. At least five different adhesins have been described that mediate adhesion of the pathogen to epithelial cells and in some cases to erythrocytes [6]. They include adhesins specific for sialic acid, the Lewis a and Lewis b antigens and sulfated glycosaminoglycans. It has been suggested that the ability of *H. pylori* to express various combinations of adhesins with distinct receptor specificity determines whether it will attach to the epithelium or remains in the mucus [2]. Adhesion to epithelial cells has been recognized as an essential step of the infectious process for virtually all bacterial pathogens and therefore much efforts are aimed to develop anti-adhesion therapy for bacterial infections [7]. It has indeed been reported that sialyllactose (NeuAc(α2-3)Gal(β1-4)Glc), an inhibitor of the sialic acid-specific adhesin of *H. pylori*, reduced significantly the load of the bacteria in monkeys [8]. The majority of the *H. pylori* population resides in the mucus which binds the organisms via specific interactions [6,9,10]. Thus, it would seem appropriate to target such therapy against *H. pylori* toward its association with the mucus before the pathogen adheres to the underlying epithelial cells and causes disease.

Tzouvelakis et al. [10] established the method to study adhesion of *H. pylori* to gastric mucus and Wadstrom et al. [6] found that this adhesion may be mediated by the sialic acid-specific adhesin. Other enteropathogens are also capable of binding to mucus constituents via specific adhesins. For example *Escherichia coli* adheres to 45 and 50 kDa glycoproteins in human mucus via its mannose-spe-
cific fimbriae [11]. Although oligosaccharides specific for the *H. pylori* lectins may potentially act as inhibitors of adhesion to mucus, their production in commercial amounts as anti-adhesion therapeutic agents is still a problem. As an alternative approach it has been suggested that dietary inhibitors may be the solution for certain infections [7]. Cranberry juice has been studied for some time in vitro [12,13]. Moreover, daily consumption of cranberry juice has been shown to reduce urinary tract infections caused by *E. coli* in humans [14]. A high molecular mass constituent isolated from cranberry was found to act as inhibitor not only of uropathogenic *E. coli* but of coaggregation of certain oral bacteria as well [15,16]. In a recent report proanthocyanidin extracts from cranberries were shown to inhibit the adhesion of P-fimbriated *E. coli* to uroepithelial cells [17].

In the present study it will be demonstrated that under the growth conditions employed, the adhesion of three strains of *H. pylori* to human gastric mucus and erythrocytes is mediated by the sialic acid-specific adhesin of the bacteria and is inhibited by a high molecular mass constituent derived from cranberry juice.

2. Materials and methods

2.1. Materials

Non-dialyzable material (NDM) was prepared as previously described [12]. Briefly, cranberry juice was dialyzed against distilled water in dialysis bags with a cutoff point of molecular mass 12 000–15 000 at 4°C for 6 days and the NDM was lyophilized. The digoxigenin labeled *Sambucus nigra* agglutinin (SNA), a lectin specific for sialic acid, anti-digoxigenin and bromophenol blue substrate were purchased from Roche Ltd., Mannheim, Germany. Extracellular matrix material from the bovine eye was obtained as described [18]. Twin 20, glucose, galactose, mannose, fucose, lactose, sialic acid were from Sigma Chemical Co. (St. Louis, MO, USA) and 3’Sialyllactose was obtained from Megafarm-Calbiochem. Solutions of the two latter compounds were adjusted to pH 7 with 0.5 N NaOH.

2.2. Bacterial strains and culture conditions

Two clinical isolates of *H. pylori* (BZMC-25 and EHL-65) were obtained from the Gastroenterology Department, Bnei-Zion Medical Center and from the Ezra Health Laboratory, Haifa, Israel. One strain 17874 was a gift from Bnei-Zion Medical Center and from the Ezra Health Laboratory, Haifa, Israel. One strain 17874 was a gift from the Gastroenterology Department, Bnei-Zion Medical Center and from the Ezra Health Laboratory, Haifa, Israel. One strain 17874 was a gift from the Gastroenterology Department, Bnei-Zion Medical Center and from the Ezra Health Laboratory, Haifa, Israel.

