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INHIBITION OF ENTEROTOXIGENIC *ESCHERICHIA COLI* (ETEC) ADHESION TO CACO-2 CELLS BY HUMAN MILK AND ITS IMMUNOGLOBULIN AND NON-IMMUNOGLOBULIN FRACTIONS

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ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) is the most common cause of diarrhea in children in developing countries and among travelers to ETEC endemic areas. ETEC diarrhea is caused by colonization of the small intestine mediated by colonization factor (CF) antigens, and subsequent elaboration of enterotoxins. Breast feeding has been related to protection against enteric infections. The protective effect of human milk can be ascribed to its immunoglobulin content, specially secretory immunoglobulin A (sIgA), and to non-immunoglobulin components such as free oligosaccharides, glycoproteins and glycolipids. In this study we investigated the effect of whole human milk and its fractions immunoglobulin and non-immunoglobulin on the adherence of ETEC strains possessing different CFs to Caco-2 cells, as well as the ability of sIgA and free secretory component (fSC) to bind to bacterial superficial proteins. Pooled human milk from three donors were fractionated by gel filtration and analyzed by SDS-PAGE. Our results revealed that whole human milk and its proteins fractions, containing sIgA and fSC, inhibited adhesion ETEC strains harboring different colonization factors antigens. We also verified that sIgA and fSC, using immunoblotting and immunogold labeling assays, bound to some fimbrial proteins and other material present in bacterial surface. Our findings suggest that whole human milk and its fractions may contribute to protection against ETEC infections by blocking bacterial adhesion mediated by different colonization antigens.

Keyword: Diarrhea; Adherence inhibition; *Escherichia coli* enterotoxigenic; Colonization factor antigen; Human milk

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is an important cause of diarrhea among children in developing countries (28,29) and among travelers to ETEC endemic areas (18). Two virulence attributes that characterize ETEC are the colonization of the small intestine surface and the production of enterotoxins. It is well-known that in ETEC strains the colonization of intestinal mucosa is related to the presence of specific fimbrial antigens, called colonization factors (CFs) (10). Twenty one different CFs have been described for human ETEC (12,26).

Among the most studied and frequently found CFs are CFA I, CS1, CS2, CS3, CS4, CS5, CS6 and CS8 (12). Most of ETEC isolated in Brazil have been shown to possess CFA I, CFA II and CFA IV (14,17,27).

Epidemiological studies of diarrhea have shown that breast feeding protects infants from intestinal infections (20,32). The protective effect of human milk is usually attributed to its high content of immunoglobulin, specially secretory immunoglobulin A (sIgA) (6,16). Some works showed that sIgA inhibit the adhesion of enteropathogenic *Escherichia coli* to HeLa cells and Hep-2 (5,6,9), and possess effect against ETEC

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virulence factors (7,8). Recently, some studies of our group demonstrated that sIgA inhibits the adherence of diffusely adherent *Escherichia coli* (DAEC), enteroaggregative *Escherichia coli* (EAEC) and *Shigella flexneri* to HeLa cells (1,31).

Protection by human milk can also be ascribed to non-immunoglobulin components such as free oligosaccharides, glycolipids and glycoproteins (2,6,24). We demonstrated that human milk glycoproteins, such as lactoferrin and free secretory component (fSC), have the ability to inhibit adhesion of ETEC CFA I⁺ strains to human erythrocytes (15).

The secretory component (SC) is found in several secretions complexed with sIgA or as a free glycoprotein (22). It has been shown that fSC is an 80 kDa glycoprotein, consisting of a single polypeptide chain largely glycosylated (20%) (19). There is little information about the role of fSC in secretions, but some workers suggest that it may have a protective role against diarrhea (4,15). Recently, using immunogold labeling, we showed the ability of fSC to interact with CFA I and CFA II (CS1 and CS3) fimbrial adhesins (25). To determine if the interaction of milk proteins to bacterial superficial structures could contribute to inhibition of ETEC adhesion, we investigated the effect of whole human milk, immunoglobulin fraction (IgF) and fSC on the adherence of ETEC strains possessing CFA I, CS1, CS2, CS3, CS4, CS5, CS6 and CS8 to intestinal cells. The binding of IgF and fSC to bacteria superficial proteins also was investigated.

MATERIAL AND METHODS

Bacterial strains

The phenotypes of all human ETEC strains used in this work are shown in Table 1.

