Inhibition of enteropathogenic *Escherichia coli* (EPEC) adherence to HEp-2 cells by bovine colostrum and milk


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**SUMMARY**

*Background:* enteropathogenic *Escherichia coli* (EPEC) is the main etiological agent of infantile diarrhea in Brazil and other developing countries. Human milk IgA protects newborn intestinal mucosa by inhibiting bacterial adhesion to epithelial cells and this effect is shown by *in vitro* assays of EPEC adherence to HEp-2 cultured cells. Bovine milk, if effective in promoting this protection, could be an useful tool in the absence of the natural breastfeeding, in high-risk nurseries or in hospital infections.

*Methods:* the effect of colostrum, milk, and serum from dairy cows on the adherence to EPEC to HEp-2 cells was investigated. Colostrum from immunized and control animals and industrialized milk formulas were fractionated through a membrane device with a molecular weight cut off 10 kDa. The high molecular weight fraction (HMWF) of bovine colostrum was depleted of IgG through an affinity column and absorbed with an EPEC adherent strain. Antibodies were searched by ELISA and immunoblotting (IB).

*Results:* colostrum and milk from EPEC-immunized animals showed and inhibitory activity on adherence similar to that of control non-immunized animals. The inhibitory effect on adhesion was related to the HMWF. IgG-depleted colostrum partially retained the inhibitory effect, whereas IgG-rich eluate lost this property. The EPEC-absorbed fraction retained the inhibitory property. Industrialized milk formulas and respective HMWF also inhibited bacterial adherence. In IB assays, colostrum and milk samples from immunized animals recognized proteins of 30-40 kDa and 94 kDa, a molecular weight consistent with the adhesin intimin, in EPEC extracts.

*Conclusions:* the inhibitory effect of EPEC adherence may be mediated by HMWF components, and IgG was not the only component responsible for this phenomenon.


**INTRODUCTION**

Colostrum and milk are important secretions with nutritional and immunological properties. Many infections could be prevented by encouraging mothers to breast-feed their newborns (1, 2).

Diarrhea represents one of the major causes of morbidity and mortality among infants of low socio-economic levels in developing countries. Enteropathogenic *Escherichia coli* (EPEC) is an important cause of acute diarrhea in the first year of life in such countries (3). In São Paulo, Brazil, EPEC is the most frequently detected pathogen in children under 12 months of age with acute diarrhea, and the serogroups O111 and O119 are most frequently isolated (4, 5).

Methods for assessing bacterial adhesion include assays performed with HeLa or HEp-2 cells in culture. In such systems, EPEC strains show a localized adherence (LA) specific pattern (6). Studies by
Cravioto et al (7) and Silva and Giampaglia (8) showed that this system is suitable to study the inhibitory effect of human milk and colostrum on the adherence of EPEC to cultured cells.

Regardless of serogroups commonly found as EPEC, virulence is determined by several factors including the presence of a 50 to 70 Mda plasmid, EAF (EPEC adherence factor) and a chromosomal pathogenicity island, LEE (locus of enterocyte effacement), which encodes a type III secretory apparatus (9).

A four-stage model of EPEC interaction with epithelial cells was recently proposed by Knutton et al (10). According to this model, growth of bacteria in tissue culture results in the expression of bundle-forming pili (BFP) encoded by EAF plasmid (11); expression of intimin, a 94-kDa outer membrane protein; and production of EspA (EPEC secreted proteins) filaments (stage 1). BFP and EspA form a direct link between the bacterium and the host cell, and among bacteria themselves, resulting in adhesion of bacteria in a localized pattern (6). Secreted proteins are translocated into host cell, including EspB and Tir (translocated intimin receptor), and probably other effector proteins yet to be identified. This in turn leads to tyrosine protein kinase (TPK) activation and display of the intimin receptor, which is a LEE-encoded bacterial protein (tyrosine phosphorylated Tir), and to actin rearrangements (stage 2). Intimin binds to phosphorylated Tir, and polymerized actin accumulates beneath intimately attached bacteria (stage 3). Further actin polymerization produces mature attaching and effacing (A/E) lesion (12). At this stage, all EspA filaments and intimin have been eliminated from the bacterial surface (stage 4). This stage comprises loss of host cell microvilli, formation of pedestals beneath the adherent bacteria, and rearrangements of host cell cytoskeleton.

