Binding of lactoferrin and free secretory component to enterotoxigenic Escherichia coli

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Abstract

The ability of two glycoproteins of human milk, lactoferrin and free secretory component, to bind to Escherichia coli colonization factors (CFAs) was investigated using immunocytochemistry assays of enriched fimbrial extracts. The results revealed that lactoferrin binds to fimbrial CFA I adhesin but not to CFA II adhesin (CS1 and CS3), while free secretory component interacts with both CFA I and CFA II adhesins. Our data indicate that lactoferrin and free secretory component, which are very abundant proteins of human milk, could play an important role against infant enteric disease by blocking bacterial adhesion.

Keywords: Diarrhea; Human milk; Lactoferrin; Free secretory component; Colonization factor antigen; Enterotoxigenic Escherichia coli

1. Introduction

Enterotoxigenic Escherichia coli (ETEC) has been considered one of the major cause of diarrhea in children living in developing countries [1] and [2]. The ability of many strains of ETEC to adhere and colonize the intestinal mucous membrane of humans has been correlated with the presence of specific fimbrial antigens, called colonization factor antigens (CFA) [3].

CFA I was the first colonization factor described from human ETEC [3] and it is composed of only one type of subunit [4]. The second colonization factor from ETEC, CFA II [5], is composed of three distinct E. coli surface antigens (CS), which may be expressed in different combinations: CS1 and CS3, CS2 and CS3, CS3 only or some rare cases CS2 only [6] and [7]. Most of the ETEC strains isolated in Brazil have been shown to possess CFA I or CFA II [8] and [9].

Epidemiological studies of diarrhea have show that breast feeding protects infants from intestinal infections [10] and [11]. The protective effect of human milk has been attributed to its immunoglobulin content, mainly to secretory immunoglobulin A (sIgA) [12] and [13], and to non-specific defence factors such as lactoferrin, free secretory component (fSC), lysozyme, bifidus factor and oligosaccharides [14], [15] and [16].

Lactoferrin is an 80-kDa glycoprotein, found in high concentrations in human milk [15]. Several workers have suggested that lactoferrin has the ability to interact with various components of bacterial surface such as the lipid-A of the lipopolysaccharide [17] and porins

[18]. In addition, a study investigating the lactoferrin binding capacity of different groups of E. coli reveals that ETEC strains had a significantly higher lactoferrin binding than others groups [19].

The SC can be found in several secretions complexed with sIgA or as free glycoprotein [20]. It has been shown that fSC is an 80-kDa glycoprotein, which consists of a single polypeptide chain and large amounts of carbohydrate (20%) [21]. There is little information about the role of fSC in secretions but some workers suggest that it may have a protective role against diarrhea [16] and [22].

Previously, we demonstrated that both lactoferrin and fSC inhibit adhesion to erythrocytes by ETEC CFA I+ strains [16]. To further analyze the role of these compounds, we investigated the ability of lactoferrin and fSC to bind to CFA I and CFA II.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The ETEC CFA I+ and CFA II+ strains used were TR50/3 (CFA I+ ST I+/LT I+ O63:H−) and PB176 (CFA II+ CS1+ CS3+ ST I+/LT I+ O6:H16), kindly provided by Dr B.C. Guth, Escola Paulista de Medicina, São Paulo, Brazil. These strains have been isolated from children suffering from diarrhea and stored frozen. The bacterial cells were grown in CFA agar [3] at 37°C overnight.

2.2. Fimbrial purification

The fimbrial purification was performed as described by Mynott et al. [23], with some modifications. CFA-positive bacteria were harvested and suspended in phosphate-buffered saline (PBS). Fimbriae were detached from bacteria by heat treatment in a shaking water bath (60 rev min−1) at 65°C for 20 min. Bacterial cells were removed and fluid supernatant was centrifuged at 39 000×g for 2 h at 4°C to remove outer-membrane contaminants. The resultant fluid supernatant was stored for 48 h at 4°C to allow fimbrial aggregation and centrifuged at 167 000×g for 2 h in a Beckman L5-50B (fixed rotor 50Ti) ultracentrifuge. The pelleted material containing the partially purified fimbriae, was suspended in 2 ml of PBS supplemented with mannose 1%.

