Human colostrum contains IgA antibodies reactive to colonization factors I and II of enterotoxigenic *Escherichia coli*

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Abstract

Diarrhea is an important cause of morbidity and mortality amongst infants of low socio-economic levels in developing countries and in travelers who visit such areas. Enterotoxigenic *E. coli* strains express two sets of virulence-associated factors: enterotoxins (heat-stable toxins or heat-labile toxins) and colonization factors. Studies have shown that breast-feeding protects infants against infectious diseases, such as diarrhea, as it presents a great variety of immunological components. The aim of this study was to analyze the reactivity of immunoglobulin A from human colostrum to colonization factor antigens I and II. The colostrum ability in preventing enterotoxigenic *E. coli* adhesion to Caco-2 cells was also evaluated. Colostrum samples were collected from 32 healthy women, and a human colostrum pool was prepared. Enterotoxigenic *E. coli* strains expressing colonization factor antigens I and II were utilized. The colostrum pool and individual samples showed variable antienterotoxigenic *E. coli* immunoglobulin A titers, that were reactive with colonization factor antigen I and CS1/CS3 (colonization factor antigen II). The human colostrum pool and individual samples inhibited enterotoxigenic *E. coli* colonization factor antigen I and II adhesion to Caco-2 cells, at variable levels, and this ability was a result of immunoglobulin A antibodies reactive to these colonization factors. The immunoglobulin A-depleted pool lost this inhibitory ability. As bacterial adhesion is the initial mechanism of enterotoxigenic *E. coli* infection, breast-feeding could protect the offspring against diarrhea caused by this agent.

Introduction

Diarrhea continues to be an important cause of morbidity and mortality amongst infants of low socio-economic levels in developing countries. Of the several bacterial agents associated with this syndrome, enterotoxigenic *Escherichia coli* (ETEC) is one of the most common causes of acute diarrhea in children less than 5 years of age and in travelers who visit such areas (Black et al., 1981, 2003). In order to cause the disease, ETEC strains must express two sets of virulence-associated factors: enterotoxins [heat-stable toxins (ST) or heat-labile toxins (LT)] and colonization factors (CFs), consisting of a set of diverse fimbrial or afimbrial adhesins conferring host and tissue specificity (Nataro & Kaper, 1998). Of the great variety of CFs described so far, colonization factor antigen I (CFA/I), colonization factor antigen II (CFA/II) and colonization factor antigen IV (CFA/IV) are the best characterized on both a structural and genetic basis. CFA/I is composed of a single antigen, whereas CFA/II comprises a family of coli surface (CS) antigens (CS1, CS2 and CS3), the latter of which has been found alone or associated with CS1 or CS2 (Smyth, 1982; Wolf, 1997; Oyofo et al., 2001). Primary experiments to test the role of these antigens in colonization have been carried out on animal models, but in vitro studies using different cell lines have been used to evaluate their properties, and the Caco-2 cell line is one of the most suitable models for this purpose, particularly for ETEC strains carrying the CFA/I and CFA/II (CS2) adhesions (Darfeuille-Michaud et al., 1990; Viboud et al., 1996).

The prevalence of CFs amongst clinical ETEC isolates differs considerably depending on the geographic region, but several epidemiological studies conducted in different countries have indicated that 23–94% of human ETEC isolates express either CFA/I, CFA/II or CFA/IV (Wolf, 1997; Kaper et al., 2004). ETEC strains are also important pathogens in São Paulo, Brazil, and are responsible for 7–20% of cases of infantile diarrhea (Reis et al., 1982; Gomes
et al., 1991). Moreover, different studies have shown that CFs occur in 29–43% of the isolated strains, and CFA/I or CFA/II accounted for the most prevalent CFs identified (Reis et al., 1982; Guth et al., 1994; Nunes, 2000).

