Original Paper

Caries Research

Caries Res 2009;43:171–178
DOI: 10.1159/000213888

Received: December 5, 2008
Accepted after revision: February 20, 2009
Published online: April 23, 2009

Human Milk Compounds Inhibiting Adhesion of Mutans Streptococci to Host Ligand-Coated Hydroxyapatite in vitro

L. Danielsson Niemi[a] O. Hernell[b] I. Johansson[a]

Departments of [a]Odontology/Cariology and [b]Clinical Sciences/Pediatrics, Umeå University, Umeå, Sweden

Key Words
Adhesion of mutans streptococci · Casein · Human milk · Saliva · *Streptococcus mutans*

Abstract
Acquisition of mutans streptococci at an early age is a risk factor for later caries development. Following our recent finding that human milk may inhibit adhesion of *Streptococcus mutans* the aim of the present study was to identify compounds in human milk preventing adhesion of mutans streptococci to saliva- or gp340-coated hydroxyapatite (s-HA and gp340-HA) using an in vitro model system. Superdex 200 fractions of human milk and purified proteins were screened for binding inhibition of the *S. mutans* strain Ingbritt. Avid inhibition was seen to both s-HA and gp340-HA for caseins, lactoferrin, IgA and IgG, and moderate inhibition for α-lactalbumin and bile salt-stimulated lipase, whereas albumin and lysozyme had no effect. The inhibitory epitope in β-casein was delineated to its C-terminal LLNQELLNPTHQIYPVTQPLAPVHNPISV stretch by use of synthetic peptides. Similarly, a peptide (SCKFDEYFSQSCA) corresponding to the human lactoferrin stretch that is highly homologous to the previously shown inhibitory stretch of bovine lactoferrin was found to inhibit *S. mutans* Ingbritt binding. Inhibition by human milk, IgA, and the inhibitory β-casein peptide was universal among 4 strains of *S. mutans* (Ingbritt, NG8, LT11, JBP) and 2 strains of *S. sobrinus* (6715 and OMZ176). IgG inhibited 4, α-lactalbumin 3 and lactoferrin 2 of these 6 strains. It was also confirmed that none of the milk components coated on HA mediated *S. mutans* Ingbritt adhesion, which was consistent with the finding that no milk protein was recognized on Western blots by gp340/DMBT1 monoclonal antibodies.

Oral infection with mutans streptococci *(Streptococcus mutans* and *S. sobrinus)* is a major etiological determinant for dental caries, and infection in infancy is associated with caries development later in childhood [Kohler et al., 1988; Straetemans et al., 1998]. Acquisition of mutans streptococci, and other oral bacteria, results from transmission from the caregiver and subsequent binding (adhesion) to proteins, glycoproteins and glycolipids in the salivary pellicles or on partner bacteria (coaggregation) [Caufield et al., 1993; Whittaker et al., 1996]. Besides early acquisition and high proportions of mutans streptococci in tooth biofilms, avid saliva-mediated adhesion of *S. mutans* is associated with caries development [Stenudd et al., 2001; Shimotoyodome et al., 2007]. Accordingly, the ability of mutans streptococci to interact

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0008–6568/09/0433–0171$26.00/0

Accessible online at:
www.karger.com/cre

Prof. I. Johansson
Department of Odontology
Umeå University
SE–901 87 Umeå (Sweden)
Tel. +46 90 785 6035, Fax +46 90 770 580, E-Mail ingegerd.johansson@odont.umu.se

with salivary constituents, such as salivary agglutinin (i.e. the cysteine-rich gp340 glycoprotein), mucins, proline-rich proteins, and IgA, but also nonsalivary proteins, such as collagen type I, laminin, fibronectin and fibrinogen has been studied [Gibbons and Hay, 1989; Prakobphol et al., 2000; Beg et al., 2002; Jonasson et al., 2007].

Three families of cell membrane-anchored proteins of oral streptococci are recognized to mediate their adhesion to various host receptors or partner bacteria: (a) the antigen I/II (AgI/II) family, (b) the Chs protein family, and (c) a family of glycoproteins [Jakubovics et al., 2005]. The major host receptor for the AgI/II proteins (the SpaP and Pac proteins) of *S. mutans,* i.e. the high-molecular-weight multi-domain gp340 glycoprotein, aggregates *S. mutans* cells when in the fluid phase and mediates binding when adsorbed to a surface [Brady et al., 1991; Jakubovics et al., 2005]. Interestingly, the avidity of adhesion versus aggregation by gp340 differs among strains of *S. mutans* [Jakubovics et al., 2005]. Since aggregation would facilitate bacteria clearance, whereas adhesion would facilitate their retention, events counteracting adhesion of *S. mutans* to saliva components may possibly postpone colonization upon transmission and therefore be beneficial for caries prevention.

We have reported that human milk inhibits adhesion of the *S. mutans* strain Ingbritt (a serotype c strain expressing the SpaP surface protein) [Brady et al., 1991] to saliva-coated hydroxyapatite (s-HA) in an individually varying fashion [Wernersson et al., 2006]. The components mediating adhesion inhibition in human milk remain unidentified, but the finding is in line with previous reports that bovine milk, in particular bovine casein and lactoferrin and specifically its amino acid 480–492 peptide domain (SCAFDEFFSQSCA), inhibits *S. mutans* adhesion to s-HA and gp340-coated HA (gp340-HA) [Vacca-Smith et al., 1994; Schüpbach et al., 1996; Oho et al., 2004]. The aim of the present study was to identify compounds in human milk preventing adhesion of *S. mutans* to s-HA or gp340-HA using an in vitro model system.

