

Secretory immunoglobulin A obtained from pooled human colostrum and milk for oral passive immunization

Carbonare CB, Carbonare SB, Carneiro-Sampaio MMS. Secretory immunoglobulin A obtained from pooled human colostrum and milk for oral passive immunization.

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Passive immunization is useful in cases of immunodeficiencies or infectious diseases, but usually seric gammaglobulin or hyperimmune sera are administered parenterally, providing good systemic immunization, though with low protection of the mucosal surfaces. The oral administration of secretory antibodies, especially surface immunoglobulin (SIg)A, which is perfectly adapted to the mucosal environment, would therefore, be preferable. The aim of the present study was to obtain a SIgA preparation from pooled human colostrum and milk, which should maintain the essential properties of the antibodies suitable for clinical oral administration. IgA preparations were obtained from colostrum and milk pools by salt precipitation. The final products were evaluated in terms of yield and purity, as well as antibody activity to bacterial antigens and toxins and inhibitory activity of bacterial adhesion to epithelial cells. The best yield and purity were achieved when the colostrum pool was used as a source of IgA; the final product showed very few bands of protein contamination. The IgA preparations preserved the antibody reactivity against various microbial antigens, well comparable with the reactivity exhibited by the original milk and colostrum pools. SIgA preparations were able to inhibit greatly the adhesion of enteropathogenic *Escherichia coli* to Hep-2 cells and the invasion of enteroinvasive *E. coli*. These promising results show the feasibility of obtaining SIgA suitable for oral use, which may in future be administered to immunodeficient patients with gastrointestinal manifestations, from human colostrum and milk.

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Passive immunization occurs naturally in humans during the gestational period, when immunoglobulin (Ig)G antibodies reactive with various pathogens are transferred to the fetus through the placenta, and during breastfeeding, when milk antibodies protect the mucosal surfaces of the newborn, thus preventing infections. Human colostrum and milk have a rich repertoire of antibodies reactive with a great variety of micro-organisms and other antigens from the environment and food (1, 2).

Secretory IgA (SIgA), the main Ig in human milk, is structurally and functionally suitable for

the mucosal environment, neutralizes antigens and viruses, promotes microorganism agglutination, facilitates antigenic exclusion and prevents the adherence of pathogens to the epithelial mucosal surfaces (3).

Passive immunization may be indicated in cases of primary or secondary immunodeficiencies, immunosuppressed patients submitted to transplantation, accidents with toxins and some of acute bacterial or viral infections. The parenteral administration of Ig results in good systemic immunization but is not equally efficient in providing protection for the mucosal surfaces,

as seric Igs are not significantly transferred to the mucous membranes in humans. Further, there are some clinical trials with the oral administration of Ig preparations obtained from human sera or bovine colostrum for the treatment or for the prevention of enteritis and necrotizing colitis.

Some therapeutic studies have been carried out on patients with infectious diseases or in situations of risk. An Ig preparation obtained from bovine colostrum was administered orally to acquired immunodeficiency syndrome (AIDS) patients with *Cryptosporidium parvum* diarrhea (4); and Igs from hyperimmune bovine colostrum to children with *Helicobacter pylori* infection (5). A preparation of Igs containing 73% of IgA and 26% of IgG from human sera was administered orally to low weight newborn children with necrotizing colitis (6), and to adult patients for the treatment of enteritis caused by different agents such as *Clostridium difficile* or *Campylobacter jejuni* (7).

There are many reports of the experimental oral administration of IgA in animal models followed by a challenge with such bacteria as *Escherichia coli* and *Helicobacter felis* or viruses such as rotavirus. Intranasal administration of IgA was tried in mice to provide protection against *Streptococcus* or Sendai virus (7).

The oral administration of fresh colostrum and milk was first reported for the treatment of diarrhea in premature newborns who had not responded to the antibiotic therapy (8). Afterwards, babies at risk received human milk or colostrum in cases of acute or prolonged infection caused by enteropathogenic *E. coli* (EPEC) and *Salmonella* (9, 10); for the prevention and treatment of gastroenteritis and necrotizing colitis (11) and of chronic diarrhea in malnourished children between 9 months and 3 yr of age (12).