Colostrum and human milk protect the neonate intestinal mucosa against EPEC infections by inhibiting bacterial adherence, could be useful sources of antibodies to be used in the absence of natural breastfeeding, in high-risk nurseries, or in preventing nosocomial infections.

MATERIALS AND METHODS

Immunization of cows

Four pregnant Friesian cows (numbered from 1 to 4) in a dairy farm in São Paulo, were immunized 7 to 9 times during the last 10 weeks of gestation. Each cow was injected subcutaneously with 1 ml of a suspension of inactivated EPEC strains of serogroups O111, O119 and O55 (10^7-10^8 bacteria/ml), in 0.5 % formol in saline solution with the adjuvant Avridine (Pfizer). All bacterial strains were AL+, BFP^-. Four non-immunized cows (numbered I to IV), and a pool of human colostrum were used as controls. Blood samples for determination of anti-EPEC antibodies were obtained from the cows prior to immunization and after the last injection.

Preparation of colostrum and milk

Colostrum from the immunized and control cows was obtained within the first 24-48 h post-partum. Subsequent milking was performed after 6 to 16 days (sample A), 21 to 32 days (sample B) and one year (sample C, from cow #2) post-partum. Colostrum and milk samples were delipidated and stored at –20 °C until assayed.

Studies on industrialized milk

The following formulas were analyzed: skim milk Molico (five cans from different lots), NAN 1, NAN HA and Alfaré (both depleted of proteins by enzymatic treatment) and soy based formula Alsoy (all from Nestlé Industrial e Comercial Ltda., São Paulo).

Industrialized milk components, including potassium caseinate (6.4 g/l), lactose (74 g/l), soy lecithin (1.3 g/l) and maltose (22.3 and 74 g/l) diluted in 1 % solution of Tween, or in PBS, were also analyzed.

Fractionation of colostrum and milk samples

Fifteen ml aliquots of colostrum samples from three immunized animals (cows #1, 3, and 4), and one non-immunized animal (II); two samples of Molico
and milk formulas NAN 1, NAN HA, Alfaré and Alsoy, were fractionated into low (LMWF) and high molecular weight fractions (HMWF), using an Amicon Centriprep (Amicon, Inc., Beverly, MA, USA), with a molecular weight cut off of 10 kDa. HMWF were reconstituted to the original volume after fractionation. Absorption of cow #1 HMWF with EPEC strain O111:H-HMJ 0041-1-85 (LA+, BFP+) was performed as previously described (14).

IgG purification on affinity column

Six ml aliquots of colostrum from cows #I and #II were depleted of IgG in a protein G-Sepharose column. Subsequent elution of IgG from the column was performed using glycine-HCl pH 2.8. The initial volume of all materials was restored after affinity chromatography to maintain the original concentration.

Adhesion assays

The EPEC strain O111:H+, HMJ 0041-1-85 (LA+, eaeA+, EAF+, bfp+), isolated from the stools of a child with acute diarrhea from São Paulo, Brazil, was used for adhesion assays. There were carried out as described by Silva and Giampaglia (8). Briefly, HEp-2 cells (ATCC-CCL 23) were grown in Lab-Tek chamber slides (Nunc Inc., Denmark), added by a mixture of colostrum or milk and a standardized bacterial suspension in medium with 2 % fetal calf serum and 10 % D-mannose to prevent non-specific adhesion. After incubation for 30 minutes, unattached bacteria were removed by washings, and a new medium without colostrum or milk was added, followed by incubation for 3 h. Cells were then washed, fixed with methanol, and stained with May-Grünwald and Giemsa. At least 300 HEp-2 cells were observed in each preparation under light microscope (×400 or ×1,000). Results were expressed as the percentage of cells with six or more attached bacteria comparing to control cells treated without colostrum or milk. Antibody titer was expressed as the dilution necessary for each sample to reach the arbitrary absorbance value of 0.2.