Materials and Methods

Bacterial Strains, Growth and Radiolabeling

S. mutans strains Ingbritt, NG8, LT11, and JBP, and *S. sobrinus* strains OMZ176 and 6715 were grown overnight at 37°C in air with 5% CO_2 on Columbia II agar supplemented with 30 ml human erythrocyte suspension per liter. For labeling, bacteria were transferred to 7 ml Jordan's broth [Jordan et al., 1960] containing 10 μl [^{35}S]methionine (100 μCi, Trans [^{35}S]-label, ICN Pharmaceuticals Inc., Irvine, Calif., USA), grown overnight, harvested and washed three times in 7 ml adhesion buffer (ADH; 50 mM KCl, 1 mM $CaCl_2 \cdot 2H_2O$, 0.1 mM $MgCl_2 \cdot 6H_2O$, 0.62 mM K_2HPO_4, 1.4 mM KH_2PO_4, pH 6.5) [Gibbons and Hay, 1988]. The isogenic Pac-defective *S. mutans* 834 mutant generated from the wild-type strain NG8 by allelic replacement [Lee et al., 1989; Brady et al., 1991] was cultured as described above but with addition of tetracycline (5 g/ml) to the media. Washed bacteria were resuspended in ADH, sonicated shortly twice, and then diluted to an appropriate concentration with 0.5% bovine serum albumin (BSA, Sigma-Aldrich Inc., St. Louis, Mo., USA). Bacterial concentration was determined by comparison with the turbidity of a standard curve for *S. mutans*.

Saliva and Milk Sampling

Milk and saliva were collected, handled, and stored as previously described [Wernersson et al., 2006]. Parotid saliva from 1 healthy adult and milk from 3 healthy mothers who had breastfed for more than 6 months were used [Wernersson et al., 2006]. The study was approved by the Ethics Committee for human experiments at the University of Linköping, Sweden.

Fractionation of Human Milk Proteins

Skimmed milk (human milk centrifuged at 40,000 *g* for 1 h at 4°C) was fractionated on a HiLoad 26/60 Superdex 200 prep grade column (Amersham Biosciences, Uppsala, Sweden) using running buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 8.0, 0.3 ml/min). Fractions of 3 ml were collected. To identify the major proteins in the Superdex 200 fractions, 15-μl samples (5–10 μg protein) from every 2nd fraction in peak areas and every 5th fraction in non-peak areas were boiled in 5 μl sample buffer (250 mM Tris, 40.4% glycerol, 8% SDS, 0.04% pyronin Y) for 3 min. The samples were then loaded onto 7.5% or 4–20% Tris-HCl Ready Gels (BioRad, Hercules, Calif., USA), and separated by electrophoresis at 20 mA (running buffer 25 mM Tris, 192 mM glycine, 0.1% SDS). Full Range Rainbow (RPN 800, Amersham Biosciences, Buckinghamshire, UK) was used as molecular weight standard, and purified human milk proteins as references, i.e. albumin, α-lactalbumin, lactoferrin, lysozyme, colostrum IgA (secretory IgA) and IgG (all Sigma), β-/κ-casein [Strömqvist et al., 1995], and bile salt-stimulated lipase (BSSL) [Bläckberg et al., 1987]. Proteins were visualized by staining with Coomassie brilliant blue. SDS-PAGE-separated proteins were transferred to PVDF membranes (Immobilon-P, 0.45 μm, Millipore Corporation, Bedford, Mass., USA) for 1 h (65 mA/membrane, max. 25 V, transfer buffer 48 mM Tris, 39 mM glycine, 0.04% SDS, 5% methanol). After blocking the membranes with 5% BSA in Tris-buffered saline with Tween 20 (TBS-T; 50 mM Tris, 150 mM NaCl, 0.05% Tween 20; pH 7.4) for 1 h at room temperature, the membranes were incubated with primary antibodies. These were rabbit anti-human IgG (γ-chain) and IgA (α-chain), rabbit anti-human albumin, rabbit anti-human lactoferrin, rabbit anti-human lysozyme (all Dako immunoglobulins or Dakopatts a/s, Glostrup, Denmark), rabbit anti-human BSSL [Bläckberg et al., 1987], mouse anti-human casein (Neomarkers, Fremont, Calif., USA), and mouse anti-human saliva gp340 (mAb143 and mAb303, gift from Prof. D. Malamud, University of Pennsylvania, Pa., USA), HYB 213-01 and HYB 213-06 (AntibodyShop, Gentofte, Denmark). After overnight incubation at +4°C the membranes were washed four times for 10 min with TBS-T, and incubated with secondary antibodies for 1 h at room temperature. The secondary antibodies were goat anti-rabbit