Nowadays the primary immunodeficiencies (PID) are frequently present in clinical practice, mainly in pediatrics. In practical, the main clinical manifestation of all the types of PID so far identified has the higher susceptibility to infection, and diarrhea is the third most frequent manifestation. The immunodeficient patients with intestinal manifestations of difficult control might be benefited by the use of oral IgA. One of the present authors (MMSCS) had the opportunity to use human colostrum as an adjuvant successfully in the treatment of agammaglobulinemic or IgA deficient children with protracted diarrhea caused by *Giardia* or of unknown etiology.

The attainment of purified Igs from colostrum and milk makes the use of a therapeutic preparation possible with controlled concentration and antibody activity and the absence of

contaminating microbes. In a previous study, the present authors compared different procedures for IgA isolation in small volumes of human colostrum and milk (13). The precipitation method with ammonium sulfate (14) was chosen as the best procedure so far in yield, purity, and in the maintenance of antibody properties. The present work describes the obtaining of Ig preparations from greater volumes of human colostrum and milk pools and such analyses of their properties show that they are suitable for oral clinical use.

Materials and methods

Colostrum and milk pools

Samples of colostrum and milk, donated by healthy women, were obtained from the Milk Bank of the University of São Paulo Hospital. All samples were previously pasteurized after collection (at 62.5°C for 30 min) and maintained frozen at -20°C. After thawing, samples of milk and colostrum were centrifuged and equal volumes of each sample were pooled, yielding 2300 ml of milk and 1200 ml of colostrum. One small aliquot of each pool was kept frozen and the remaining volume was used for the IgA isolation procedure.

IgA isolation by ammonium sulfate precipitation

The IgA precipitation with ammonium sulfate salt was performed by a method previously described, with some modifications (14, 15). The liquid phase of milk or colostrum was adjusted to the neutral pH and enough ammonium sulfate salt was added, with constant stirring, until a 50% concentration was reached. The mixture was kept overnight under refrigeration and centrifuged at 7600 *g* for 40 min. The pellet was resuspended and dialyzed against saline solution with several changes. The final product was divided into aliquots and maintained at -20°C for further analysis.

Total protein and IgA determination

All preparations of IgA isolated were submitted to the determination of total protein concentration by the Bicinchoninic acid method (Pierce, Rockford, IL, USA). Determination of IgA concentration was undertaken using the quantitative radial immunodiffusion assay with anti-human α -chain antibody (Sigma, St Louis, MO, USA).

SDS-PAGE

The sample extracts were prepared according to the total protein content and were diluted in

sample buffer to a final protein concentration of 1 μg of protein/ μl . The antigenic extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in Mini-Protean II apparatus (Bio-Rad, Hercules, CA, USA). The gels were stained with Coomassie blue to analyze the distribution pattern of proteins. Molecular weight standards (Pharmacia, Upsala, Sweden) were run on each gel.

Bacterial strains

The EPEC strain serotype O111:H-, Shiga toxin-producing *E. coli* (STEC) strain serotype O111ac:H- (Stx1 producer), enterotoxigenic *E. coli* (ETEC); enteroinvasive *E. coli* (EIEC), *Salmonella enterica typhimurium*, were from the Butantan Laboratory Collection, Sao Paulo. Bacteria were grown in Tryptic Soy Broth medium (Difco Laboratories, Detroit, MI, USA) and bacterial suspensions of known concentration, determined by optical density (OD) readings, have been used for the sensibilization of enzyme-linked immunosorbent assay (ELISA) plate (Costar, Cambridge, MA, USA), and for the crude extract preparation used in SDS-PAGE.

ELISA

Anti-EPEC antibody titers were determined by ELISA using a whole-cell assay as previously described (16). Microtiter 96-well plates (Costar) were coated with standardized bacterial suspension. After blocking with skimmed milk, the antigen-antibody reaction was carried out with appropriate dilutions of samples, followed by incubation with anti-human IgA peroxidase conjugate (Sigma) and developed with the substrate H_2O_2 in o-phenylenediamine dihydrochloride (OPD) (Sigma). OD values were obtained at 492 nm (Multiskan Labsystems, Helsinki, Finland). Titer was determined as the reciprocal of dilution corresponding to the OD 0.5. Anti-rotavirus antibodies were detected by an ELISA similar to that described above, using plates coated with rotavirus antigen obtained by infection of MA104 cell culture and posterior ultracentrifugation of supernatant in sacrose gradient.