Breast milk samples

Human milk was obtained from the Human Milk Bank of the Hospital Universitário de Brasília (Brasília, Brazil). Samples were obtained from lactating mothers up to one month after delivery and kept frozen -20°C. Aliquots of individual milk samples were taken from at least three donors and pooled.

Fractionation of human milk

The fractionation of pooled human milk was performed as described previously by Giugliano *et al.* (15). Briefly, lipids and casein were removed, and proteins concentrated by adding ammonium sulfate to 70% saturation. The sample obtained was dialyzed and applied to a Sephacryl S-200 HR column (2.6 x 80 cm; Pharmacia Biotech AB, Uppsala, Sweden) eluted with Tris-HCl buffer, pH 7.6. Thereafter, the fractions eluted from each peak were pooled and proteins were concentrated, dissolved and dialyzed overnight against Tris-HCl buffer, pH 7.6. This material was used in the adhesion inhibition assays.

Table 1. Bacterial samples.

Strains	Serotype	CFs	Enterotoxins
H10407A ^c	O78:H11	CFA I, longus	LT/ST
PB176 ^b	O6:H16	CS1, CS3	LT/ST
E4833 ^b	O6:H16	CS2, CS3	LT/ST
58R957 ^b	O6:H16	CS2	LT
E2539C1 ^c	O25:NM	CS3, longus	LT
61.1 ^a	O9:H10	CS8	LT
E11881E ^c	ND	CS4, CS6, longus	LT/ST
118299 ^c	ND	CS5, CS6, longus	ND
B7A ^c	O148:H28	CS6, longus	LT/ST

^aStrain from our bacterial collection; ^bStrains kindly provided by Dr. B.C Guth, Universidade Federal de São Paulo, Brazil; ^cStrains kindly provide by Dr. J.A. Girón, Universidad Autonoma de Puebla, Mexico; ND- not determined.

Inhibition of bacterial adhesion assay

Caco-2 (8 x 10⁴ cells/mL) cells (11), were grown in 24-well tissue culture plates containing Dulbecco's modified Eagle's medium (DMEM), supplemented with 15% fetal bovine serum at 37°C in 4% CO₂, for at least 10 days. Bacterial strains were grown in Luria Broth (LB) for 18 h at 37°C. To study the effect of human milk upon ETEC adherence, 40 µL bacterial culture were previously exposed to 40 µL human milk or its fractions for 30 min at room temperature. This mixture was added to the wells of the tissue culture plates, containing confluent Caco-2 cells grown in fresh DMEM supplemented with 0.5% D-mannose. The plates were incubated for 1 h at 37°C. The cells were then washed three times with sterile phosphate buffered saline (PBS). After treating the Caco-2 cells with a 0.02% trypsin-EDTA solution, the cells were resuspended and counted in a Neubauer chamber. The suspension, containing bacterial cells, was diluted in PBS and inoculated in LB agar, in order to determine the number of colony forming units (CFU) per cell. All tests were performed in triplicate, and at least in three different occasions. The effect of human milk and its fractions was determined by calculating the mean number of bacteria attached per cell. Bacterial cells incubated with PBS were used as controls.

Statistical analysis

The results were expressed as mean number ± standard deviations (SD) of bacteria adhering per cell. Statistical significance was determined by using independent sample t- Student test. The SPSS 10.0 program for Windows was used.

Fimbriae purification

Fimbriae purification was performed as described by Mynott *et al.* (23), with some modifications. To improve CFs expression,

ETEC strains were grown in CFA agar (10) at 37°C for 18 h. In this conditions longus factor is not expressed since it requires special culture conditions (13). Bacterial cells were harvested and suspended in PBS. Fimbriae were detached from bacteria by heat treatment in a shaking water bath at 65°C for 20 min. Bacterial cells were removed and the supernatant was centrifuged at 39000g for 2 h at 4°C, to remove outer-membrane contaminants. The resulting supernatant was stored overnight at 4°C, to allow fimbrial aggregation and then centrifuged at 190387g for 2 h in a Hitachi (fixed rotor RP70T) ultracentrifuge (Hitachi, Japan). The pellet, corresponding to the partially purified fimbriae, was suspended in 500 µL of PBS (enriched fimbrial preparation).