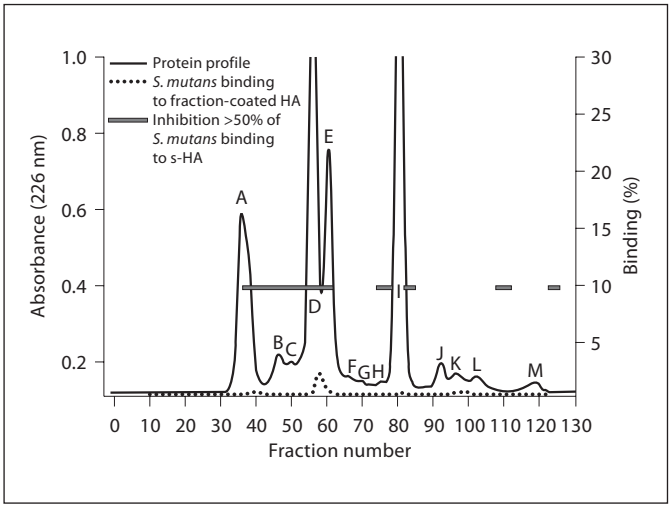

Fig. 1. Protein profile after Superdex 200 fractionation of human skim milk, with fractions inhibiting *S. mutans* strain Ingbritt binding to s-HA, exceeding 50%. Control binding to s-HA was 35% and background binding, i.e. binding to albumin-coated HA, <2%. No binding of *S. mutans* strain Ingbritt occurred to milk fraction-coated HA.

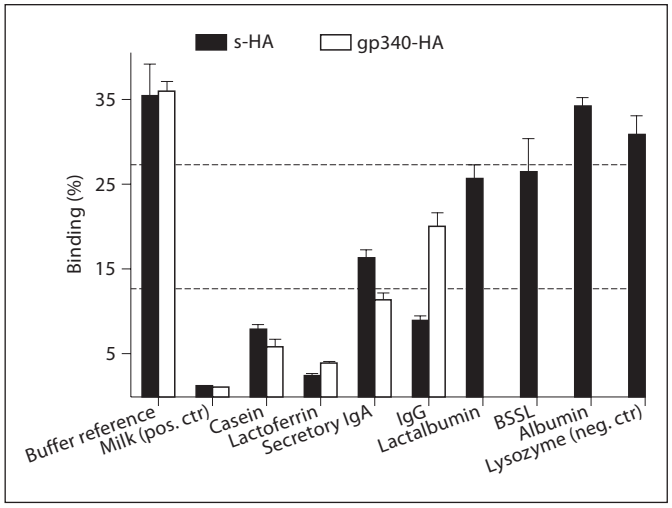

Fig. 2. Binding of *S. mutans* strain Ingbritt (percent of added bacteria) to s-HA or gp340-HA, respectively when purified human milk proteins were added simultaneously as the bacteria. The bars represent the mean and SE of triplicate measures, and the dotted lines 20 and 60% inhibition, respectively, compared with binding to ligand-coated HA when buffer was added instead of milk protein.

IgG-HRP (Dakopatts a/s) and goat anti-mouse IgG [Fab]-HRP (Nordic Biosite, Täby, Sweden). After the membranes had been washed four times for 10 min in TBS-T, antibody binding was detected by Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, Ill., USA).

Purified Proteins and Synthetic Peptides for Binding Inhibition

For further mapping of proteins and protein epitopes responsible for binding inhibition purified proteins (listed above) and a set of synthetic custom peptides with unmodified terminals (>95% purity) were used (fig. 2, 3; table 1). The peptides were SCKFDEYFSQSCA (lf) corresponding to the 502 to 514 amino acid stretch of human lactoferrin, and 7 peptides covering the sequences of human β-casein from the N- to the C-terminus. These were (bc1) RETIESLSSSEESITEYKKVEKVKHEDQQQ, (bc2) GEDEHQDKIYPSFQPQPLIYPFVEPIPYGF, (bc3) LPQNILPLAQPAVVLPVPQPEIMEVPKAKD, (bc4) TVYTKGRVMPVLKSPTIPFFDPQIPKLTDL, (bc5) ENLHLPLPLLQPLMQQVPQPIPQTLALPPQ, (bc6) PLWSVPQPKVLPIPQQVVPYPQRAVPVQAL, and (bc7) LLNQELLLNPTHQIYPVTQPLAPVHNPISV. The peptides, which were synthesized and quality tested by mass spectrometry and high-performance liquid chromatography by Genscript Corp. (Piscataway, N.J., USA), were dissolved in sterile water (bc1–bc7) or DMSO/water (lf), and pH was adjusted to 7.0–7.1. Aliquots were stored at –20°C until used.

S. mutans *Adhesion and Adhesion Inhibition*

Binding (adhesion) of *S. mutans* to HA was performed as described previously [Gibbons and Hay, 1988; Wernersson et al., 2006]. Briefly, 5 mg HA (Macro-Prep Ceramic Hydroxyapatite Type II, 80 μm, Bio-Rad, Hercules, Calif., USA) was incubated with human parotid saliva (diluted 2:3 with ADH, s-HA), purified gp340 (6 μg/ml, gp340-HA), or Superdex 200 milk fractions during end-over-end agitation for 1 h at room temperature. After two washes, and blocking with BSA, the beads were incubated with radiolabeled bacteria (62.5 μl ADH + 62.5 μl of a 1×10^9 cells/ml suspension) for 1 h at room temperature. Binding inhibition was tested by comparing the number of *S. mutans* cells binding to s-HA/gp340-HA with binding when the 62.5 μl ADH buffer was substituted for by milk, milk fractions, purified proteins, or peptides. The concentration of milk proteins was 1 mg/ml in the assay, unless otherwise stated in the figures. The numbers of bacteria attached, after unbound bacteria were washed away, were determined by scintillation counting. Samples were run in triplicate.