Extensive studies support the concept that breast-feeding is effective in protecting infants against infectious diseases, such as diarrhea (Kovar et al., 1984; Blake et al., 1993). Several factors in human milk, including antibodies and nonspecific factors with antibacterial and antiviral activity, may be important in conferring this protective role (Taylor & Dimmock, 1985; Mazanec et al., 1993). Immunoglobulin A (IgA) accounts for 70% of total body immunoglobulin production and has been found to be the main immunoglobulin in human milk, accounting for more than 90% of the immunoglobulins (Xanthou, 1998; Macpherson & Uhr, 2004). Human milk contains antibodies directed against a variety of pathogens, to which the mother has been exposed during her lifetime, that will protect the offspring against the prevalent pathogens in the environment (Kolb, 2001). The main function of IgA is to bind and block the adherence of microbial pathogens to the intestinal epithelial surface and neutralize their toxins (Cravioto et al., 1991). Several studies have already demonstrated that secretory IgA antibodies of colostrum obtained from Brazilian women strongly inhibited the in vitro adhesion of enteropathogenic and Shiga toxin-producing E. coli to human epithelial cells (Carbonare et al., 1997; Palmeira et al., 2005). Moreover, Nathavitharana et al. (1995) reported the presence of specific secretory IgAs reactive to CFA/I in the milk and saliva of Sri Lankan and Asian women.

The importance of CFs in ETEC pathogenesis is well known, and thus it can be proposed that the presence of specific anti-CF IgA in human colostrum will block the adhesion capacity of ETEC strains. Therefore, the aim of this study was to analyze the reactivity of IgA from different samples of human colostrum against CFA/I and CFA/II, the major CFs identified in our community. The ability of colostrum in preventing ETEC adhesion to Caco-2 cells was also evaluated for the first time.

**Materials and methods**

**Bacterial strains and culture conditions**

The prototype ETEC strains used in this study were TR69/1 (CFA/I\(^+\), O63:H\(^+\), LT/ST), isolated from a child with diarrhea in São Paulo (Reis et al., 1982), TR69/1-P, a plasmidless mutant lacking CFA/I (CFA/I\(^-\)), 258903 (CFA/I\(^+\)–CS2\(^+\) (58R957) or CFA/I plasmidless mutant (CFA/I\(^-\), TR69/1-P) by adding the specific ETEC culture to the colostrum as described by Carbonare et al. (1997). Another aliquot of colostrum pool was also totally IgA depleted. Briefly, the colostrum pool was decaseinated by acidification with acetic acid, and then applied to an anti-human IgA CNBr-activated Sepharose (Sigma, St Louis, MO) column to obtain an IgA-depleted fraction and an IgA-enriched preparation (eluate), as described previously (Fernandes et al., 2001). The absorbed supernatants and IgA-depleted and IgA-enriched preparations were maintained at \(-20^\circ\text{C}\).

**Colostrum pool absorptions**

Different aliquots of the colostrum pool were absorbed with ETEC strains expressing CFA/I\(^+\) (258903), CFA/I\(^-\)–CS2\(^+\) (58R957) or CFA/I plasmidless mutant (CFA/I\(^-\), TR69/1-P) and CFA/II\(^+\) by adding the specific ETEC culture to the colostrum as described by Carbonare et al. (1997). Another aliquot of colostrum pool was also totally IgA depleted. Briefly, the colostrum pool was decaseinated by acidification with acetic acid, and then applied to an anti-human IgA CNBr-activated Sepharose (Sigma, St Louis, MO) column to obtain an IgA-depleted fraction and an IgA-enriched preparation (eluate), as described previously (Fernandes et al., 2001). The absorbed supernatants and IgA-depleted and IgA-enriched preparations were maintained at \(-20^\circ\text{C}\).

**Adhesion to Caco-2 cells**

Caco-2 cells, a human cell line derived from a carcinoma of the colon that exhibits structural and functional differentiation patterns characteristic of mature enterocytes in post-confluent cultures, were grown for 10 days in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma) containing 4.5 g L\(^{-1}\) glucose, 1% MEM nonessential amino acids, 25 mM HEPES, 2 mM L-glutamine, 0.5% of 50 mg mL\(^{-1}\) gentamycin (all from Sigma) and 10% fetal bovine serum (Cultilab, Campinas, Brazil) in chamber slides for tissue culture (Nunc Inc., Glostrup, Denmark). Suspensions of CFA/I\(^+\) and CFA/II\(^+\) (CS2) containing \(10^8\) CFU mL\(^{-1}\) were added to the medium of the tissue culture supplemented with 2% D-mannose, and the mixture was incubated at 37 °C for 3 h. After four washes with phosphate-buffered saline (PBS), cells were fixed with methanol and stained with 20% Giemsa stain (adherence positive control). Individual colo-
strum samples and the original or absorbed colostrum pools were examined in inhibition assays, as described by Silva & Giampaglia (1992) with some modifications. Colostrum diluted 1:50, 1:100 and 1:200 was added to the mixture of bacterial suspension and tissue culture, and the test was performed as mentioned above. At least 400 cells were observed in each preparation under a light microscope (×400 or ×1000 magnification). The amount of adhesion was determined by calculating the percentage of cells with six or more adhering bacteria on the cell surface. The inhibitory effect of colostrum was calculated as the difference between the percentage of bacterial adhesion in control wells without colostrum and that in wells containing the colostrum samples ×100 (%). The values represented the mean of at least three experiments for individual colostrum samples and the mean of seven experiments for whole pooled colostrum.