Immunoblotting

Immunoblotting was used to detect antibodies to EPEC antigens, as described by Towbin et al (20) with modifications. Bacterial components were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After transferring to nitrocellulose membranes, strips were incubated with colostrum or milk samples and revealed with peroxidase conjugated anti-bovine IgG or IgA and 3,3’-diaminobenzidine (DAB) (Sigma) as substrate. Molecular weight standards (Pharmacia, Uppsala, Sweden) were run in each gel.

Antibody determination

Levels of IgG anti-EPEC antibodies in colostrum and milk samples were evaluated by enzyme-linked immunosorbent assay (ELISA), using a method described in Carbonare et al (19). Microtiter ELISA plates (Costar, Cambridge, MA) were coated with a standardized bacterial suspension of E. coli O111. The antigen-antibody reaction was carried out overnight with appropriate dilutions of human and bovine colostrum, bovine formula milk, and all HMWF and LMWF, and revealed by peroxidase conjugated anti-bovine IgG (Sigma, St. Louis, USA) and o-phenylenediamine dihydrochloride (OPD) (Sigma) substrate. Immunoblotting was used to detect antibodies to EPEC antigens, as described by Towbin et al (20) with modifications. Bacterial components were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After transferring to nitrocellulose membranes, strips were incubated with colostrum or milk samples and revealed with peroxidase conjugated anti-bovine IgG or IgA and 3,3’-diaminobenzidine (DAB) (Sigma) as substrate. Molecular weight standards (Pharmacia, Uppsala, Sweden) were run in each gel.
All milk samples but sample B from cow #1 had high inhibitors levels. The percentage inhibition of EPEC adherence varied greatly among different materials, but all levels were slightly lower than those observed with the pool of human colostrum.

**Effect of cow serum on the adherence of EPEC to the HEp-2 cells**

All serum samples presented high degrees of inhibition of bacterial adherence. No significant differences were observed in adherence assay results between cows’ serum samples collected before and after immunization with EPEC (t student test). However, post-immunization anti-EPEC IgG titers were higher than pre-immunization ones in 3 of 4 animals (table I).

**Effect of milk formulas and milk components on the adherence of EPEC to the HEp-2 cells**

At least 69 % of inhibition of EPEC adherence was observed when milk formulas were used in the assays (table II). This effect was observed when bovine milk formulas Molico, NAN 1, NAN HA, and Alfaré were used, as well as when the soy-based formula Alsoy was used. Milk components (maltose 22.3 g/l and 74 g/l; lactose 74 g/l; and potassium caseinate 6.4 g/l) showed low levels of adherence inhibition, ranging from 17 to 24 %. Results with soy lecithin as well as all other components diluted in 1 % Tween 80 were not considered due to the drastic effect of this detergent upon the bacterial adherence. Differences between inhibition of milk formulas group and milk components group were statistically significant when compared by the Tukey method.
Results of the fractionation experiments revealed that the component responsible for the inhibition of bacterial adherence was present in the HMWF, of both colostrum from immunized and non-immunized animals, NAN 1, NAN HA, and Alfaré. In the cases of Molico and Alsoy, HMWF showed significantly lower values of adhesion inhibition compared to original formulas. HMWF of Molico showed significantly higher value compared to its LMWF, while for Alsoy HMWF and LMWF had equally low values (figure 2). The LMWF had little effect in the assays in all cases.

Effect of original colostrum, IgG-depleted colostrum, and IgG-enriched eluate from immunized (#1) and non-immunized cow (#II) on the adherence of EPEC to HEp-2 cells

Figure 3 shows that the levels of adherence inhibition by original colostrum, IgG-depleted colostrum, and IgG-enriched eluate from immunized cows were similar to those from non-immunized animals. Greater inhibitory levels were observed with original colostrum. IgG depletion caused a significantly loss in inhibitory activity compared to original colostrum. On the other hand, results using the IgG-enriched eluate showed less than 5% inhibition of adherence.