The gastric mucus was from human stomachs taken after post-mortem surgeries from the pathological laboratories in Rambam Meical Center, Haifa, Israel, and partially purified as previously described [23]. Briefly, 10% (v/v) of the crude mucus in 0.1 M NaCl was adjusted to pH 7 with NaOH and stirred overnight at 4°C. Insoluble material was removed by centrifugation at 8000×g for 10 min and the dissolved mucus was precipitated by adding ethanol to a final concentration of 60% (v/v). The precipitate was pelleted by centrifugation, as before, then lyophilized and kept at −20°C. For binding studies the mucus was dissolved (150 µg ml⁻¹) in carbonate–bicarbonate buffer, (20 mM, pH 9.6) and 100 µl of the mucus suspension was dispensed into wells of a polystyrene microplate (Dynatech, McLean, VA, USA). The mucus was allowed to dry on the bottom of the wells by incubation overnight at 37°C. After three washes of unbound material with PBS, the mucus-coated wells were blocked with 10 mg ml⁻¹ of bovine serum albumin (Sigma) for 90 min at room temperature with continuous shaking (100 rpm).
2.4. Mucus treatments

Sialidase (neuraminidase): mucus was desialylated by adding 50 mU ml⁻¹ of *Vibrio cholerae* sialidase (Sigma) to 2.4 mg ml⁻¹ of mucus in PBS containing 1 mM Ca²⁺, pH 5.5. After incubation for 1 h at 37°C, the mixture was chilled on ice for 5 min and then centrifuged for 10 min at 12000×g to remove insoluble material [24]. The asialomucus was lyophilized and kept in −20°C. Removal of sialic acid was confirmed by the assay of sialic acid-specific lectin binding to the treated mucus following the manufacturer’s instructions.

Sodium metaperiodate: sodium metaperiodate (100 μl, 10 mM) was added to the mucus-coated wells and incubated for 1 h at 37°C. The wells were then washed three times with PBS, blocked by incubation with bovine serum albumin (10 mg ml⁻¹) overnight at 4°C and used in the adhesion assay [25].

2.5. Determination of bacteria bound to mucus

2.5.1. By the urease test

A 100-μl suspension of *H. pylori* at the desired density was added to each mucus-coated well. After 90 min incubation at 37°C, the wells were washed with PBS to remove unbound bacteria. To estimate the number of bound bacteria, 100 μl of urea reagents solution consisting of 2.5 ml PBS (3 mM, pH 6.8), 0.4 ml urea solution (2% in PBS, 330 μmol l⁻¹) and 0.1 ml phenol red (7 μg ml⁻¹, pH 5.0) was added to each well [22]. The color development was monitored at 560 nm with enzyme-linked immunosorbent assay (ELISA) reader MR-5000 at 37°C every 30 min for 2 h. To convert the absorbance readings into CFU ml⁻¹, a standard curve was constructed as follows: to 5 μl of serial 2-fold dilutions of known amounts of bacteria (typically from 10⁶ to 10⁰) in each microtiter well, 95 μl of the urease reagents solution was added. The microtiter plate was incubated for 90 min at 37°C, and the absorbance at 560 nm was then recorded. The absorbance of urease in control wells consisting of 95 μl urea reagents solution and 5 μl PBS was subtracted from all values to obtain net absorbance as an indicator of bacterial urease activity. Each determination was performed in quadruplicates.

Inhibition of bacterial binding was performed by preincubation of the bacteria with different concentrations of the test compounds for 30 min at 37°C after which the mixture was added to mucus-coated wells. The percent inhibition was calculated by the following equation: % inhibition = no. of treated bacteria bound/no. of untreated bacteria bound×100 or no. of bacteria bound to treated mucus/no. of bacteria bound to untreated mucus×100.

2.5.2. By ELISA

To mucus-coated wells containing bound bacteria as described above, 35 μl of rabbit anti-*H. pylori* antiserum (Virostat, Portland, ME, USA) diluted to 1/5000 in antibody buffer (PBS+tween 20, 0.05%) was added. Following incubation for 90 min at 37°C the wells were washed five times with washing solution (NaCl 0.15 M+tween 20, 0.05%) and 100 μl of horseradish peroxidase-labeled anti-rabbit immunoglobulin-G (Sigma) diluted 1/2500 in antibody buffer was added to each well. After further incubation for 90 min at 37°C, the wells were washed five times as above, 150 μl substrate solution (one tablet of O-phenylenediamine (Sigma)/12 ml citrate-phosphate buffer+ H₂O₂, 0.03%, pH 5) was added to each well. The color was allowed to develop for 10–20 min at 37°C and then 50 μl of 4 N H₂SO₄ was added to stop the reaction. The OD was recorded at 490 nm with ELISA reader MR-5000. Controls consisted of wells without bacteria [26].