**Detection of ETEC antibodies**

Anti-ETEC CFA/I^+^, anti-ETEC CFA/I^-^ and anti-ETEC CFA/II^+^ (CS1/CS3 and CS2) IgA titers were determined by enzyme-linked immunosorbent assay (ELISA), as described by Carbonare et al. (1995). Briefly, ELISA plates (Costar) were coated with whole cell suspension adjusted to an optical density (OD) of 0.7 at λ = 540 nm. Individual colostrum samples and the original or absorbed colostrum pool were evaluated in serial dilutions, and antihuman IgA peroxidase conjugate (Sigma) was used for the detection of IgA. Fimbrial extracts were diluted to a concentration of 1 µg µL^-1^ and loaded on to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and stained with Coomassie Brilliant Blue G or, for immunoblotting assays, were blotted on to nitrocellulose membranes (Towbin et al., 1979). The strips were incubated overnight with appropriate dilutions of colostrum, and antihuman IgA peroxidase conjugate, diluted 1:4000, was used in the reaction (Sigma). The reaction was developed with the substrate in 3,3',5-diaminobenzidine (DAB) (Sigma) (Carbonare et al., 2003).

**Results**

**Human colostrum inhibits ETEC adhesion to Caco-2 cells**

The adhesion of ETEC CFA/I^+^ and ETEC CFA/II^+^ (CS2^+) to Caco-2 cells was strongly inhibited by the colostrum pool and all individual samples. Figure 1 shows the adhesion pattern of the ETEC CFA/I^+^ strain (258903), and Table 1 summarizes all the results of the adhesion assays. IgA-depleted colostrum lost its inhibitory effect, whereas the IgA-enriched preparation maintained its inhibitory activity. The pooled colostrum lost its inhibitory activity on adhesion of the homologous strain after absorption with ETEC.

**IgA antibodies from colostrum samples**

The fimbrial extracts analyzed by SDS-PAGE stained with Coomassie Brilliant Blue G demonstrated a typical CFA/I

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Fig. 1. Micrograph showing the adhesion of enterotoxigenic *Escherichia coli* (ETEC) colonization factor antigen I-positive (CFA/I^+) strain 258903 (O128:H^-, ST/ST) to the Caco-2 cell line. (a) Control adhesion without colostrum; (b) adhesion inhibition in the presence of pooled human colostrum; (c) absence of adhesion inhibition in the presence of immunoglobulin A (IgA)-depleted pooled human colostrum. Cells and bacteria were stained with 20% Giemsa stain and were observed at ×1000 magnification.
band of 16 kDa (Fig. 2, lane A), which was absent in lane B (the fimbrial extract of the plasmidless mutant strain lacking CFA/I), and typical bands of CS1, CS3 and CS2 of CFA/II with molecular weights of 18 and 16 kDa (lane C) and 15.3 kDa (lane D), respectively. Immunoblotting assays using anti-IgA immunoglobulins detected several proteins, including CFA/I and CS1, CS3 and CS2 of CFA/II, in pooled colostrum (Fig. 3a) as well as in the IgA-enriched preparation (Fig. 3c). No recognition of these CFAs was observed when IgA-depleted colostrum was assayed (Fig. 3b). A differential pattern of recognition to CFA/I and CFA/II was observed amongst the individual colostrum samples tested (Fig. 4). Of 17 individual colostrum samples tested, four presented IgA antibodies against CFA/I, CS1/CS3 of CFA/II and CS2 of CFA/II (Fig. 4a), 12 recognized just CFA/II (Fig. 4b) and, in five samples, no IgA antibodies against these CFAs were identified (Fig. 4c).