SDS-PAGE and immunoblotting analysis

The protein content was measured by the method described by Bradford (3) and analysed by SDS-PAGE on acrylamide 12% gels, as described by Laemmli (21). Gels were stained with Coomassie brilliant blue G (Bio-Rad Laboratories, Hercules, CA, USA).

After electrophoresis, proteins were transferred to nitrocellulose membranes (Trans-Blot SD Semi-Dry CELL, Bio-Rad Laboratories, Hercules, CA, USA) by the method described by Towbin *et al.* (30). To verify the binding of IgF and fSC from human milk to the fimbrial proteins, the membranes were previously treated with human milk fractions overnight at 4°C. Subsequently, the membranes were incubated with specific anti-SC, anti-goat IgG peroxidase conjugated and the reaction was developed with 4-chloro-1-naphthol.

Immunolabeling assay and electron microscopy

The binding of human milk components to CFs were determined by an immunological labeling assay, as described

previously (25). Briefly, enriched CFs fimbrial preparations were exposed to human milk fractions for 1h at room temperature. Afterwards, the fimbrial suspensions were placed on Formvar-carbon-coated grids (200 mesh) (Electron Microscopy Sciences, Fort Washington, USA). The grids were previously treated with a blocking solution, containing 1% bovine serum albumin, 0.85% NaCl, in 10 mM Tris-HCl buffer, pH 8.0. To verify the binding of IgA and fSC to different colonization factors, the grids were incubated with goat anti-SC serum, followed by anti-goat IgG gold labeled (10 nm gold) and negatively stained with 2% phosphotungstic acid, pH 7.2. Subsequently, the grids were examined with a Jeol Jem 100 C or 1011 electron microscope (Jeol, Japan).

Reagents, antibodies and culture medium

Reagents and antibodies were from Sigma (St. Louis, MO, USA) and culture medium from Gibco (Grand Island, NY, USA), Difco (Detroit, Michigan, USA) and VETEC (Rio de Janeiro, RJ, Brazil).

RESULTS

Inhibition of the adhesion of ETEC strains to Caco-2 cells

Adhesion inhibition assays showed that whole human milk inhibited the adhesion of seven of the nine ETEC strains to Caco-2 cells (Table 2). The adherence of the strains possessing CS2/CS3 and CS2 was not affected by whole milk, while in all other adherent strains tested it was inhibited ($P < 0.05$). To verify the effect of the human milk immunoglobulin (IgF) and non-immunoglobulin (NIgF) fractions in the ETEC adhesion, concentrated proteins of the whey were separated on a Sephacryl S-200 HR column. A typical profile showing three protein peaks was obtained, as previously reported (15).

Table 2. Inhibition of ETEC strains adherence to Caco-2 cells by whole human milk and its fractions.

CFs	Whole human milk			IgF			NIgF		
	Control Mean ± SD	Treated Mean ± SD	<i>P</i>	Control Mean ± SD	Treated Mean ± SD	<i>P</i>	Control Mean ± SD	Treated Mean ± SD	<i>P</i>
CFA I	28.81 ± 8.17^a	12.66 ± 13.04^a	>0.01	0.69±0.72	0.35±0.29	0.17	0.89 ± 0.70^a	0.34 ± 0.18^a	0.04
CS1/CS3	7.62 ± 9.10^a	1.80 ± 1.04^a	0.03	1.69 ± 1.62^a	0.57 ± 0.72^a	0.02	1.13 ± 0.50^a	0.51 ± 0.46^a	0.01
CS2/CS3	12.36±20.47	18.18±29.38	0.85	0.35±0.16	0.44±0.15	0.24	0.30±0.16	0.40±0.24	0.34
CS2	8.66±12.16	2.25 ± 5.29	0.51	0.16±0.08	0.21±0.18	0.45	0.26±0.20	0.39±0.36	0.39
CS3	7.89 ± 10.45^a	15.2 ± 0.78^a	0.02	2.77±5.07	1.09±1.16	0.54	1.04±0.50	1.31±1.13	0.69
CS4/CS6	0.83 ± 0.71^a	0.11 ± 0.08^a	>0.01	0.30 ± 0.21^a	0.14 ± 0.06^a	0.06	0.73±0.94	0.42±0.39	0.54
CS5/CS6	0.63 ± 0.50^a	0.17 ± 0.19^a	0.05	0.96 ± 0.70^a	0.42 ± 0.30^a	0.03	3.32±2.07	2.13±0.80	0.27
CS6	0.82 ± 0.93^a	0.15 ± 0.13^a	0.01	0.17 ± 0.08^a	0.09 ± 0.08^a	0.02	0.44±0.15	0.34±0.14	0.31
CS8	57.79 ± 44.26^a	18.19 ± 27.29^a	0.03	0.61 ± 0.35^a	0.31 ± 0.25^a	0.04	3.05 ± 2.81^a	1.87 ± 0.63^a	0.03