Immunoblotting

Immunoblotting assays were carried out on all materials, colostrum and milk pools and IgA preparations, using crude extracts of bacteria, as described elsewhere (17). Bacterial components of crude extracts were separated by SDS-PAGE in 12.5% acrylamide gels in Mini-Protean II

apparatus. After transferring to nitrocellulose membranes in a semidry Trans-Blot SD apparatus (Bio-Rad), strips were incubated with appropriate dilutions of colostrum or IgA preparations, followed by anti-human IgA peroxidase conjugate (Sigma) and substrate H_2O_2 in 3,3'-diaminobenzidine (Sigma).

Bacterial adhesion and invasion inhibition

Original pools and final products were submitted to EPEC adhesion assays to HEP-2 cells, according to the technique previously described by Silva and Giampaglia (18) in Lab-Tek chamber slides for tissue culture (Nunc Inc., Naperville, IL, USA). HEP-2 cell monolayer was incubated with a mixture of cell culture medium, exponential phase bacterial suspension and colostrum or IgA preparation. Control chambers with bacteria and medium or the medium alone were included. Phosphate-buffered saline washings removed the unattached bacteria and fresh medium without colostrum or IgA was added for a second incubation. The cells were washed, fixed with methanol (Merck, Darmstadt, Germany) and stained with May-Grünwald and Giemsa (Merck). At least 300 cells were observed in each preparation under the light microscope ($\times 400$ or $\times 1000$). The inhibitory effect was determined as the percentage of cells with six or more adhered bacteria on the cell surface when compared with the control under the same conditions but without colostrum. Invasion assays were performed on all materials, as described elsewhere (16). The invasion assay is similar to the adhesion assay, with some modifications, including the addition of gentamicin in the medium used during the second incubation period.

Toxin neutralization assays

The anti-tetanic and anti-diphtheric antibody were determined by the ToBI test (19). This method is based on the previous neutralization of the toxin by the sample antibodies and later detection of the remaining toxin by a sandwich ELISA, using a plate coated with capture anti-toxin antibody and development with a conjugated antitoxin antibody. The readings are compared with standard curves obtained by the assay performed with standard samples with known concentrations of antibodies.

Cytotoxicity assays on Vero cells

The cytotoxicity assays of STEC toxins Stx1 and Stx2 were undertaken by the method described

elsewhere (20) on Vero cells, with some modifications. The neutralizing ability of the antibodies present in the original pools and the IgA preparations was evaluated by pre-incubating the samples with a known concentration of the toxin, followed by culture on Vero cells.

Results

Evaluation of the IgA preparations

All preparations were analyzed as regards their yield, recovery, and purity. The intermediate and final products were submitted to protein and IgA determination. The best results were achieved by the colostrum pool (Table 1), but the lower yield of the milk pool is compensated by the greater volume available. The SDS-PAGE in 12.5% acrylamide gel (Fig. 1a) showed three well-defined bands corresponding to the secretory component (approximately 78 kDa), high molecular weight (MW) chain (55–60 kDa) and low MW chain (25–28 kDa). Some weaker bands might be due to the contaminating proteins or fractions resulting from IgA denaturation. The J chain is not visible (15–16 kDa). The non-reducing 6% acrylamide gel confirmed the presence of a very high MW band, similar to that of the standard SIgA. This band probably corresponds to the whole SIgA molecule, with a MW of approximately 378 kDa (Fig. 1b).