2.6. Hemagglutination assay

Equal volumes of 2-fold dilutions of *H. pylori* cell suspensions and 5% suspensions of human red blood cells (RBC) (25 μl) type O were mixed in wells of microtiter plates (U-shaped) and allowed to settle at room temperature for 1–2 h. Hemagglutination was visualized by the aggregated cells setting on the bottom of the well forming a loose carpet [27,28].

2.7. Hemagglutination inhibition assay

Bacterial suspensions were diluted with PBS to give ++++ HAU (4-hemagglutination units). Inhibition tests were performed by mixing 25 μl bacterial suspension with 25 μl of different concentrations of inhibitor for 30 min at room temperature, after which 25 μl of a 5% RBC suspension was added to each well and allowed to settle at room temperature for 1–2 h.

3. Results

3.1. Binding characteristics of *H. pylori* to human gastric mucus

To estimate the binding of *H. pylori* to human gastric mucus in the solid phase assay standard curves were constructed for each bacterial suspension employed in the particular experiment relating the level of urease activity to the number of bacteria (CFU ml⁻¹). Typical examples are shown in Fig. 1A,B. The urease activity of the bound bacteria reached plateau in most of the experiments after 2 h incubation (data not shown) and adhesion was highest for bacteria harvested after 2 days culture in broth. Urease activity was low being 0.0212, 0.03 and 0.016 OD for *H. pylori* remaining bound to bovine submaxillary glands mucin, gastric porcine mucin, and bovine extracellular matrix, respectively, as compared to 0.1865 OD for bacteria bound to human gastric mucin.
3.2. Effect of NDM on adhesion of H. pylori

NDM at 100 µg ml⁻¹ concentrations inhibited markedly the adhesion of H. pylori BZMC-25 to human gastric mucus (Fig. 2A). This inhibition was noted when the adhesion of the bound bacteria was monitored by urease activity assay or by ELISA using anti-H. pylori antiserum ruling out the possibility that the inhibition is confined to urease-active bacteria. In separate experiments we found that 100 µg ml⁻¹ NDM did not affect the urease activity of H. pylori (data not shown). The inhibition of adhesion by NDM is dose dependent and the 50% inhibitory concentration is strain dependent being 37, 125 and 305 µg ml⁻¹ of NDM for H. pylori strains BZMC-25, EHL-65 and 17874, respectively (Fig. 2B).

The adhesion of strains BZMC-25 and 17874 was not affected when the mucus was first treated with NDM nor when the NDM was added after the bacteria were allowed to adhere to the mucus (Fig. 3A,C). It was, however, abrogated when the mucus was pretreated with sodium metaperiodate, which oxidizes vicinal hydroxyl groups in carbohydrate residues, and with sialidase, which removes terminal non-reducing sialic acid residues. Confirmation that the latter treatment removed the bulk of the sialic acid was obtained by the use of SNA lectin: the sialidase-treated mucus bound only 20% of the lectin, as compared to the untreated mucus. Adhesion of H. pylori strains to mucus was inhibited specifically by 3′-sialyllactose, but not by lactose or three other simple sugars tested (Fig. 3B,D). In separate experiments we found that 6-sialyllactose was without effect (data not shown).

H. pylori is known to cause hemagglutination of human erythrocytes either via its sialic acid-specific adhesin or via its ability to bind to the Lewis b antigen [9,28]. NDM

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**Fig. 1.** Binding characteristics of H. pylori BZMC-25 to immobilized human gastric mucus. Adhesion of H. pylori (A) grown in broth for 2 days to human gastric mucus measured after 90 min incubation with the urease reagents solution as a function of the bacterial density (B) grown in broth for 1 or 2 days after 90 min and assayed as above. The number of mucus-adherent H. pylori harvested from 2 days broth culture was 2.4×10⁸±2.9×10⁷ and considered as 100% to derive the relative adhesion of bacteria as a function of their culturing age. Data are means and standard deviations of at least quadruplicate experiments.