2.3. SDS–PAGE and immunoblot analyses

The protein content was measured by method of Bradford [24] and analyzed by SDS–PAGE on acrylamide 12% as described by Laemmli [25]. 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 method of Towbin et al. [26]. The membranes were incubated with specific anti-CFA IgG, anti-rabbit IgG peroxidase-conjugated (Sigma, St. Louis, MO, USA) and reaction was analyzed using ECL detection system (Amersham Life Science, Buckinghamshire, England, UK).

Quantitative analyses of electrophoretic protein profiles were obtained by scanning the gels at 340 nm in a CS-9301 PC SHIMADZU computing densitometer.

2.4. Antibodies

Polyclonal monospecific rabbit antiserum against CFA I and CFA II (CS1CS3) were also kindly provided by Dr. B.C. Guth, Escola Paulista de Medicina, São Paulo, Brazil. Rabbit anti-lactoferrin serum, goat anti-fSC serum and the secondary antibodies peroxidase-conjugated and gold-labeled were purchased (Sigma, St. Louis, MO, USA).

2.5. Purification of human milk fSC

Fractionation and purification of fSC was performed as described previously [16]. Briefly, lipids and casein were removed, and proteins were concentrated by adding ammonium sulfate to 70% final saturation. The sample obtained was dialyzed and then applied to a Sephacryl S-200 HR column (2.6×80 cm; Pharmacia Biotech AB, Uppsala, Sweden) equilibrated with Tris–HCl buffer, pH 7.6. Thereafter, the fractions of the second peak were applied to a DEAE cellulose column (2.6×30.0 cm; Sigma, St. Louis, MO, USA) equilibrated with the same buffer. For further purification of fSC, the material eluted in the first peak after DEAE cellulose chromatography was concentrated, dialyzed and applied to a Heparin–Sepharose affinity column (1.0×10.0 cm; Pharmacia Biotech AB, Uppsala, Sweden) equilibrated with Tris–HCl buffer, pH 8.0 [16].

2.6. Immunolabeling assay and electron microscopy

For immunogold labeling, the CFA I and CFA II fimbrial preparations were placed on Formvar-carbon-coated grids (200 mesh) (Electron Microscopy Sciences, Fort Washington, USA). Then, grids were treated with antibody anti-CFA I or anti-CFA II and sequentially incubated with anti-rabbit IgG gold-labeled (10 nm gold; Sigma, St. Louis, MO, USA). Thereafter, fimbrial preparations were examined by negative staining.

Binding of glycoproteins to CFAs were determined by an adapted immunological labeling assay using lactoferrin commercially acquired or fSC from human milk at 0.2 mg ml−1 and 0.08 mg ml−1, respectively. Enriched CFA I (0.7 mg ml−1) and CFA II (1.6 mg ml−1) fimbrial preparations were previously exposed to lactoferrin (Sigma, St. Louis, MO, USA) or fSC for 1 h at room temperature. Subsequently, fimbrial suspension were extensively washed with 10 mM Tris–HCl buffer, pH 8.0, to remove lactoferrin or fSC that did not bind. Prior to immunolabeling assay, coated grids were incubated for 1 h at room temperature with a blocking solution containing bovine serum albumin 1%, NaCl 0.85% and Tween 20 1% in 10 mM Tris–HCl buffer. Thereafter, the grids were placed on a drop of each fimbrial preparation, incubated with rabbit anti-lactoferrin serum or goat anti-fSC serum (Sigma, St. Louis, MO, USA), followed by anti-rabbit IgG gold-labeled or anti-goat IgG gold-labeled (10 nm gold; Sigma, St. Louis, MO, USA) and negatively stained with phosphotungstic acid 2%, pH 7.2. Subsequently, the grids were examined with a Jeol Jem 100 C electron microscope (Jeol, Japan).