Table 1. Quantitative and qualitative evaluation of milk and colostrum immunoglobulin (Ig)A preparations compared with the values for the original pools

	Milk		Colostrum	
	Pool	IgA	Pool	IgA
Purity (%)	4.8	10.1	27.8	60.9
Recuperation (%)	—	81.81	—	106.32
Yield (mg of IgA/ml of pool)	—	0.72	—	8.2
IgA concentration (mg/ml)	0.88	1.87	7.71	42.40
Anti-EPEC antibodies (titer)	72	183	80	483
Anti-rotavirus antibodies (titer)	9	129	42	160
EPEC adhesion inhibition (%)	89	70	91	91
EIEC invasion inhibition (%)	92	76	79	76
Tetanic toxin neutralization (UI/ml)*	0.18	0.16	0.28	0.27
Diphtheric toxin neutralization (UI/ml)†	ND	ND	0.06	0.08
Stx neutralization	ND	ND	ND	ND

ND, not detected (below the minimum detected by the method); EPEC, enteropathogenic *Escherichia coli*; EIEC, enteroinvasive *E. coli*.

$$\text{Purity (\%)} = \frac{\text{IgA content of the preparation} \times 100}{\text{Total protein content of the preparation}}$$

$$\text{Recuperation (\%)} = \frac{\text{IgA content of the preparation} \times 100}{\text{IgA content of the pool}}$$

$$\text{Yield (mg of IgA/ml of pool)} = \frac{\text{IgA content of the preparation}}{\text{Total volume of the pool}}$$

*Minimum protective level is 0.01 UI/ml of circulating antitoxin.

†Minimum protective level is 0.02 UI/ml of circulating antitoxin.

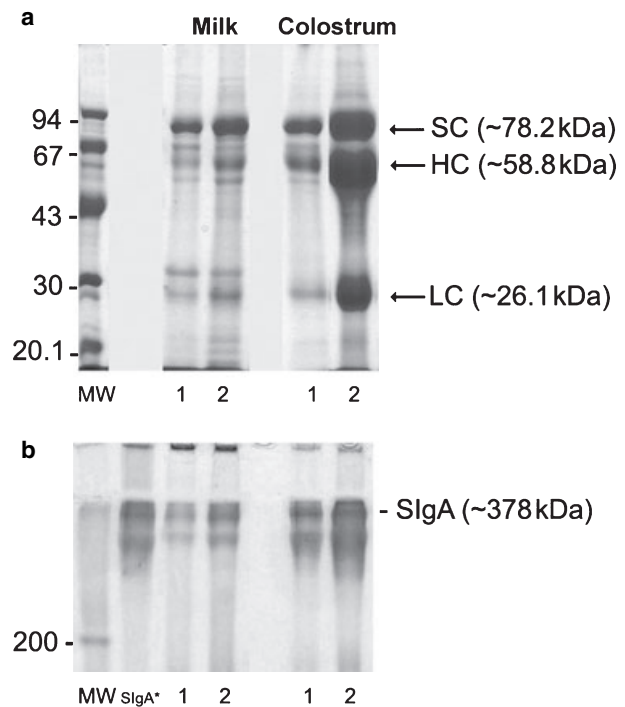


Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of immunoglobulin (Ig)A preparations compared with those of the original pools: (a) 12.5% acrylamide gel; (b) 6% acrylamide non-reducing gel. 1, milk or colostrum pools; 2, IgA preparations; MW, molecular mass standards; SC, secretor component; HC, heavy chain; LC, light chain; SIgA*, secretory IgA standard (Sigma).

Evaluation of the antibody reactivity of the preparations

The levels of IgA antibodies directed to EPEC and rotavirus antigens were evaluated by ELISA. The results given in Table 1 show that the levels of anti-EPEC and anti-rotavirus antibodies of the IgA preparations are higher than those of the original pools. This result was to be expected because of the relative concentration of IgA and confirms the maintenance of antibody reactivity after the AS precipitation procedure.

This was confirmed by immunoblotting assays performed with the bacterial strains EPEC, enterohemorrhagic *E. coli* (EHEC), ETEC, EIEC, *Streptococcus pneumoniae*, *Salmonella typhimurium*, and *Haemophilus influenzae*. Figs 2 and 3 show that all preparations were able to recognize the antigenic bacterial fraction, of the same pattern as those of their original pools.