Effect of LA⁺ EPEC-absorbed HMWF on bacterial adherence to HEp-2 cells

Absorption of HMWF with LA⁺ EPEC strain did not prevent inhibitory effect on bacterial adhesion: original colostrum, 78% inhibition; HMWF undiluted and diluted 1:2, 77% and 81% inhibition, respectively; HMWF pre-absorbed with LA⁺ EPEC strain, 91% inhibition.

Antibody determination

ELISA results showed higher titers of anti-EPEC IgG in colostrum as compared to milk, in 3 or 4 ani-

**Table I**

<table>
<thead>
<tr>
<th>Immunized cow</th>
<th>% Inhibition*</th>
<th>Anti-EPEC IgG Titer** (x 10⁴)</th>
<th>% Inhibition*</th>
<th>Anti-EPEC IgG Titer** (x 10⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow #1</td>
<td>80.7 ± 1.4</td>
<td>1.1</td>
<td>76.4 ± 1.7</td>
<td>2.8***</td>
</tr>
<tr>
<td>Cow #2</td>
<td>88.5 ± 0.4</td>
<td>2.6</td>
<td>91.8 ± 0.9</td>
<td>5.4***</td>
</tr>
<tr>
<td>Cow #3</td>
<td>92.2 ± 0.6</td>
<td>1.3</td>
<td>85.6 ± 0.2</td>
<td>4.8***</td>
</tr>
<tr>
<td>Cow #4</td>
<td>86.8 ± 0.8</td>
<td>0.8</td>
<td>75.2 ± 1.8</td>
<td>0.7</td>
</tr>
</tbody>
</table>

*% inhibition of EPEC adhesion to HEp-2 cells, in relation to control assay without sera.

**Titer = reciprocal of dilution equivalent to OD = 0.2 in ELISA.

***p < 0.05, Student t-test.

**Table II**

<table>
<thead>
<tr>
<th>Milk or component</th>
<th>Concentration* (g/l)</th>
<th>%Inhibition (average ± SE)</th>
<th>n**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molico lot #1</td>
<td>100</td>
<td>76.9 ± 5.1</td>
<td>4</td>
</tr>
<tr>
<td>Molico lot #2</td>
<td>100</td>
<td>81.3 ± 4.2</td>
<td>4</td>
</tr>
<tr>
<td>Molico lot #3</td>
<td>100</td>
<td>69.4 ± 3.8</td>
<td>4</td>
</tr>
<tr>
<td>Molico lot #4</td>
<td>100</td>
<td>78.2 ± 5.4</td>
<td>2</td>
</tr>
<tr>
<td>Molico lot #5</td>
<td>100</td>
<td>77.6 ± 0.5</td>
<td>4</td>
</tr>
<tr>
<td>NANN 1</td>
<td>146.5</td>
<td>85.3 ± 1.2</td>
<td>6</td>
</tr>
<tr>
<td>NANN HA</td>
<td>145.5</td>
<td>91.6 ± 1.0</td>
<td>7</td>
</tr>
<tr>
<td>Alfaré</td>
<td>150</td>
<td>94.5 ± 0.6</td>
<td>6</td>
</tr>
<tr>
<td>Alsoy</td>
<td>147.5</td>
<td>79.8 ± 3.3</td>
<td>5</td>
</tr>
<tr>
<td>Potassium caseinate</td>
<td>6.4</td>
<td>16.9 ± 0.4***</td>
<td>2</td>
</tr>
<tr>
<td>Maltose</td>
<td>22.3</td>
<td>21.6 ± 8.2***</td>
<td>2</td>
</tr>
<tr>
<td>Lactose</td>
<td>74</td>
<td>19.7 ± 0.5***</td>
<td>2</td>
</tr>
<tr>
<td>Soy lecithin</td>
<td>1.3</td>
<td>23.8 ± 2.5***</td>
<td>2</td>
</tr>
</tbody>
</table>

*Concentration according to milk reconstitution guidelines. Components concentrations according to milk formulation.