Results represent mean number ± SD of bacteria adhering per cell at least six determinations; ^a The treatment was significantly different ($P < 0.05$) from the control. IgF - immunoglobulin fraction; NIgF - non-immunoglobulin fraction.

Electrophoretic analysis of first peak revealed a typical profile of sIgA, showing protein bands corresponding to the secretory component, the heavy, light and J chains. SDS-PAGE of the second peak showed a main protein band corresponding to the free secretory component. Immunoglobulins were not detected in this peak, since the band corresponding to the heavy chain was absent. The presence of sIgA and fSC in these peaks was confirmed by immunoblotting.

As shown in Table 2, the first peak, containing 0.5 mg/mL of IgF, inhibited adherence of ETEC strains harbouring colonization factor antigens CS1/CS3, CS4/CS6, CS5/CS6, CS6 and CS8 to Caco-2 cells. Although, the *P* value for effect of IgF over CS4/CS6 strain was 0.06 (Table 2), we considered that this fraction to be effective in decreasing the adhesion of this strain. This decision was taken because in statistical tests accepting the null hypothesis in such cases could lead to a type II error higher than rejecting it. Furthermore, as shown below, we have other evidences of the action of IgF on this bacterial strain structure. The NIgF eluted in the second peak, corresponding to 0.6 mg/mL of fSC and others milk proteins, inhibited adhesion strains possessing antigen I (CFA I), CS1/CS3, and CS8. The proteins corresponding to the third eluted peak had no effect on bacterial adherence (data not shown). Both IgF and NIgF were not able to influence bacterial growth (31).

Binding of IgF and fSC to superficial bacterial proteins

To determine the binding of IgF and fSC to the ETEC CFs, immobilized superficial bacterial proteins were exposed to immunoglobulin or non-immunoglobulin fractions. Binding of

IgF and fSC were detected by immunoblotting analysis. The binding profiles of the IgF and fSC to the superficial proteins preparations were similar (Fig. 1B,C). We observed that IgF and fSC bound strongly to protein bands with molecular masses corresponding to CFA I (15.7 kDa), CS1 (17.3 kDa), CS3 (15.1 kDa), CS5 (21.8 kDa), CS6 (15.7 kDa) and bound weakly with a protein band corresponding to CS8 antigen (18.7 kDa). Furthermore, immunoblotting analysis revealed that both, IgF and fSC, interacted with a superficial protein of ~40 kDa extracted from the strains expressing in CFA I, CS4/CS6, CS5/CS6, CS6, and CS8. Weak bindings on others protein bands also were detected.

Binding of IgF and fSC to fimbrial structures

Immunogold labeling assays, performed with different CFs preparations exposed to IgF, showed that both CS1/CS3 and CS8 fimbriae preparations were labeled. We also observed some scattered gold beads not bound to the fimbriae structure in CS1/CS3, CS4/CS6, and CS8 fimbrial preparations. Our results also revealed that IgF interacts with the extracellular amorphous mass in CS4/CS6 preparation. Fig. 2 illustrates a representative binding, observed in this study.

As previously reported (25), fSC binds to CFA I, CS1 and CS3 fimbriae. In this work, fimbrial preparations exposed to fSC revealed immunolabeling on fimbriae CS4/CS6 and CS8 preparations. We also observed that fSC has ability to scattering binding on other bacterial surface material, similar to IgF, present in both fimbrial preparations from CS4/CS6 and CS8 strains.