**Discussion**

Breast-feeding is undoubtedly associated with protection against a great variety of neonatal infections and, considering that diarrhea is still of concern with regard to child mortality, especially in developing countries, the role of human milk in protecting infants against diarrhea has been investigated by several authors (Martins Filho et al., 1980; Silva & Giampaglia, 1992; Carneiro-Sampaio et al., 1996; Fernandes et al., 2001). Breast-fed infants are more resistant to infectious diseases, especially those of the gastrointestinal tract, when compared with bottle-fed children (Martins Filho et al., 1980).

Enterotoxigenic *Escherichia coli* is a major cause of acute diarrhea and is responsible for a high rate of infantile mortality in developing countries, including Brazil (Guth et al., 1994; Nunes, 2000).

In this study, we showed that a human colostrum pool, as well as individual colostrum samples, was able to strongly inhibit the *in vitro* adhesion of CFA/I- and CFA/II-producing ETEC strains to the Caco-2 cell line. It was also observed that the colostrum pool and several individual

### Table 1. Inhibition of enterotoxigenic *Escherichia coli* (ETEC) adhesion to Caco-2 cells by human colostrum

| Material† | CFA/I † | CFA/II †
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>Whole pooled colostrum</td>
<td>91.4</td>
<td>92.3</td>
</tr>
<tr>
<td>IgA-depleted pooled colostrum</td>
<td>19.3</td>
<td>3.8</td>
</tr>
<tr>
<td>Purified IgA from pooled colostrum</td>
<td>91.6</td>
<td>94.4</td>
</tr>
<tr>
<td>Pooled colostrum absorbed with CFA/I† (CS2) strain</td>
<td>47.0</td>
<td>3.3</td>
</tr>
<tr>
<td>Pooled colostrum absorbed with CFA/I† strain</td>
<td>3.1</td>
<td>43.0</td>
</tr>
<tr>
<td>Pooled colostrum absorbed with CFA/I† plasmidless mutant</td>
<td>90.6</td>
<td>50.8</td>
</tr>
<tr>
<td>Sample 4</td>
<td>97.7</td>
<td>95.1</td>
</tr>
<tr>
<td>Sample 5</td>
<td>98.0</td>
<td>97.4</td>
</tr>
<tr>
<td>Sample 15</td>
<td>98.0</td>
<td>97.4</td>
</tr>
<tr>
<td>Sample 23</td>
<td>89.9</td>
<td>43.6</td>
</tr>
</tbody>
</table>

CFA/I, colonization factor antigen I; CFA/II, colonization factor antigen II; IgA, immunoglobulin A; LT, heat-labile toxin; ST, heat-stable toxin.

†Difference between the percentage of adhesion in control (without colostrum) after observation of 400 cells and the percentage of bacterial adhesion in wells containing the colostrum samples x100 (%). Values represent the mean of at least three experiments with each individual colostrum sample and the mean of seven experiments with whole pooled colostrum.

CFA/I † strain: 258903 (O128:H-, ST/LT).

CFA/II † (CS2) strain: 58R957 (O6:H16, LT/ST).

Colostrum samples were diluted 1:50.

Titer of IgA antibodies raised to ETEC proteins in colostrum

ETEC antibody levels in human colostrum are summarized in Fig. 5. ELISA performed with 11 individual colostrum samples showed variable anti-ETEC CFA/I (Fig. 5a) and anti-ETEC CFA/II (Fig. 5b) IgA titers. The colostrum pool absorbed with either ETEC CFA/I+ or ETEC CFA/II+ demonstrated a large decrease in anti-CF IgA titers. Absorption with the CFA/I plasmidless mutant (CFA/I*/C0) also resulted in a decrease in IgA titers reactive to CFA/I+.

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on acrylamide 15% gel of fimbrial extracts of prototype enterotoxigenic *Escherichia coli* (ETEC) strains. Lanes: A, CFA/I†; B, CFA/I†; C, C51 and C53 of CFA/II†; D, C52 of CFA/II†. The arrows indicate the band of CFA/I in lane A, C51 and C53 in lane C and C52 in lane D. CFA/I, colonization factor antigen I; CFA/II, colonization factor antigen II.
samples contained IgA antibodies that were reactive to these ETEC CFs, thus suggesting that IgA may be responsible for the presently identified inhibitory effect of colostrum. Indeed, this seems to be the case, as IgA-depleted colostrum lost almost 100% of its inhibitory capacity, whereas IgA-enriched eluate maintained this ability.