Evaluation of the biological activity of the preparations

The IgA preparation obtained from the colostrum pool was able to inhibit the EPEC adhesion to HEp-2 cells or the EIEC invasion of them (Fig. 4), in the same degree as in the original pool

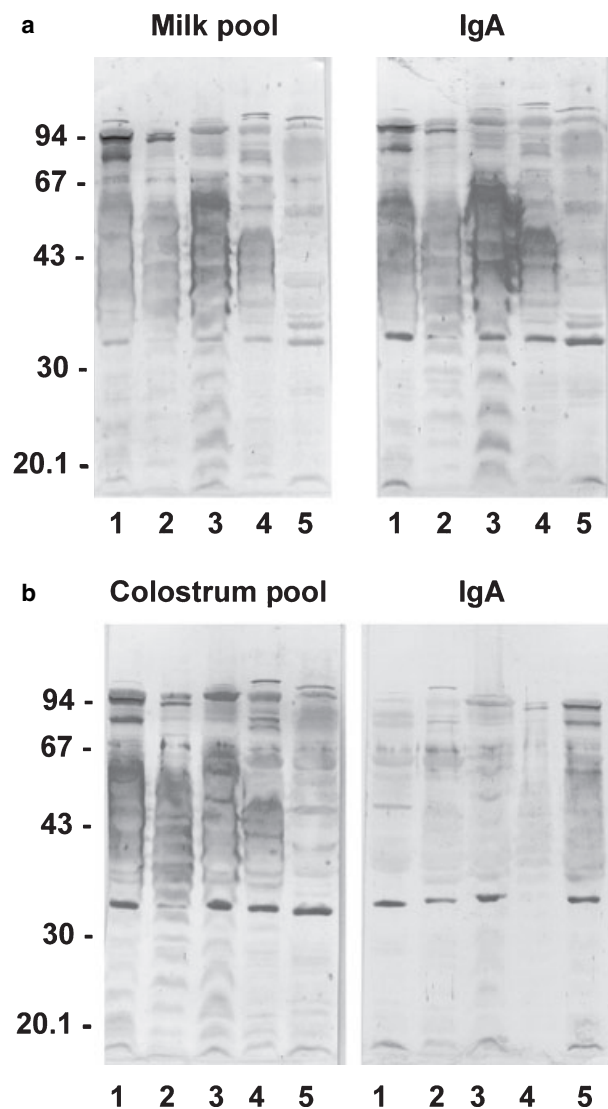


Fig. 2. Immunoblotting with crude extracts of: 1, enteropathogenic *Escherichia coli*; 2, Shiga toxin-producing *E. coli*; 3, enterotoxigenic *E. coli*; 4, enteroinvasive *E. coli*; 5, *Salmonella typhimurium*. Samples: (a) milk pool, 1:40 dilution; IgA from milk, 1:300 dilution; (b) colostrum pool, 1:100 dilution; IgA from colostrum, 1:300 dilution; peroxidase conjugated anti-human IgA, 1:4000 dilution.

(Table 1). The IgA preparations obtained from the milk pool had their inhibitory ability slightly lessened when compared with the original pool, but the inhibition values was above 70% (Table 1).

All preparations exhibit the neutralizing ability of tetanic toxin at levels equivalent to those of the original pools and 16–28 times higher than the minimum protective level. The IgA preparation obtained from the colostrum pool showed the neutralizing ability of diphtheric toxin to the same degree as that of the original pool, three to four times higher than the minimum protective level. Nevertheless, this was not observed for the