**n = number of experiments.

***p < 0.05, in variance analysis with 1 factor followed by multiple comparison by Tukey method.

ND: Not done due to insolubility of soy lecithin in PBS.
mals. Samples obtained from cow #2 showed some protein denaturation (table III). High molecular weight fractions (HMWF) from immunized animals contained titers of anti-EPEC antibodies equivalent to the respective original colostrum, whereas all LMWF showed negative results (data not shown). Colostrum samples from non-immunized animals showed very low titers of anti-EPEC antibodies < 100.

**Immunoblotting**

Immunoblotting assays with colostrum from immunized animals and developed with anti-bovine IgG showed strong reactivity with several EPEC antigenic fractions, including the 94 kDa adhesin Intimin, whereas milk from immunized animals and colostrum samples from non-immunized animals exhibited weaker reactions (figure 4). The pool of human colostrum, developed with anti-human IgA conjugate, recognized several EPEC antigenic fractions, as expected. Immunoblotting assays of bovine colostrum samples developed with anti-bovine IgA did not show any reaction (data not shown). Negative reactions were also obtained with industrialized milk and all the LMWFs.

**DISCUSSION**

EPEC can cause severe aqueous diarrhea by attaching to human enterocytes, inducing the effacement of their brush border (12). The in vitro model utilized in the present study simulates the interactions between EPEC and epithelial cells. Using this model, we showed that bovine colostrum and milk samples strongly inhibit EPEC adhesion to HEp-2 cells.

Both IgA anti-EPEC 94-kDa outer-membrane protein (7, 14, 15) and oligosaccharides (7) present in human breast milk are capable of inhibiting EPEC adherence to HeLa and HEp-2 cells. The inhibition of bacterial adhesion or invasion of enterocytes is con-
sidered one for the protective effects of human milk against gastrointestinal infections in infants (13).

Bovine milk with some of the protective properties found in human milk would be very useful in certain situations, such as patients with immunodeficiencies, bottle-fed infants and in the prevention of nosocomial gastrointestinal infections in nurseries. In a study in which an immunoglobulin preparation obtained from bovine milk from vaccinated animals was given to children with EPEC diarrhea, 84.3% of the stool cultures became negative (17). Similar results were obtained in 19 of 25 children with rotavirus gastroenteritis (16).

The mechanism involved in the inhibition of EPEC adhesion to HEp-2 cells by bovine colostrum and milk must be somewhat different from that accepted for inhibition by human colostrum, because the inhibitory activity occurred irrespective of the presence of anti-EPEC antibodies in bovine colostrum or milk.

In the present study, anti-EPEC IgG antibody levels detected by ELISA were higher in colostrum or milk from immunized animals than from non-immunized animals. However, both samples were able to inhibit EPEC adhesion to HEp-2 cell, suggesting that this ability is not related to anti-EPEC IgG antibody titers. Our results are in agreement with those by Rump et al (18). These authors investigate the effect of treating immunodeficient patients with Lactobin-R, an immunoglobulin concentrate obtained from non-immunized cows’ colostrum. They showed remission of diarrhea in 51.3% of the patients after 4 weeks of treatment.

Immunoblotting assays confirmed the presence of specific anti-EPEC antibodies in colostrum samples from immunized animals, including antibodies to a 94 kDa band consistent with Intimin. Weaker bands were detected using samples of milk and colostrum from non-immunized animals.

Serum samples obtained before and after immunization showed an increase in antibody titers detected by ELISA, however there were no changes in the inhibitory levels assessed by adhesion assays. These results confirm that this ability is not related to anti-EPEC antibodies.

Since we observed inhibitory activity in colostrum from non-immunized animals, we performed adhesion assays using industrialized milk formulas which are frequently given to infants in Brazil. All formulas strongly inhibited EPEC adhesion to HEp-2 cells, in the absence of EPEC antibodies, as shown by ELISA or Immunoblotting.