S. mutans *Aggregation and Aggregation Inhibition*

Aggregation of bacteria by parotid saliva was carried out by mixing equal volumes of saliva and bacterial cells in ADH buffer (5×10^9 cells/ml). Inhibition by milk, milk proteins or peptides was measured by adding the test substance (1 mg/ml in assay) simultaneously as saliva to the bacteria. ADH buffer was used in controls. Aggregate formation was scored visually after 5 min at room temperature on a scale from 0 to 5, where 0 = no visible aggregates; 1 = small uniform aggregates but no change in suspension turbidity; 2 = more aggregates of slightly larger size but no change in suspension turbidity; 3 = more and slightly larger aggregates than those that scored 2 and a slight decrease in turbidity; 4 = larger aggregates than those that scored 3 but no change in number and a solution becoming clear, and 5 = larger, but fewer aggregates in a clear solution [Hallberg et al., 1998].

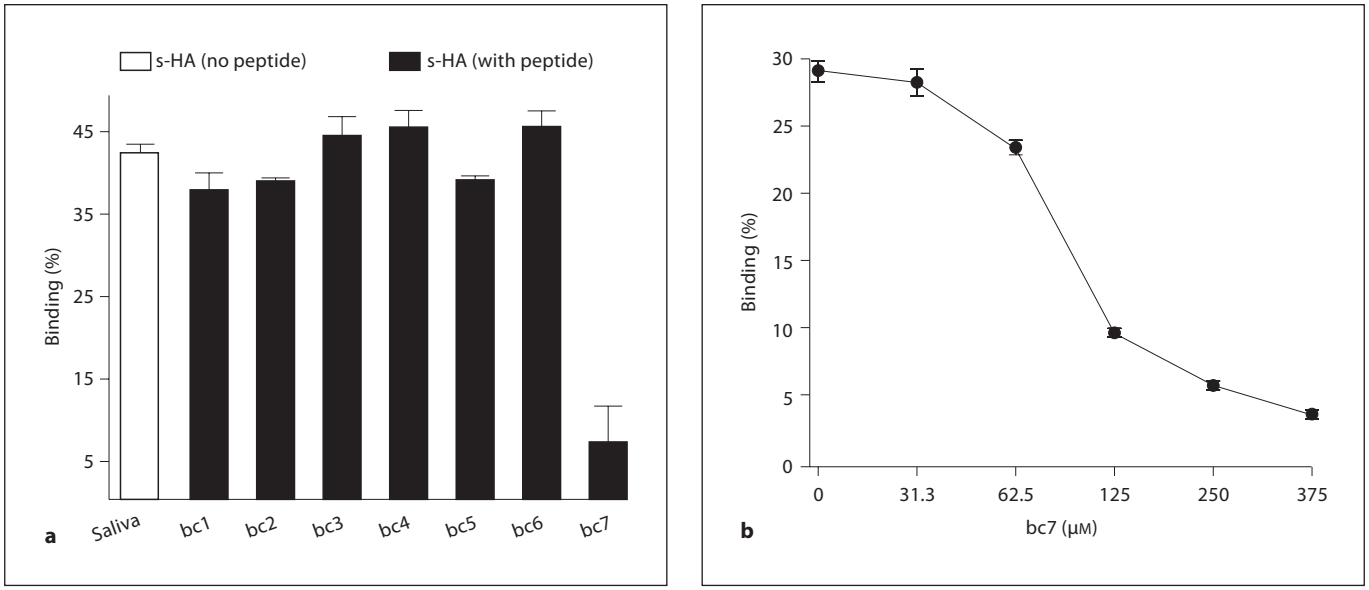

Fig. 3. Binding of *S. mutans* strain Ingbritt (percent of added bacteria) to s-HA when synthetic peptides covering the entire β-casein sequence (**a**), and a serial dilution of the inhibitory peptide bc7 (**b**) were added simultaneously as the bacteria. Data represent mean and SE from triplicate measures.

Statistics

SPSS (version 16.0.1, SPSS Inc., Chicago, Ill., USA) was used for descriptive statistics. To avoid overinterpretation of findings, a binding difference ≥15% was taken as a true difference. This was based on the finding that in 94% of triplicate assays where *S. mutans* were bound to s-HA (positive controls) the coefficient of variation among triple values was <7%, and in the remaining 6% the coefficient of variation was <11%.

Results

Adhesion Inhibition of S. mutans *Ingbritt by Human Milk Fractions*

Superdex 200 chromatography of human milk yielded a protein profile of 4 large (A, D, E and I) and 9 small peaks (B, C, F–H, J–M) (fig. 1). The major proteins were identified as IgG, IgA and albumin in peak A; IgG, IgA and BSSL in peak B; BSSL, albumin and an unidentified 80-kDa protein in peak C; caseins and 2 unidentified 17- and 18-kDa proteins in peak D; lactoferrin in peaks E–H; α-lactalbumin in peak I, and lysozyme in peak J. No milk protein was recognized on Western blots by the 4 gp340/DMBT1 monoclonal antibodies. None of the milk fractions coated on HA-mediated *S. mutans* Ingbritt adhesion, except at very low levels (<2.5%) to protein(s) in peak D (fig. 1).

