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Breast Milk Anti-*Escherichia coli* Heat-Labile Toxin IgA Antibodies Protect against Toxin-Induced Infantile Diarrhea

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ABSTRACT. Cruz, J. R., Gil, L., Cano, F., Cáceres, P. and Pareja, G. (Program on Infection, Nutrition and Immunology, Institute of Nutrition of Central America and Panama, Guatemala, Guatemala). Breast milk anti-*Escherichia coli* heat-labile toxin IgA antibodies protect against toxin-induced infantile diarrhea. Acta Paediatr Scand 77, 658, 1988.

A prospective study to assess whether milk IgA antibodies against *Escherichia coli* heat labile-toxin protect breast-fed children against labile toxin-induced gastroenteritis was carried out among infants of a marginal urban area in Guatemala. One hundred and thirty children were kept under surveillance for diarrhea by periodic home visits. Stool specimens were collected from each child routinely every 2–3 weeks and during diarrheal episodes, to study the excretion of labile toxin-producing *Escherichia coli*. Milk samples from the children's mothers were obtained concomitantly with the fecal specimens of the infants to be analyzed for anti-labile toxin antibodies. Twenty infections by heat-labile toxin-producing *Escherichia coli* as a sole agent were documented among breast-fed infants. Nine of these infections resulted in gastroenteritis, while the remaining 11 were asymptomatic. At the time of infection children who became sick were ingesting breast milk with significantly (p=0.028) lower titers of antilabile toxin IgA than those who remained healthy. Only one of the 8 infected children receiving breast milk with high titers (≥256) of anti labile toxin IgA developed diarrhea, compared to 8 of the 12 subjects being fed milk with low titers (≤64) (p=0.025). This is the first report documenting protection by IgA antibodies in milk against labile toxin-induced gastroenteritis in infected breast-fed infants. *Key words: Escherichia coli heat-labile toxin, human milk, IgA antibodies, diarrhea, protection.*

Breast milk contains several defense factors that may be involved in the protection of the breast-fed infant against infectious diseases (1–3). The main immunoglobulin in human milk is the secretory IgA (SIgA), which is resistant to enzymatic degradation and, therefore, functional in the gastrointestinal tract (4–5). SIgA antibodies against a variety of microorganisms have been detected in breast milk (6–8), but only those directed against cholera toxin or *Vibrio cholera* lipopolysaccharide have been shown to be protective in naturally occurring human infections (9). In view of the importance of enterotoxigenic *Escherichia coli* as a diarrheogenic agent among children worldwide (10–12), the present study was undertaken with the purpose of assessing whether milk IgA anti-*Escherichia coli* heat-labile toxin (LT) antibodies protect the breast-fed infant against LT-induced gastroenteritis.

METHODS

Population and field methodology. The study was undertaken in "Colonia El Limón", a marginal urban area of Guatemala City with 7308 inhabitants. One hundred and thirty families with children 0–8 months old were randomly selected and enrolled in the project. In our study population, 95% of the mothers and 37% of the fathers had no formal education. Fifty percent of the families earned less than US $ 600 per year. With parental consent, children aged 0–8 months were enrolled in the study and followed for 3–9 months. Auxiliary nurses visited the homes of the children once a week to obtain information on the presence of diarrhea. When a case of gastroenteritis was detected, a fecal sample for microbiological studies was obtained from the sick child and if he/she was being breast-fed, milk was collected from the mother at the same time. Additionally, stool and milk specimens were obtained routinely from