**Fig. 2.** Effect of NDM on adhesion of H. pylori to human gastric mucus. Immobilized human mucus was exposed to a density of 0.3 OD of H. pylori strain BZMC-25 suspension obtained from 2 days broth culture in the absence (open bars) and in the presence of 100 µg ml⁻¹ NDM (closed bars) for 30 min, washed free of non-adherent bacteria and the activity of the adherent bacteria was determined either by recording the urease activity of the bacteria or by adding anti-H. pylori antibodies in an ELISA system (A). The percent inhibition of adhesion to mucus (B) of H. pylori strains BZMC-25 (close squares), EHL-65 (open squares) and 17874 (triangles) as a function of NDM concentration in the reaction mixture was determined using the urease activity assay to monitor adhesion of the bacteria. The values are the mean and standard deviation of at least quadruplicate experiments.
inhibited in a dose dependent fashion the hemagglutination of human erythrocytes caused by strains 25 and 17874 (Table 1). The hemagglutination was sensitive to sialyllactose but resistant to inhibition by Lewis antigen or acetyl lactosamine, suggesting that it is mediated by the sialic acid-specific adhesion of the bacteria employed.

4. Discussion

The adhesion of *H. pylori* to mucus constituents in the human stomach is a prerequisite for the colonization of this unique niche, which has become the major habitat of the pathogen on this planet [5,9]. While a number of specific adhesins and their cognate receptors on human cell membrane have been identified [29–33] little is known on the specificity of *H. pylori* adhesion to human gastric mucus. We employed the urease activity of bacteria bound to immobilized mucus used by other investigators to measure bacterial adhesion [10]. This adhesion is human-specific because the bacteria did not adhere to bovine extracellular matrix nor to porcine gastric mucus. Furthermore, the adhesion of the *H. pylori* strains used to human gastric mucus is abrogated by periodate oxidation or desialylation of the mucus and is inhibited specifically by sialyllactose but not by lactose or various monosaccharides. This sialic acid-specific adhesion is best expressed by bacteria grown in broth for 48 h. Our results confirm those obtained by others showing that *H. pylori* binds to sialic acid glycoconjugates in human gastric mucus via its sialic acid adhesin [10,34]. They are also consistent with previous findings showing that gastric mucus inhibited sialic acid-specific adhesion of *H. pylori* to epithelial cell lines [35,36]. Human gastric mucus, however, may contain sulfated glycoconjugates which act as inhibitors of adhesion of *H. pylori* to epithelial cells [34,37]. In preliminary studies we found that sulfated sugars (e.g. heparin) did not inhibit adhesion of *H. pylori* strain BZMC-25 to human mucus, suggesting that this carbohydrate is not involved in the adhesion system employed.

Our data show that the sialic acid-specific adhesion to human gastric mucus and to erythrocytes is inhibited by NDM constituents derived from cranberry. The 50% inhibitory concentration of NDM was 37, 125 and 305 µg ml⁻¹ of NDM for *H. pylori* strains BZMC-25, EHL-65 and 17874, respectively. Selective inhibition of bacterial adhesion by NDM was noted in previous studies. Thus, NDM inhibited adhesion of P-fimbriated *E. coli* to erythrocytes but not of type 1 fimbriated *E. coli* nor of diarrheogenic *E. coli* [15]. It also inhibited the coaggregation of certain but not all oral bacterial pairs [16]. In all these cases the target for the NDM action is the bacteria and this is also the case for *H. pylori* because preincubation of NDM with mucus has no effect on adhesion of this organism. NDM at 100 µg ml⁻¹ did not cause detachment of the bacteria from mucus. It is well known that detachment of bacteria is difficult to achieve. For example, sialyllactose detached less than 10% of bacteria bound to epithelial cell monolayer preexposed to 5 × 10⁷ CFU per ml density [35].

It has been argued that the most common habitat of *H. pylori* is gastric mucus and to cause a disease, generations
of H. pylori must detach from mucus and reach the underlying epithelium [9]. Cranberry juice may inhibit such de novo adhesion by virtue of its NDM constituent. Previous clinical trials have shown that consumption of cranberry over a defined period of time may prevent recurrent urinary tract infections probably by inhibiting the adhesion of uropathogens to intestinal cells, the source of uropathogens [14,15]. Similarly, consumption of cranberry over a defined period may inhibit the sialic acid-specific adhesion of H. pylori to new sites either in the mucus or on the epithelium and may form a possible therapeutic approach.

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