Milk fractions added together with *S. mutans* cells (strain Ingbritt) to s-HA instead of buffer inhibited binding by more than 50% for fractions 34–64 (peaks A–F), fractions 74–78 (peak H), fractions 82 and 84 (peak I), and fractions 108–113 and 125 (not corresponding to an evident peak) (fig. 1). The results were similar when milk fractions from 2 mothers were used separately. Based on the identification of the major proteins in the peaks, IgG and s-IgA (peak A), BSSL (peak B), albumin (peak C), casein (peak D), lactoferrin (peaks E and H), and α-lactalbumin (peak I) were hypothesized as inhibitory components. The median inhibition by undiluted, skimmed milk was 96% among all experiments.

Adhesion Inhibition of S. mutans *Ingbritt by Purified Milk Proteins*

In vitro adherence of *S. mutans* Ingbritt to s-HA beads was avidly reduced in the presence of purified caseins, lactoferrin, IgA, and IgG instead of buffer (fig. 2), and moderately in the presence of α-lactalbumin and BSSL, whereas albumin and lysozyme had no inhibitory effect (fig. 2). Similar results were obtained when binding inhibition by casein, lactoferrin, s-IgA and IgG to gp340-HA was tested (fig. 2). Notably, binding inhibition to s-HA by BSSL varied significantly (from 26% inhibition to no inhibition) among batches.

Table 1. Binding inhibition of strains of *S. mutans* and *S. sobrinus* to s-HA by human milk, purified human milk proteins and a 30 amino acid synthetic peptide representing the C-terminal part of human β-casein (bc7)

Species/strains	Binding[a]	Binding inhibition[b]					
		human milk	IgA	casein peptide bc7	IgG	α-lactalbumin	lactoferrin
S. mutans							
Ingbritt	++	+++	+++	+++	+++	++	+++
NG8	++	+++	++	+++	+	–	++
JBP	+	+++	+++	++	–	–	–
LT11	+	+++	+++	+	–	+	–
S. sobrinus							
OMZ176	+	+++	+++	+++	++	++	–
6715	+	++	+++	++	++	–	–

[a] + = Binding <30%; ++ = binding ≥30% of added bacterial cells.
[b] Inhibition is indicated with + if ≥15 to <30%, ++ if ≥30 to <50%, and +++ if ≥50% reduction in binding compared to when buffer was used. Inhibition was calculated as 100 × [(a – b)/a)] where a stands for number of bacteria bound to s-HA and b for number of bacteria bound to s-HA with test substance. The protein/peptide concentration in the assay was 1 mg/ml, which for the bc7 peptide corresponds to 500 μM. Data represent median values from repeated experiments with triplicate measurements in each experiment.

Parotid saliva aggregation of *S. mutans* cells (strain Ingbritt) was significantly reduced by lactoferrin from 4+ (large aggregates in a clear solution) to 1+ (small aggregates in an unclear solution), whereas the other tested proteins had minor effects (data not shown). For comparison, skimmed milk reduced saliva-induced aggregation from score 4+ to score 2+ (moderate aggregates in unclear solution).

Adhesion Inhibition of S. mutans *Ingbritt to s-HA and gp340-HA by β-Casein Peptides*

β-Casein is the dominant casein variant (>85% of the caseins) in human milk [Hansson et al., 1993; Strömqvist et al., 1995]. To investigate if β-casein carries an adhesion inhibitory epitope, a set of 7 synthetic peptides covering the entire sequence of β-casein was tested for in vitro adhesion inhibition of *S. mutans* Ingbritt to s-HA. None of the 6 N-terminal peptides (bc1–bc6, 500 μM) added with bacteria to s-HA instead of buffer influenced binding substantially, whereas the LLNQELLNP THQIYPVTQP LAPVHNPISV peptide (bc7) corresponding to the C-terminal part of β-casein inhibited binding of *S. mutans* Ingbritt avidly (fig. 3a). Maximal inhibition by peptide bc7 (86%) was reached at 375 μM, which was similar to the median inhibition by undiluted, skimmed milk. Extrapolation from the curve indicated a 50% binding reduction at 100 μM of peptide bc7 (fig. 3b). The bc7 peptide also reduced saliva-induced aggregation of *S. mutans* Ingbritt from score 4+ to score 3+, whereas none of the other peptides affected saliva-induced aggregation (data not shown).

In addition, in vitro adhesion inhibition of *S. mutans* Ingbritt to s-HA was tested for a synthetic SCKFDEYF-SQSCA peptide (lf) derived from human lactoferrin and highly homologous with the inhibitory stretch of bovine lactoferrin [Oho et al., 2004]. The lf peptide (500 μM) inhibited binding by 49% (median value from repeated experiments).

Adhesion Inhibition of Different S. mutans *and* S. sobrinus *Strains by Milk Proteins*

In vitro adhesion inhibition by human milk, the bc7 β-casein peptide, lactoferrin, secretory IgA, IgG and α-lactalbumin was compared among 6 strains of mutans streptococci, i.e. 4 strains of *S. mutans* (Ingbritt, NG8, LT11, JBP) and 2 strains of *S. sobrinus* (6715 and OMZ176) (table 1). Human milk, IgA and the bc7 peptide inhibited s-HA binding for all 6 strains, whereas IgG inhibited 4 and α-lactalbumin 3 of the 6 strains, and lactoferrin inhibited binding of the strains Ingbritt and NG8 only. An isogenic mutant of strain NG8 lacking the AgI/II family adhesion (834) bound in very low numbers to s-HA and gp340-HA (<5% of input bacterial cells) and therefore inhibition was not evaluated.