Soluble oligosaccharides present in human milk could be responsible for the inhibition of bacterial adherence, by binding to cellular receptors, or preventing the interactions of fimbrial adhesins to human cells (21, 22). Fractionation on bovine colostrum samples to HMWF and LMWF showed that the active component is present in the HMWF, and this is not related to free oligosaccharides, as previously shown for human milk in other systems (7). This is keeping with the observation that bovine milk has a lower content of oligosaccharides, as compared to human milk (23). The presence of anti-EPEC antibodies in HMWF and their absence in LMWF, was confirmed by ELISA and Immunoblotting assays.

Industrialized milk formulas were also submitted to fractionation, and the inhibitory activity remained intact.

**Table III**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Colostrum</th>
<th>Milk #1</th>
<th>Milk #2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunized cow #1</td>
<td>1.5 x 10⁶</td>
<td>5 x 10⁵</td>
<td>4.5 x 10²</td>
</tr>
<tr>
<td>Immunized cow #2</td>
<td>8 x 10⁴</td>
<td>4 x 10⁵</td>
<td>4 x 10²</td>
</tr>
<tr>
<td>Immunized cow #3</td>
<td>8 x 10⁴</td>
<td>3 x 10⁵</td>
<td>3 x 10²</td>
</tr>
<tr>
<td>Immunized cow #4</td>
<td>5 x 10⁴</td>
<td>3 x 10⁵</td>
<td>2 x 10²</td>
</tr>
<tr>
<td>Non-immunized cows #1-IV</td>
<td>1 x 10⁴</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Titers are expressed as OD/1 dilution determined in ELISA.

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Allergol et Immunopathol 2001; 29(6): 229-237
ACKNOWLEDGEMENTS

The authors thank FAPESP for financial support, Nestlé Industrial e Comercial Ltda., for the cows immunization and the Obstetrics Clinic of University Hospital, from the human colostrum samples.

RESUMEN

Antecedentes: Escherichia coli enteropatogénica (EPEC) es el principal agente etiológico de diarrea infantil en el Brasil y en otros países en desarrollo. La IgA presente en la leche humana protege la mucosa intestinal del recién nacido por inhibición de la acción bacteriana a las células epiteliales. Este efecto es observado por ensayos in vitro de adhesión de EPEC a células HEp-2. La leche bovina, de ser efectiva en estos mecanismos de protección, podría ser una herramienta importante en ausencia de lactantes infantiles en el Brasil y en otros países en desarrollo.

Métodos: fue investigado el efecto del calostro, leche y suero de vacas en la adherencia de la EPEC a la pared de células HEp-2. Calostro de animales inmunizados y animales control, como también leche de formulación industrial fueron fraccionadas a través de una membrana con un corte para un peso molecular de 10 kDa. La fracción de mayor peso molecular (HMWF) de calostro bovino fue liberada de IgG a través de una columna de afinidad y absorbida con una cepa adherente de EPEC. Fueron investigados anticuerpos por técnicas de ELISA e immunoblotting (IB).

Resultados: el calostro y leche de animales inmunizados con EPEC mostraron una actividad inhibitoria en la adherencia similar a la de los animales control no inmunizados. El efecto en la adhesión fue asociado a la HMWF. El calostro libre de IgG re-
tuvo parcialmente el efecto inhibitorio, mientras que la fracción eluida rica en IgG perdió esta propiedad. La fracción absorbida en EPEC retuvo la propiedad inhibitoria. La leche de formulación industrial y la HMWF también inhibieron la adherencia bacteriana. En ensayos de IB, muestras de calostro y leche de animales inmunizados reconocieron proteínas de 30-40 kDa y 94 kDa, peso molecular coincidente con el de la adhesina intimina en los extractos de EPEC.

**Conclusiones:** el efecto inhibitorio de la adherencia de la EPEC podría estar mediado por HMWF y la IgG no sería el único componente responsable por este fenómeno.

**Palabras clave:** *Escherichia coli* enteropatógena. Leche bovina. Calostro bovino. Leche humana. Diarrea infantil. Adhesión bacteriana.

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**REFERENCES**