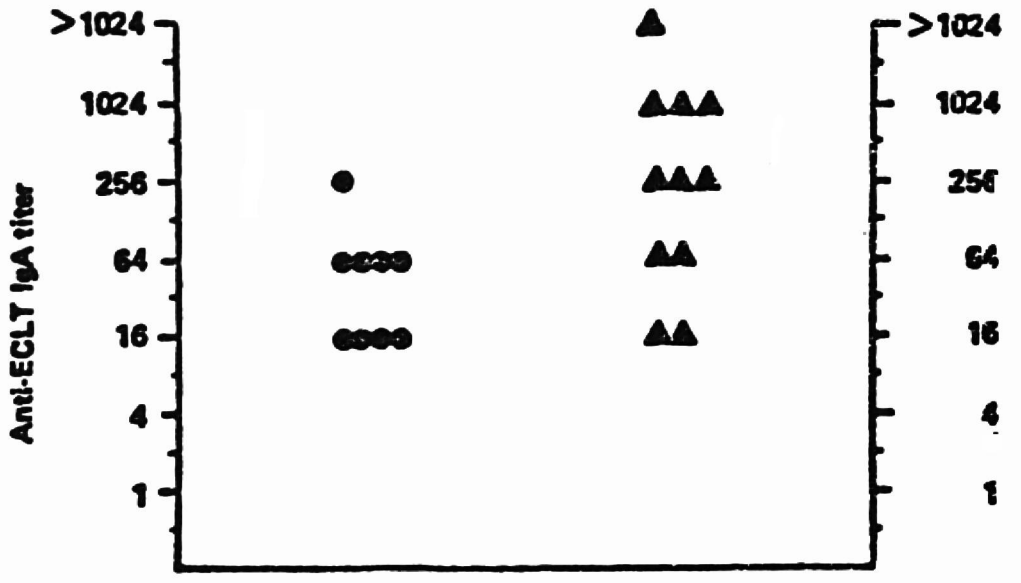

Fig. 1. Levels of anti-LT IgA antibodies in breast milk in relation to asymptomatic (▲) or symptomatic (●) infections.

each infant–mother pair every 2–3 weeks. Both stool and milk samples collected from the mother–infant pair on a given day were identified with the same number.

Laboratory methodology for the isolation of E. coli. The fecal material in Cary-Blair transport medium was streaked on Salmonella-Shigella and McConkey agar plates. After 24 h of incubation, lactose-fermenting colonies were picked and identified as *Escherichia coli* by biochemical tests (15). Production of LT was investigated by the adrenal mouse tumor (Y-1) cell assay, using an extract in casaminoacids broth (14). The presence of other bacterial pathogens, rotaviruses and parasites in the stools was determined as described (15).

Milk specimens, collected with a Marshal breast pump and infant nurser (Marshal Electronics, Cleveland, Ohio, USA) were kept frozen at −20°C in glass containers. At the end of the field phase, when microbiological studies had been completed, a list of the samples in which LT-producing *E. coli* had been detected was obtained from the computer files. The only identifiers used were the number of the specimen and whether the subject was being breast-fed or not; technicians were blinded to occurrence of symptomatic versus asymptomatic infection. Analysis of anti-LT antibodies in those milk specimens that corresponded to LT-producing *E. coli* positive cultures was done. Immediately before their analysis, the samples were thawed and centrifuged (400×*g*) to remove the fat and cells. Anti-*Escherichia coli* LT IgA antibodies were detected by means of a modification (16) of the enzyme-linked immunosorbent assay (ELISA) described by Yolken et al. (17). This assay is based on the antigenic cross reaction between *Escherichia coli* LT and cholera toxin and can be used to measure anti-LT antibodies in geographic areas where cholera does not exist, as in Guatemala. Briefly, polypropylene 96-well ELISA plates (Dynatech Labs., Maryland, USA) were coated with burro anti-cholera toxin antiserum and purified cholera toxin (provided by Dr John Robbins, Federal Drug Administration, USA). Serial dilutions (1:1 to 1:2048) of the milk specimens were then allowed to react with the antigen, and the bound IgA was detected with goat anti-human α chain antiserum conjugated with alkaline phosphatase (TAGO, Burlingame, California, USA), using p-nitro-phenyl-phosphate (SIGMA, St. Louis, Missouri, USA) as substrate. The optical density of each well was determined with an ELISA Minireader® (Dynatech). The levels of anti-LT IgA antibodies are expressed as titers, that is the reciprocal of the highest dilution with a positive reaction (O.D. test dilution/O.D. buffer blank ≥2).