Discussion

The present study identified human casein, and specifically the C-terminal section of β-casein, and secretory IgA from human milk to inhibit attachment of all tested strains of mutans streptococci to s-HA (a model for tooth enamel), whereas the effect of other milk components, such as lactoferrin, IgG, and α-lactalbumin varied among strains.

To identify candidate components in human milk for *S. mutans* adhesion inhibition Superdex 200 fractions of skimmed milk were screened. Skimmed milk was obtained by centrifugation of pH-unadjusted milk and harvesting the middle clear phase after removal of the top fat layer and a minor pellet. This intermediary phase contains casein and whey proteins, in contrast to acidified milk where the main part of the caseins precipitate and are in the pellet [Kunz and Lönnerdal, 1990]. Several of the Superdex protein peaks inhibited binding of the *S. mutans* strain Ingbritt by more than 50%, and purified key proteins of these peaks, i.e. caseins, lactoferrin, s-IgA, IgG, α-lactalbumin, and BSSL also inhibited binding avidly to moderately. However, it cannot be excluded that nonidentified proteins in the peaks contribute to the inhibitory effect of milk for the tested strains of mutans streptococci.

The finding that purified human milk casein and secretory IgA inhibit adhesion of mutans streptococci to s-HA is in line with previous reports that bovine caseins inhibit adhesion of *S. mutans* [Vacca-Smith et al., 1994] and *S. sobrinus* [Schüpbach et al., 1996], and that mutans-specific secretory IgA is present in human milk [Camling et al., 1987]. The strain diversity of lactoferrin inhibition was, however, unexpected. It was previously reported that bovine lactoferrin and its 480 to 492 amino acid stretch (SCAFDEFFSQSCA) inhibit adhesion and aggregation of the *S. mutans* strain MT8148 (expressing the Pac surface protein) by blocking the host ligand (gp340) receptor binding site [Oho et al., 2002, 2004]. In the present study, human lactoferrin inhibited the *S. mutans* strains Ingbritt and NG8 (expressing the SpaP and Pac surface proteins, respectively), but none of the other 4 tested strains of mutans streptococci. Interestingly, human lactoferrin, which inhibited binding of *S. mutans* Ingbritt avidly when added simultaneously as bacteria (present study), also inhibited binding when ligand-coated beads (saliva and gp340, respectively) were preincubated with lactoferrin (73 and 65%, respectively), whereas no inhibition occurred when bacterial cells were preincubated (additional data not shown). We therefore hypothesized that human lactoferrin, like bovine lactoferrin, inhibits binding by blocking the gp340 receptor site. This remains to be delineated further, but the hypothesis is supported by our finding that the SCKFDEYFSQSCA peptide from human lactoferrin (highly homologous to the bovine lactoferrin SCAFDEFFSQSCA peptide blocking recombinant Pac from *S. mutans* strain MT8148 by binding to gp340) inhibits both adhesion and aggregation of *S. mutans* strain Ingbritt.

Human milk contains the nonglycosylated, multiphosphorylated β-casein, and the heavily glycosylated, mono-phosphorylated κ-casein [Hansson et al., 1993; Strömqvist et al., 1995]. In late lactation milk, the total protein content is approximately 10 mg/ml, of which 30–50% are caseins (approximately 85% β-casein and 15% κ-casein) [Kunz and Lönnerdal, 1990]. The β- and κ-caseins are tedious to separate, and therefore 7 peptides representing the dominant β-casein sequence were synthesized and tested for inhibition. An inhibitory epitope within the C-terminal 30 amino acid stretch of β-casein had a universal inhibitory effect on s-HA adhesion for all 6 tested strains of mutans streptococci. The mechanism for casein inhibition cannot be defined by the present study. On the one hand, phosphorylation of native β-caseins confers high affinity for HA, which, similarly to bovine casein micelles, may lead to replacement of saliva pellicle proteins [Schüpbach et al., 1996]. On the other hand, the inhibitory effect of the nonphosphorylated synthetic β-casein peptide bc7 indicates a protein-protein interaction with a bacterial surface or saliva pellicle protein.

Several innate immunity molecules identified in saliva are also present in milk, such as lysozyme, lactoferrin, lactoperoxidase, ll37 and defensins [Amerongen and Veerman, 2002]. The innate immunity glycoprotein gp340, a receptor for mutans streptococci, is secreted by the salivary glands [Prakobphol et al., 2000]. gp340, and the spliced DMBT1, is also expressed by other cells and tissues, such as lachrymal glands, macrophages, lung and brain tissues, and gastric and intestinal mucosa [Holmskov et al., 1999; Schulz et al., 2002]. We speculated that gp340 would be expressed during lactation and secreted with the milk, since low amounts of gp340/DMBT1 RNA has been detected in mammary tissues [Holmskov et al., 1999]. However, as none of the 4 monoclonal antibodies detected gp340 in milk and confirmed the hypothesis we concluded that the low levels of RNA reported in mammary glands were not reflected by detectable levels of a gp340/DMBT1 protein in human milk. The finding supports the statement by Braidotti et al. [2004] that hetero-

geneity of DMBT1 immunoreactivity in breast tissues suggested lack of constitutive expression of gp340/DMBT1 in the mammary gland epithelium. The finding is also supported by the fact that human milk does not mediate binding of *S. mutans* [Wernersson et al., 2006].