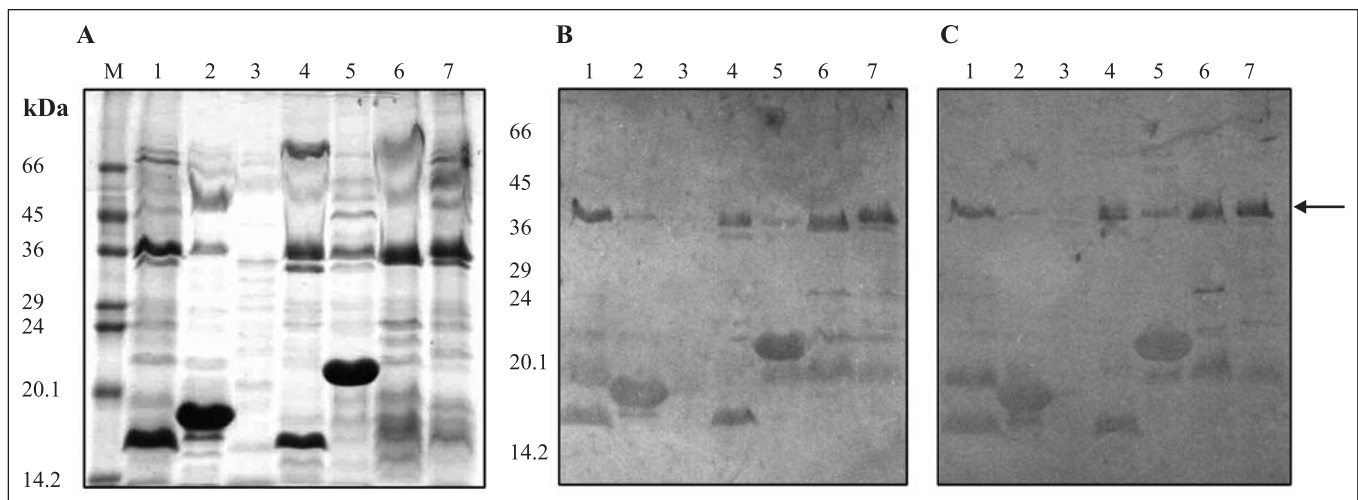


Figure 1. SDS-PAGE (A) and immunoblots of fimbriae preparations reacted with secretory IgA (B) and fSC (C). Binding of IgA and fSC to different colonization factors was revealed using specific anti-SC antibody. M, molecular mass markers; lane 1, CFA I; lane 2, CS1/CS3; lane 3, CS3; lane 4, CS4/CS6; lane 5, CS5/CS6; lane 6, CS6; lane 7, CS8. Arrow indicates the ~40 kDa protein. CFA I (15.7 kDa), CS1 (17.3 kDa), CS3 (15.1 kDa), CS5 (21.8 kDa), CS6 (15.7), CS8 (18.7 kDa).