Statistical methods. The Kendall correlation coefficient, the Mann-Whitney and the Fisher's exact tests (18, 19), were used for the statistical analyses. Before performing the Fisher's test, the children were divided into two categories according to the titer of antibodies in milk, with the cut-off point being the median.

RESULTS

Twenty-three infections by LT-producing *Escherichia coli* (LTEC) were documented among the breast-fed children. Twelve of the infections resulted in diarrhea and 11 were asymptomatic. Two of these episodes of diarrhea were also associated with HEp-2 adherent *Escherichia coli*, and one with rotaviruses; these three cases were excluded from the analysis. Only one child was being exclusively breast-fed at the time of infection by LTEC; the remaining ones were receiving either liquids or solid foods in addition to breast milk.

Among the 20 infected children included in the analysis, the nine who developed diarrhea

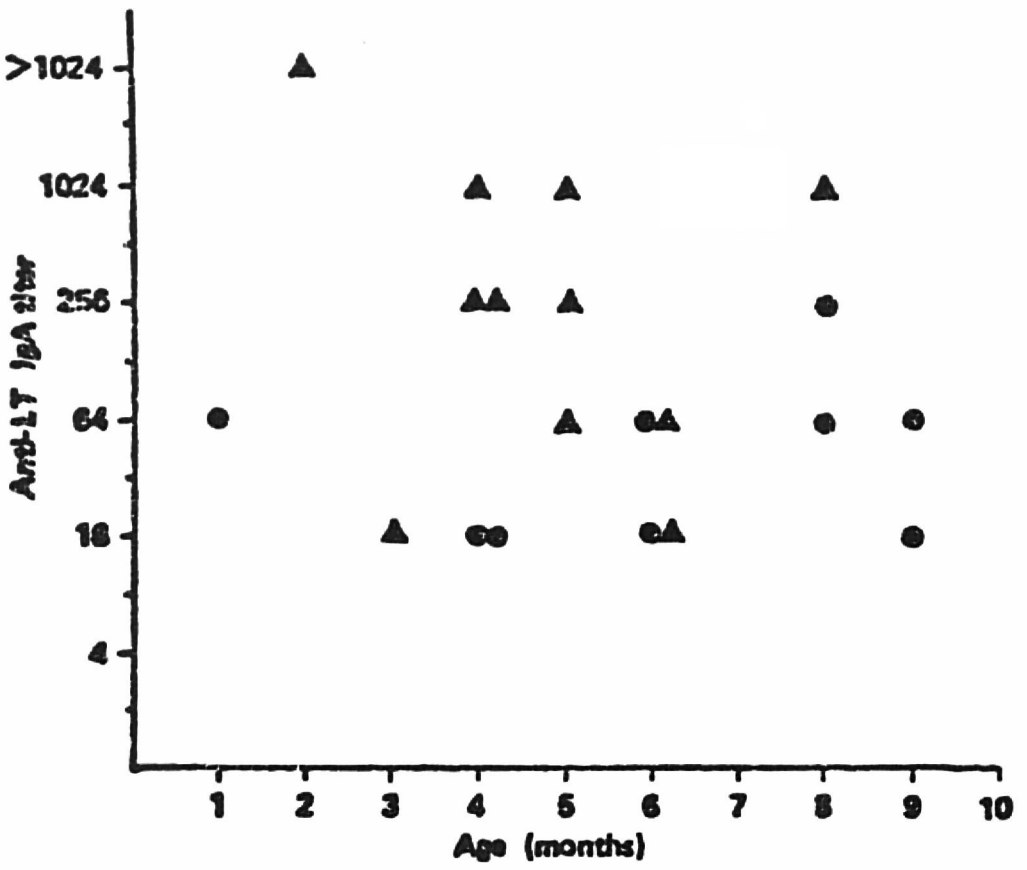

Fig. 2. Levels of anti-LT IgA antibodies in breast milk in relation to asymptomatic (▲) or symptomatic (●) infections.

were ingesting breast milk with lower titers of anti-LT IgA antibodies than the 11 who remained healthy (p=0.028, Mann-Whitney test, Fig. 1). The risk of developing diarrhea was 0.125 for those who were fed high-titered (≥256) breast milk and 0.667 for those who were fed milk with low titers (≤64) of anti-LT, meaning that children in the latter group were 5.3 times as likely to have diarrhea as children in the former category (p=0.025, Fisher's exact test).