The literature on the in vivo relationship between breastfeeding and infection with mutans streptococci or dental caries is contradictory [Li et al., 2000; Valaitis et al., 2000], whereas recent literature shows breastfeeding per se not to be associated with increased caries development [Iida et al., 2007]. The present finding that whole milk and milk components, such as casein and IgA, seem to have a universal inhibitory effect on adhesion to s-HA among mutans streptococci may support the idea that breastfeeding could counteract acquisition of mutans streptococci in childhood. Albeit isolated in small numbers in very young infants [Wan et al., 2001; Tanner et al., 2002], substantial acquisition of mutans streptococci is suggested not to occur until around 2 years of age, after teeth have erupted [Caufield et al., 1993]. Thus, a direct effect of breastfeeding on significant acquisition of mutans streptococci to tooth tissues may be relevant in countries where prolonged breastfeeding is common. In Western countries, where partial breastfeeding is common for 6–12 months, breastfeeding may still have a direct effect since the first teeth generally erupt around 6 months of age. A complementary mechanism would be that breastfeeding supports acquisition of health-associated oral microorganisms, such as *Actinomyces naeslundii* and *S. sanguis* together with the 'anti-mutans' effect. Once established polymicrobial biofilm communities are characterized by a certain degree of stability at the specific niches [Nyvad and Kilian 1987; Marsh, 2006], and long-lasting effects, i.e. favoring commensal over opportunistic bacteria as reported for the gut [Wold and Adlerberth, 2000], might therefore be an in vivo effect associated with oral health.

Acknowledgements

This study was supported by grants from the County Council of Västerbotten, TUA, Sweden, the Swedish Dental Society, and the Swedish Nutritional Foundation. Josephine Wernersson and Elisabeth Zingmark are acknowledged for skillful laboratory work.

References

Amerongen AV, Veerman EC: Saliva – The defender of the oral cavity. Oral Dis 2002;8:12–22.

Beg AM, Jones MN, Miller-Torbert T, Holt RG: Binding of *Streptococcus mutans* to extracellular matrix molecules and fibrinogen. Biochem Biophys Res Commun 2002;298:75–79.

Bläckberg L, Ängquist KA, Hernell O: Bile salt-stimulated lipase in human milk: evidence for its synthesis in the lactating mammary gland. FEBS Lett 1987;217:37–41.

Brady LJ, Crowley PJ, Ma JK, Kelly C, Lee SF, Lehner T, Bleiweis AS: Restriction fragment length polymorphisms and sequence variation within the spaP gene of *Streptococcus mutans* serotype c isolates. Infect Immun 1991;59:1803–1810.

Braidotti P, Nuciforo PG, Mollenhauer J, Poustka A, Pellegrini C, Moro A, Bulfamante G, Coggi G, Bosari S, Pietra GG: DMBT1 expression is down-regulated in breast cancer. BMC Cancer 2004;4:46–50.

Camling E, Gahnberg L, Krasse B: The relationship between IgA antibodies to *Streptococcus mutans* antigens in human saliva and breast milk and the numbers of indigenous oral *Streptococcus mutans*. Arch Oral Biol 1987;32:21–25.

Caufield PW, Cutter GR, Dasanayake AP: Initial acquisition of mutans streptococci by infants: evidence for a discrete window of infectivity. J Dent Res 1993;72:37–45.

Gibbons RJ, Hay DI: Human salivary acidic proline-rich proteins and statherin promote the attachment of *Actinomyces viscosus* LY7 to apatitic surfaces. Infect Immun 1988;56:439–445.

Gibbons RJ, Hay DI: Adsorbed salivary acidic proline-rich proteins contribute to the adhesion of *Streptococcus mutans* JBP to apatitic surfaces. J Dent Res 1989;68:1303–1307.

Hallberg K, Hammarström KJ, Falsen E, Dahlén G, Gibbons RJ, Hay DI, Strömberg N: *Actinomyces naeslundii* genospecies 1 and 2 express different binding specificities to N-acetyl-β-D-galactosamine, whereas *Actinomyces odontolyticus* expresses a different binding specificity in colonizing the human mouth. Oral Microbiol Immunol 1998;13:327–336.

Hansson L, Bergström S, Hernell O, Lönnerdal B, Nilsson AK, Strömqvist M: Expression of human milk beta-casein in *Escherichia coli:* comparison of recombinant protein with native isoforms. Protein Expr Purif 1993;4:373–381.

Holmskov U, Mollenhauer J, Madsen J, Vitved L, Gronlund J, Tornoe I, Kliem A, Reid KB, Poustka A, Skjodt K: Cloning of gp340, a putative opsonin receptor for lung surfactant protein D. Proc Natl Acad Sci USA 1999;96:10794–10799.

Iida H, Auinger P, Billings RJ, Weitzman M: Association between infant breastfeeding and early childhood caries in the United States. Pediatrics 2007;120:944–952.

Jakubovics NS, Stromberg N, van Dolleweerd CJ, Kelly CG, Jenkinson HF: Differential binding specificities of oral streptococcal antigen I/II family adhesins for human or bacterial ligands. Mol Microbiol 2005;55:1591–1605.

Jonasson A, Eriksson C, Jenkinson HF, Källestål C, Johansson I, Strömberg N: Innate immunity glycoprotein gp-340 variants may modulate human susceptibility to dental caries. BMC Infect Dis 2007;7:57–64.