3. Results and discussion

Electrophoretic analyses of ultracentrifuge extract obtained from CFA I strain exhibited a major protein band (Fig. 1A, lane 1), with a molecular mass similar to that of CFA I with a purity of about 93%. The identity of the CFA I band was verified by immunoblotting assay. Results demonstrated that the protein band with a molecular mass of 16 kDa was recognized by CFA I antiserum (Fig. 1A, lane 2). SDS–PAGE and immunoblot analyses of enriched fimbrial extract CFA II strain (Fig. 1B, lanes 1 and 2, respectively) revealed that CFA II antiserum reacted with proteins of 18.8 kDa and 16.4 kDa, showing that these proteins correspond, respectively, to the CS1 and CS3 components of CFA II. The purity was about 53% to the CS1 component and 3.4% to the protein band corresponding to the CS3 component.

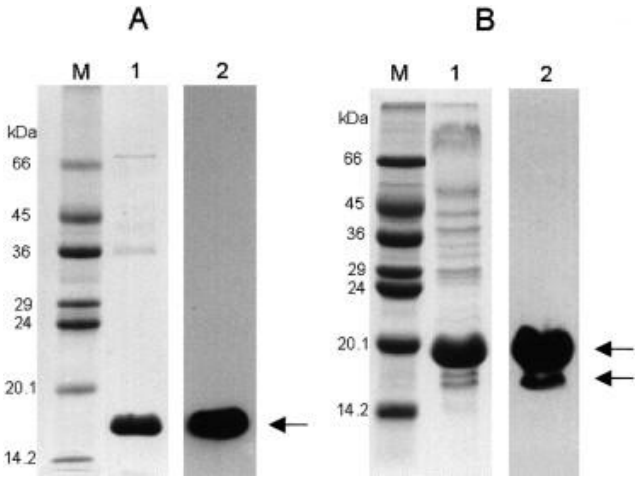

Fig. 1. Electrophoretic and immunoblot analyses of fimbrial preparations. A: Fimbrial extracts from TR50/3 CFA I+ strain: M, molecular mass markers; lane 1, SDS–PAGE; lane 2, immunoblot using anti-CFA I serum. B: Fimbrial extracts from PB176: M, molecular mass markers; lane 1, SDS–PAGE; lane 2, immunoblot using anti-CFA II serum. CFA I and CFA II (CS1 and CS3) are indicated (arrows).

In this study, to determine if lactoferrin binds to CFA I and CFA II, both fimbrial preparations were exposed to lactoferrin and afterwards treated with rabbit anti-lactoferrin serum. The CFA I fimbrial preparation showed a scattered labeling on the fimbriae (Fig. 2C). This binding pattern was similar to those obtained after the treatment of fimbriae with CFA I antiserum (Fig. 2A). When whole bacteria were exposed to lactoferrin, similar results were observed. These results revealed that lactoferrin have ability to bind to CFA I fimbriae, indicating that this compound may act as a receptor analogue in the inhibition of adhesion of CFA I to the host cell. On the other hand, the binding of lactoferrin to CFA II fimbriae (CS1 and CS3) was not demonstrated (Fig. 2D).

Immunogold assay performed with CFA I and CFA II fimbrial preparations exposed to fSC, showed that both CFA I and CFA II fimbriae were labeled (Fig. 2E,F). The observed pattern of gold beads was similar to that found with specific fimbrial antibody (Fig. 2B). A similar result was obtained when the whole bacteria was exposed to fSC. Previously, it was shown that fSC inhibits the adhesion of E. coli CFA I to host cells [16]. These findings indicate that this glycoprotein could prevent CFA I and CFA II binding to host cells. Furthermore, the ability of fSC to act as a bacterial cell receptor analogue has been reported by Falk et al. [27] and Dallas and Rolfe [28], working with, respectively, Helicobacter pylori and Clostridium difficile.