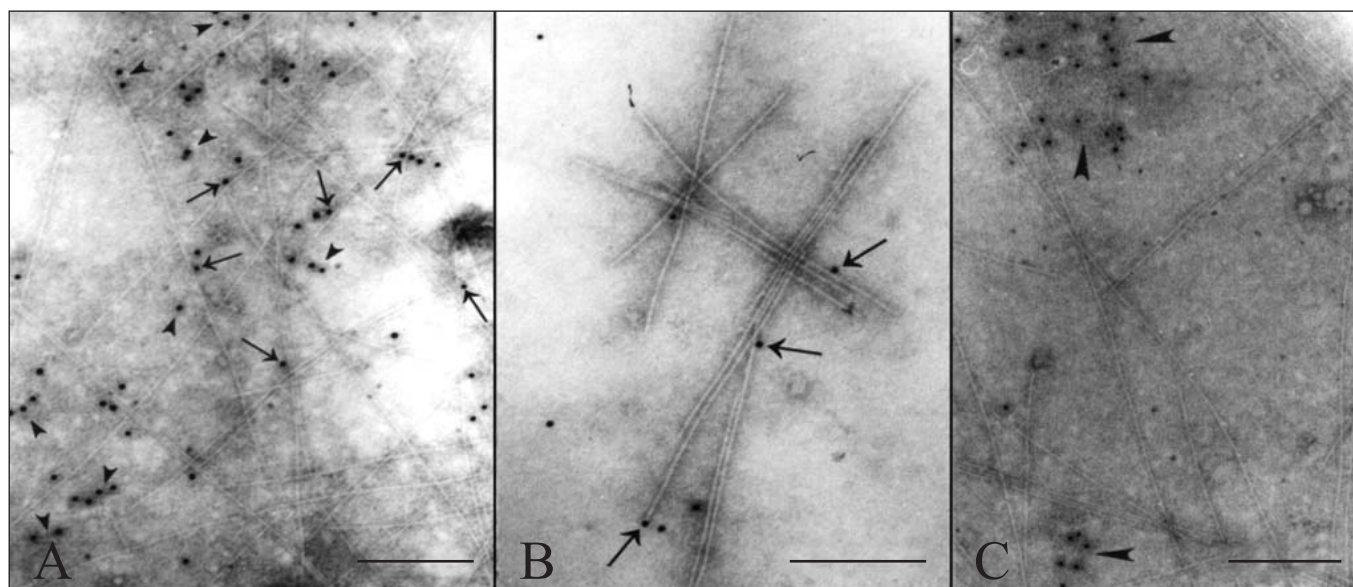


Figure 2. Representative immunogold labeling assay of the enriched fimbrial preparations. Binding pattern of fSC on CS4/CS6 (A), CS8 (B) and on extracellular amorphous mass in CS4/CS6 preparation (C). Binding of fSC to different colonization factors was revealed using specific anti-SC antibody. The arrows indicate the gold particles on fimbriae. The small arrowhead and large arrowhead indicate scattered labeling and binding on amorphous mass, respectively. Bar, 0.2 mm.

DISCUSSION

Continuing our studies on the adherence inhibition of ETEC strains by human milk, we investigated the effect of whole milk and its IgF and NIgF on the adhesion mediated by CFA I, CS1, CS2, CS3, CS4, CS5, CS6, CS8 and their combinations, on tissue culture.

Our results revealed that the whole human milk affected the adherence of most of the strains tested. Adhesion of the strain possessing CFA I fimbriae, was inhibited by both, whole milk and NIgF, but not by the IgF, indicating the importance of NIgF on the adhesion of CFA I strains. These findings are corroborated by our previous studies (15,25), showing that lactoferrin and fSC present in the NIgF inhibit bacterial adhesion to erythrocytes, binding to bacterial fimbriae.

In the cases of strains possessing CS1/CS3 and CS8 both, IgF and NIgF, affected their adhesion, probably acting synergistically. On the other hand, CS2/CS3 and CS2 strains were not affected by whole milk, while the CS3 strain was inhibited. Taken together, these results suggest a main role for CS2, since the strains possessing this antigen alone or in combination were not inhibited.

Interestingly, IgF and NIgF had no inhibitory effect on the CS3 strain adhesion, indicating that non-protein components of the milk, such as glycolipids and oligosaccharides, could be involved on the inhibition of the adhesion of this strain.

We also observed that strains CS4/CS6, CS5/CS6 and CS6 were inhibited by whole milk and IgF. The incapacity of IgF to

inhibit four of the nine strains tested may be related the need of a previous exposure of the mother to each pathogen. In this sense, probably our milk pool did not have specific antibodies directed to all of the colonization factors studied.

The mechanism by which milk compounds affect ETEC adhesion may be related to their ability to interact with bacterial surface molecules involved in adhesion. Therefore, to investigate the molecular target of immunoglobulin of milk, and the putative binding of fSC, immobilized bacterial surface proteins were exposed to IgF and NIgF.

A similar binding profile of IgF and fSC was found with both compounds, which bind to six colonization factors, indicating that these antigens are important molecular targets involved in the adhesion process. Immunoblotting analysis revealed that both, IgF and fSC, bound to CFA I, although only fSC seems to affect the adhesion of the CFA I strain, since the IgF did not present any inhibitory effect on the adhesion of this strain. The adhesion of the CS8 strain was inhibited by IgF and NIgF, probably by the ability of immunoglobulin and fSC to bind to CS8 fimbriae. We also observed that IgF and fSC bind to both, CS5 and CS6 fimbriae from CS4/CS6 preparation; however, the adhesion of the strains possessing CS4/CS6 and CS5/CS6 combinations was inhibited only by IgF. Binding of IgF and fSC to the protein band corresponding to CS6 from CS5/CS6 and CS6 strains fimbrial preparations was not detected. It is possible that these preparations were not enriched enough with CS6 antigen, preventing its recognition by milk proteins isolated in

this study. We also did not observe binding of IgF and fSC to CS4, suggesting that these proteins do not recognize this antigen, and to CS3 strain, reinforcing our hypothesis that other milk components could act as inhibitors of the bacterial adhesion to Caco-2 cells.