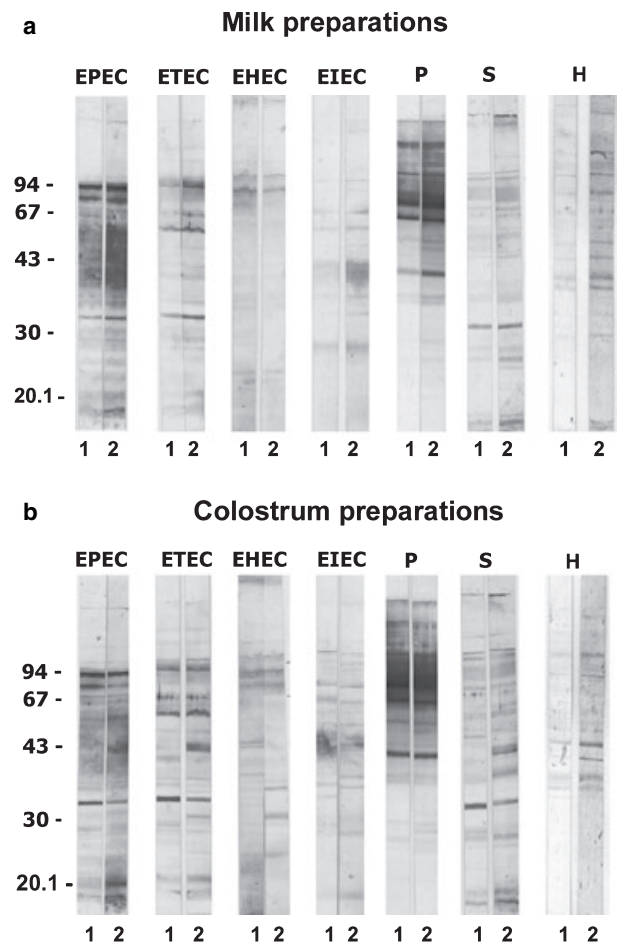


Fig. 3. Immunoblotting with crude extracts of: EPEC, enteropathogenic *Escherichia coli*; ETEC, enterotoxigenic *E. coli*; EHEC, enterohemorrhagic *E. coli*; EIEC enteroinvasive *E. coli*; P, *Streptococcus pneumoniae*; S, *Salmonella typhimurium*; H, *Haemophilus influenzae*. (a) Milk preparations: 1, milk pool 1:25 dilution; 2, milk immunoglobulin (IgA), 1:20 dilution; (b) colostrum preparations: 1, colostrum pool, 1:25 dilution; 2, colostrum IgA, 1:10 dilution; peroxidase conjugated anti-human IgA, 1:4000 dilution.

milk IgA preparation or for either of the IgA preparations in relation to the Stx1 or Stx2 toxins (Table 1).

Discussion

The Ig preparations commercially available contain predominantly seric IgG, are applied mainly for the reposition of seric antibodies in cases of primary or secondary immunodeficiencies and, in some instances, in autoimmune diseases. Nevertheless, in certain cases of gastrointestinal disorders, the parenteral administration of IgG is not significantly useful, as seric IgG is poorly transferred to the mucous membranes and IgG is not appropriate to the mucosal environment. The

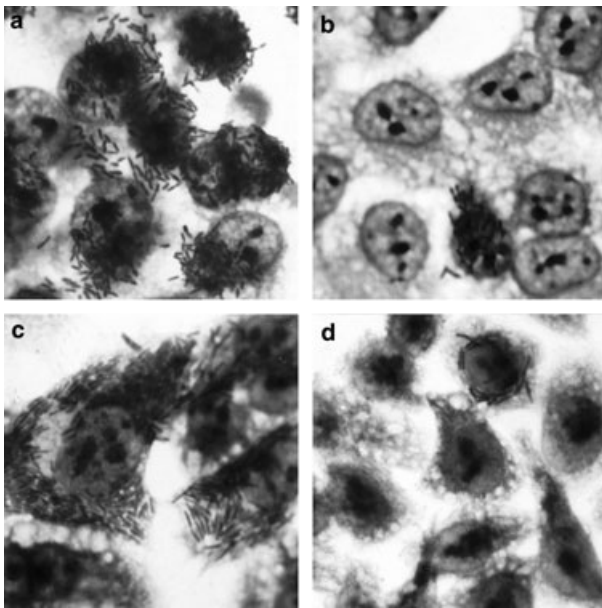


Fig. 4. Inhibition ability of original pools and final products in adhesion assays of enteropathogenic *Escherichia coli* (EPEC) and invasion assays of enteroinvasive *E. coli* (EIEC) to HEp-2 cells: (a) adhesion of EPEC to HEp-2 cells; (b) inhibition of EPEC by milk or colostrum preparations; (c) invasion of EIEC to HEp-2 cells; (d) inhibition of EIEC by milk or colostrum preparations.

development of Ig preparations based on SIgA is an important acquisition nowadays, when new cases of immunodeficiency are identified and great efforts are being made to enhance the viability of very low weight pre-term newborns.