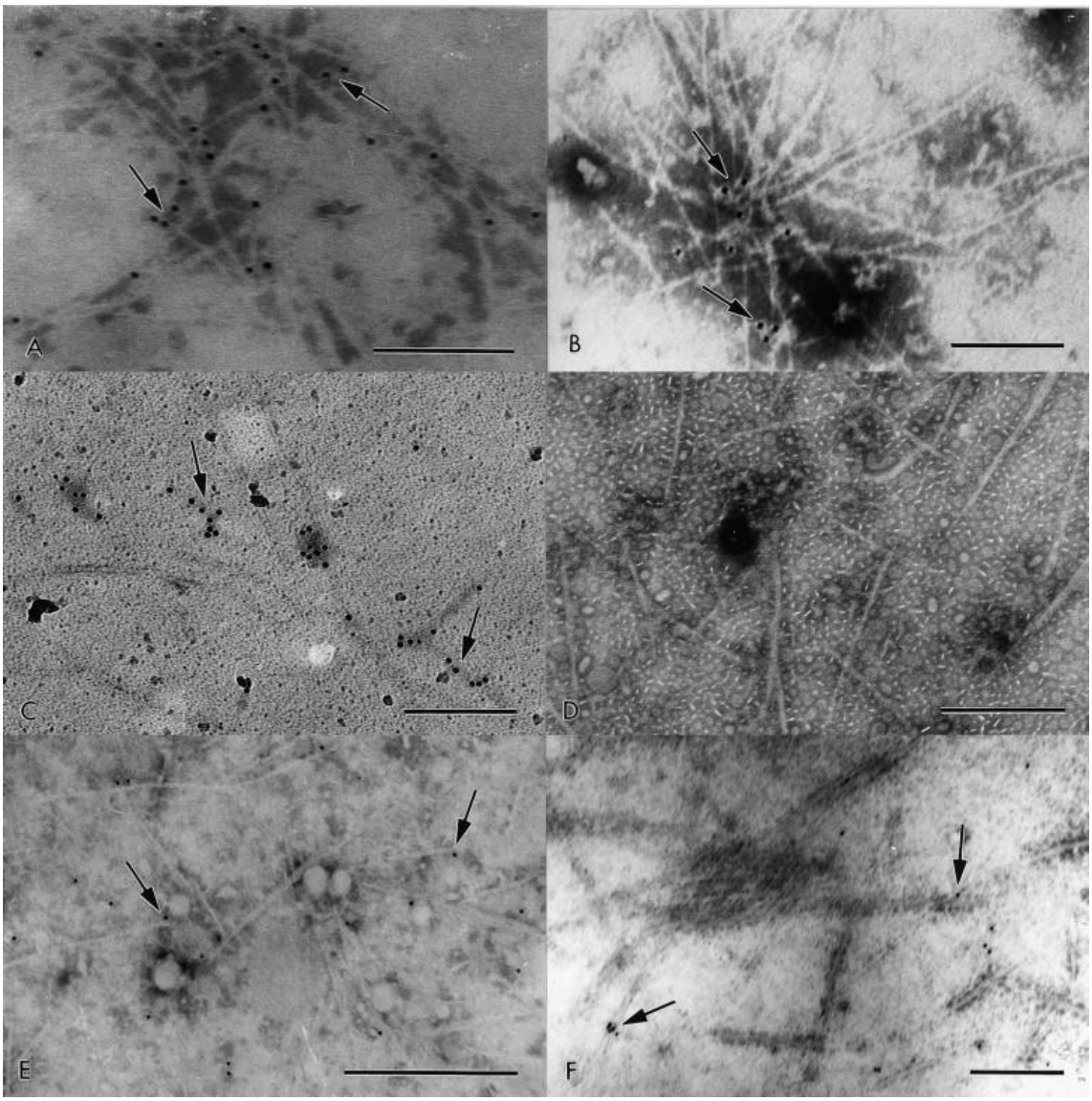

Fig. 2. Immunogold labeling assay of the enriched fimbrial preparations. CFA I (A) and CFA II fimbriae (B) recognized by anti-CFA I and anti-CFA II serum, respectively. Binding of lactoferrin on CFA I (C). Lactoferrin did not bind to CFA II fimbriae (D). The fSC binds on CFA I (E) and CFA II (F) fimbrial preparations. The arrows indicate the gold particles on fimbriae. Bar, 0.2 μm (A,B,C); bar, 0.5 μm (D,E,F).

In conclusion, our results indicate that lactoferrin and fSC, which are very abundant proteins of human milk, could contribute to protection against infant enteric disease by blocking bacterial adhesion, therefore supporting the eventual use of these compounds for prophylactic or therapeutic purposes.

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