Jordan HV, Fitzgerald RJ, Bowler AE: Inhibition of experimental caries by sodium metabisulfite and its effect on the growth and metabolism of selected bacteria. J Dent Res 1960;39:116–123.

Kohler B, Andreen I, Jonsson B: The earlier the colonization by mutans streptococci, the higher the caries prevalence at 4 years of age. Oral Microbiol Immunol 1988;3:14–17.

Kunz C, Lönnerdal B: Human-milk proteins: analysis of casein and casein subunits by anion-exchange chromatography, gel electrophoresis, and specific staining methods. Am J Clin Nutr 1990;51:37–46.

Lee SF, Progulske-Fox A, Erdos GW, Piacentini DA, Ayakawa GY, Crowley PJ, Bleiweis AS: Construction and characterization of isogenic mutants of *Streptococcus mutans* deficient in major surface protein antigen P1 (I/II). Infect Immun 1989;57:3306–3313.

Li Y, Wang W, Caufield PW: The fidelity of mutans streptococci transmission and caries status correlate with breast-feeding experience among Chinese families. Caries Res 2000;34:123–132.

Marsh PD: Dental plaque as a microbial community – Implications for health and disease. BMC Oral Health 2006;6(suppl 1):S14.

Nyvad B, Kilian M: Microbiology of the early colonization of human enamel and root surfaces in vivo. Scand J Dent Res 1987;95:369–380.

Oho T, Bikker FJ, Nieuw Amerongen AV, Groenink J: A peptide domain of bovine milk lactoferrin inhibits the interaction between streptococcal surface protein antigen and a salivary agglutinin peptide domain. Infect Immun 2004;72:6181–6184.

Oho T, Mitoma M, Koga T: Functional domain of bovine milk lactoferrin which inhibits the adherence of *Streptococcus mutans* cells to a salivary film. Infect Immun 2002;70:5279–5282.

Prakobphol A, Xu F, Hoang VM, Larsson T, Bergstrom J, Johansson I, Frängsmyr L, Holmskov U, Leffler H, Nilsson C, Borén T, Wright JR, Strömberg N, Fisher SJ: Salivary agglutinin, which binds *Streptococcus mutans* and *Helicobacter pylori,* is the lung scavenger receptor cysteine-rich protein gp340. J Biol Chem 2000;275:39860–39866.

Schulz BL, Oxley D, Packer NH, Karlsson NG: Identification of two highly sialylated human tear-fluid DMBT1 isoforms: the major high-molecular-mass glycoproteins in human tears. Biochem J 2002;366:511–520.

Schüpbach P, Neeser JR, Golliard M, Rouvet M, Guggenheim B: Incorporation of caseinoglycomacropeptide and caseinophosphopeptide into the salivary pellicle inhibits adherence of mutans streptococci. J Dent Res 1996;75:1779–1788.

Shimotoyodome A, Kobayashi H, Tokimitsu I, Hase T, Inoue T, Matsukubo T, Takaesu Y: Saliva-promoted adhesion of *Streptococcus mutans* MT8148 associates with dental plaque and caries experience. Caries Res 2007;41:212–218.

Stenudd C, Nordlund Å, Ryberg M, Johansson I, Källestål C, Strömberg N: The association of bacterial adhesion with dental caries. J Dent Res 2001;80:2005–2010.

Straetemans MM, van Loveren C, de Soet JJ, de Graaff J, ten Cate JM: Colonization with mutans streptococci and lactobacilli and the caries experience of children after the age of five. J Dent Res 1998;77:1851–1855.

Strömqvist M, Falk P, Bergström S, Hansson L, Lönnerdal B, Normark S, Hernell O: Human milk κ-casein and inhibition of *Helicobacter pylori* adhesion to human gastric mucosa. J Pediatr Gastroenterol Nutr 1995;21:288–296.

Tanner AC, Milgrom PM, Kent R, Mokeem SA, Page RC, Riedy CA, Weinstein P, Bruss J: The microbiota of young children from tooth and tongue samples. J Dent Res 2002;81:53–57.

Vacca-Smith AM, Van Wuyckhuyse BC, Tabak LA, Bowen WH: The effect of milk and casein proteins on the adherence of *Streptococcus mutans* to saliva-coated hydroxyapatite. Arch Oral Biol 1994;39:1063–1069.

Valaitis R, Hesch R, Passareli C, Sheehan D, Sinton J: A systematic review of the relationship between breast-feeding and early childhood caries. Can J Public Health 2000;91:411–417.

Wernersson J, Danielsson Niemi L, Einarson S, Hernell O, Johansson I: Effects of human milk on adhesion of *Streptococcus mutans* to saliva-coated hydroxyapatite in vitro. Caries Res 2006;40:412–417.

Wan AK, Seow WK, Purdie DM, Bird PS, Walsh LJ, Tudehope DI: Oral colonization of *Streptococcus mutans* in six-month-old predentate infants. J Dent Res 2001;80:2060–2065.

Whittaker CJ, Klier CM, Kohlenbrander PE: Mechanisms of adhesion by oral bacteria. Annu Rev Microbiol 1996;50:513–552.

Wold AE, Adlerberth I: Breast feeding and the intestinal microflora of the infant-implications for protection against infectious diseases. Adv Exp Med Biol 2000;478:77–93.