In this present study, Igs were isolated from milk and colostrum pools by ammonium sulfate precipitation, and the SDS-PAGE analyses of the final products showed three bands equivalent to the secretor component and the heavy and light chains. The non-reductor gel showed high MW bands suggestive of integral SIgA molecules in the IgA preparations as well as in the respective milk and colostrum pools.

Immunoblotting assays showed that antibodies in IgA preparations present reactivity with bacterial antigenic fractions according to the same pattern as their respective original pools, indicating that the ammonium sulfate precipitation method did not affect the reactivity of the antibodies. Our results are in accordance with those of Manjarrez-Hernandez et al. (21) who observed that SIgA from human milk isolated by ammonium sulfate react with proteins expressed by EPEC isolated from cases of infant diarrhea.

The levels of anti-EPEC antibodies detected by ELISA were higher in milk and colostrum IgA preparations than those in their respective milk and colostrum pools. This was to be expected because of the higher total IgA concentration in prepara-

tions in comparison with that of the original pools. Antibodies reactive with diarrheagenic *E. coli* were detected in the colostrum of Brazilian, Mexican, and American women (16, 22–27).

The biological activity of antibodies was evaluated by adhesion assays of EPEC to HEp-2 cells. IgA preparations maintained the same inhibitory ability as their respective original pools. The inhibitory activity of colostrum in adhesion or invasion assays with culture cells has been demonstrated elsewhere with such various diarrheagenic *E. coli* as EPEC (22, 23, 28, 29), EIEC (16); enteroaggregative *E. coli* (EAEC) (24); EHEC (25), and ETEC (30). The same ability has been demonstrated by colostrum SIgA isolated by affinity chromatography in a Sepharose anti-IgA column (Amersham Pharmacia Biotech, Uppsala, Sweden), for invasion of EIEC, adhesion of EPEC (16, 22), EAEC (24), and STEC (25). Cravioto et al. (28) verified that milk SIgA isolated by a Sephacryl S-200 (Amersham Pharmacia Biotech, Uppsala, Sweden) column was able to inhibit adhesion of *E. coli* strains with different patterns such as localized, aggregative or diffuse adhesion.

The lack of neutralization of Stx1 or Stx2 was to be expected for IgA preparations from colostrum and milk as the respective original pools did not exhibit that ability. Individual colostrum samples collected from women living in the same region were not able to neutralize Stx1 or Stx2 (25). This result is probably a reflection of the low frequency of the isolation of STEC Stx-positive bacteria in Brazil, resulting in a low frequency of anti-Stx antibodies among the Brazilian population.

The ability of IgA to neutralize tetanic and diphtherial toxins was not affected by the ammonium sulfate precipitation. Protective levels of anti-tetanic toxin antibodies were found in IgA preparations as well as in the pools, and anti-diphtherial toxin antibodies were found both in the colostrum pool and in its respective IgA preparation.

The yield of IgA originating in the colostrum pool is greater than that of milk. Great volumes of milk are easily available in human milk banks, but colostrum is difficult to obtain. Nevertheless, the small volume with high concentration yields a good final amount of IgA.

There are some authors who have reported the successful treatment of *E. coli* and *Salmonella* diarrhea by the oral administration of fresh human colostrum or milk to infants (8, 10, 12), the prevention of diarrhea and necrotizing colitis in pre-term newborns (9, 11) and also in studies on animal models (7).

There are some reports of the oral administration of Ig preparations rich in IgA (76%) obtained from human blood, consisting of the monomeric IgA molecule (IgAbulin; Immuno, Viena, Austria) (6, 7). The oral use of SIgA isolated from human colostrum or milk has not been reported but we believe that it would be the best way to achieve passive immunization of patients who need mucosal protection, seen that the dimeric molecule equipped with the J chain and secretor component is well adapted in acting on mucosal surfaces.

The ideal dose and frequency of administration call for further studies. The IgA preparation would be indicated in such of immunodeficient cases as agammaglobulinemia, hipogammaglobulinemia and AIDS with intestinal disorders, during the critical phase of the infectious disease and in association with the specific therapy.

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