Interestingly, we verified that both IgF and fSC bound to other protein bands present in bacterial superficial protein preparations, predominantly to a ~40 kDa protein, which seems to be a common protein, possibly involved in the adhesion of ETEC strains to eukaryotic cells.

In this study we have also investigated the binding of both, IgF and fSC, to fimbrial structures, using immunocytochemistry assays. Our results revealed that IgF interacts with CS1/CS3 and CS8 fimbriae, which seem to be important to the adhesion process since both were inhibited in the adhesion assays. The same result was observed with CFA I, CS1/CS3 (25) and CS8 exposed to fSC, indicating that this glycoprotein could play an important role against ETEC strains harbouring these CFs. We also verified that fSC binds to fimbrial structures present in CS4/CS6 preparation, although this binding did not affect CS4/CS6 strain adhesion to Caco-2 cells.

We also observed some gold beads showing a scattered or aggregative labeling pattern on other cellular material present in some fimbrial preparations. This binding profile may be related to the ~40 kDa protein and other bacterial surface proteins recognized by IgF and fSC in the immunoblotting assay. Taken together, our results suggest that bacterial adhesion to host cells may involve fimbrial proteins, and probably other bacterial superficial molecules. Further studies are necessary to determine the actual role of these molecules present on bacterial surface.

Our findings reveal that active compounds from the human milk, such as immunoglobulins and non-immunoglobulin proteins can inhibit the adherence of several ETEC strains to Caco-2 cells, with this mechanism probably playing an important role in the protection of infants against enteric infections.

CONCLUSIONS

This study indicates that whole human milk and its immunoglobulin and non-immunoglobulin fractions inhibit ETEC strains adhesion to eukaryotic cells, a process mediated by different colonization factors. Our findings suggest that IgF and fSC can bind to different colonization factors from ETEC, implicating these factors as important molecular targets involved in the adhesion process. Moreover, we also verified that both, IgF and fSC, bind to other bacterial superficial proteins, specially to a ~40 kDa protein, which may be involved in ETEC strains adhesion. The ability of IgF and fSC to interact with fimbrial proteins and other superficial cellular material indicates that specific antibodies and non-specific defense factors from human milk may block ETEC strains adhesion to host cell receptors.

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RESUMO

Inibição da adesão de *Escherichia coli* enterotoxigênica (ETEC) as células CACO-2 pelo leite humano e suas frações imunoglobulínica e não imunoglobulínica

Escherichia coli enterotoxigênica (ETEC) é uma importante causa de diarreia infantil em países em desenvolvimento e entre os viajantes em áreas endêmicas. A diarreia por ETEC é causada pela colonização do intestino delgado, mediada pelos fatores antigênicos de colonização (CFs), e a subsequente elaboração de enterotoxinas. A amamentação tem sido relacionada com a proteção contra infecções entéricas. O efeito protetor do leite humano pode ser atribuído ao seu conteúdo imunoglobulínico, especialmente a imunoglobulina A secretória (sIgA), e aos componentes não imunoglobulínicos tais como oligossacarídeos livres, glicoproteínas e glicolipídeos. Neste estudo nós investigamos o efeito do leite humano total e suas frações imunoglobulínica e não imunoglobulínica sobre a adesão de cepas ETEC possuidoras de diferentes fatores de colonização as células Caco-2, bem como a habilidade da sIgA e do componente secretor livre (fSC) de se ligarem as proteínas da superfície bacteriana. Uma mistura de leite de três doadoras foi fracionada por filtração em gel e analisada por SDS-PAGE. Nossos resultados revelaram que o leite humano total e suas frações protéicas, contendo sIgA e fSC, inibiu a adesão de cepas ETEC albergando diferentes fatores antigênicos de colonização. Nós também verificamos que a sIgA e o fSC, usando ensaios de imunoeletrortransferência e imunomarcção, ligaram-se a algumas proteínas fimbriais e a outro material presente na superfície bacteriana. Nossos dados sugerem que o leite humano total e suas frações podem contribuir para proteção contra as infecções por ETEC bloqueando a adesão bacteriana mediada pelos diferentes antígenos de colonização.

Palavras-chave: Diarreia; Inibição da adesão; *Escherichia coli* enterotoxigênica; Fator antigênico de colonização; Leite humano

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