The inhibitory activity of human colostrum and breast milk to the adherence of other categories of diarrheagenic *Escherichia coli* has been reported previously (Cravioto et al., 1991; Carbonare et al., 1995; Wold & Adlerberth, 2000; Fernandes et al., 2001). Several constituents capable of binding to pathogenic bacteria and of inhibiting adhesion are present in human milk. In one study, it was found that fucosylated saccharides prevented the adhesion of enteropathogenic *E. coli* (EPEC) (Cravioto et al., 1991), whereas others stressed the important role of IgA antibodies in blocking this bacterial adherence (Silva & Giampaglia, 1992; Câmara et al., 1994; Carbonare et al., 1997).

In relation to ETEC, the only two studies described so far have reported the role of lactoferrin and the secretory component, as well as colostrum and human milk oligosaccharides, in blocking ETEC CFA/I and CFA/II hemagglutination (Giugliano et al., 1995; Martín-Sosa et al., 2002). Thus, the important role of IgA antibodies in preventing CF-mediated ETEC adherence to cultured cells has not been reported previously.

We also investigated the presence of specific antibodies reactive to CFA/I and CFA/II by absorbing aliquots of the human colostrum pool with each one of these strains. Adherence and ELISA assays performed with the colostrum pool absorbed with ETEC CFA/I or CFA/II strains demonstrated that its inhibitory activity was lost, and that the antibody titers were drastically reduced when the homologous strain was tested, probably as a result of the removal of polyclonal antibodies contained in the human colostrum pool that could react with common epitopes amongst these bacteria to different degrees. Absorption with the CFA/I plasmidless mutant (CFA/I°) revealed that the colostrum pool maintained its inhibitory activity, indicating the great importance of anti-CFA/I antibodies in the adhesion inhibitory phenomenon identified in this study.

The variable titers of IgA reactive to ETEC CFA/I and CFA/II observed in individual colostrum samples by ELISA were also confirmed through immunoblotting assays, in which different degrees of CFA/I and CFA/II (CS1/CS3 or CS2) antigen recognition were identified. Despite this
variability, all colostrum samples inhibited ETEC adhesion to Caco-2 cells, indicating that even low anti-ETEC IgA levels detected by ELISA were sufficient to promote the inhibition of bacterial adhesion. Moreover, in the individual samples which did not present detectable anti-CF IgA antibodies by these techniques, the role of other colostrum components, such as oligosaccharides and lactoferrin, in blocking ETEC adhesion could be suggested.

It was interesting to observe that a larger number of samples recognized CFA/II (CS1/CS3) more strongly than CFA/I by immunoblotting assays. Taking into consideration previous epidemiological studies conducted in São Paulo (Reis et al., 1982; Guth et al., 1994; Nunes, 2000), which showed that CFA/I occurred more frequently than CFA/II, these findings could suggest that CFA/II is a stronger immunogen than CFA/I, rather than reflecting a larger environmental exposure to ETEC CFA/II strains in our population.

Quadri et al. (2003) studied the importance of the mucosal immune response when 158 children were vaccinated with oral formalin-inactivated ETEC vaccine containing six CFs (CFA/I, CS1, CS2, CS3, CS4 and CS5) and recombinant cholera toxin B subunit. They verified that the children presented significant increases of CF-specific antibody-secreting cells of the IgA isotype after the intake of the first dose of the vaccine, and these results confirmed the importance of the mucosal immune response in preventing ETEC diarrhea. Moreover, Freedman et al. (Freedman et al., 1998) verified that CFA/I is a potent immunogen when compared with LT toxin and purified lipopolysaccharide in cattle immunization. Anti-CFA/I titers were 32 times higher than those of the other antigens tested. In addition, CFA/I antibodies alone were sufficient for protection when 25 volunteers were orally challenged with ETEC CFA/I after receiving IgG concentrate derived from immunized cows.

In conclusion, this study showed, for the first time, that colostrum obtained from Brazilian mothers living in low socio-economic conditions was able to inhibit ETEC CFA/I and CFA/II adhesion to Caco-2 cells, at variable levels, and this ability was a result of the IgA antibodies reactive to these antigens. As bacterial adhesion is the initial mechanism of ETEC infection, breast-feeding could protect the infant against diarrhea caused by this agent, confirming once again the great importance of this natural practice in preserving the good health of infants.

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