The children who developed diarrhea tended to be older (6.1±2.7 months) than those who were colonized but remained symptom-free (4.7±1.6 months); this tendency, however, did not attain statistical significance (p>0.05, Mann-Whitney test). Although there was no significant correlation of age with antibody titers (r=−0.113, p>0.1, Kendall correlation) younger infants (<5 months old) tended to be more likely (6/11) to ingest milk with high titers of anti-LT IgA than the older ones (2/9).

DISCUSSION

Although the anti-microbial activity of various defense factors in human milk has been demonstrated in vitro, only anti-cholera toxin and anti-*Vibrio cholera* lipopolisaccharide specific IgA human milk antibodies have been shown to protect the breast-fed child against illness in natural infections (9). The present study suggests that specific IgA milk antibodies directed against *Escherichia coli* LT play a determining role in the outcome of an infection by LTEC in breast-fed infants.

The children who developed diarrhea in association with LT-producing *E. coli* were fed milk with significantly lower titers of anti-LT antibody than those infected but remaining healthy. Among the infants with anti-LT antibody titers <64, the risk of developing diarrhea during LTEC infection was 0.667; this risk was reduced to 0.125 in those children who were fed milk with a higher content of anti-LT IgA antibody. Our finding is in complete agreement with that of Glass and colleagues (9) regarding anti-cholera toxin IgA antibodies in milk and the development of *Vibrio cholera*-associated diarrhea. These two studies taken together indicate that specific IgA antibodies in human milk protect the breast-fed infant against toxin-induced gastroenteritis.

In our study subjects, the infected children who developed diarrhea tended to be older than those who remained asymptomatic after colonization by LTEC. The possibility that there is a difference in susceptibility to LT at different ages could be a determining factor in the outcome of LTEC infection. However, among the children who were fed low-titered breast milk, the risk of developing diarrhea was similar (p=0.594, Fisher's exact test) in the children less than 5 months old and in those above that age (Fig. 2). This finding suggests that the higher risk of developing diarrhea among children older than 5 months of age was associated with the intake of milk with low anti-LT titers.

The protection afforded by high antibody levels of anti-LT IgA antibodies was not an all-or-none phenomenon. Factors that may be important in this regard are the infectious burden of LTEC to which each child was exposed, and the volume of milk ingested. Although we did not estimate milk intake of the children, on the basis of previous observations (20) in the Guatemalan population we would not expect significant differences in the average milk intake among our 2–9 month old subjects. The mean daily intake of milk by children of the same socioeconomic and geographic characteristics at 1, 3, 6 and 9 months of age was 519, 548, 586 and 587 ml, respectively (20). Among our subjects only one child, one month old, was exclusively breast-fed and therefore probably ingesting more milk than the others, who were only partially breast-fed. This infant, who was among the low-titer group, developed diarrhea during his LTEC infection.

We have previously shown that the levels of specific antibodies in milk, including anti-LT IgA, fluctuate in time, with no correlation with either other specific IgA antibodies or total milk secretory IgA (16, 21). The practical implications of these changes in the content of milk IgA antibodies have remained unclear. Nevertheless, on the basis of the present findings and those of Glass and coauthors (9), it is now possible to suggest that a decline in the levels of IgA antibodies in milk may play a determining role in the outcome of enteric infections in the breast-fed infant. A similar relation has been presented for the development of allergy to cow's milk and breast-milk antibody levels (22). The factors that influence the appearance and persistence of specific IgA antibodies in human milk need to be investigated. Time of the day or nutritional status of the mother do not seem to influence the levels of specific IgA antibodies in human milk (23). Antigenic exposure at the intestinal level of the lactopositive mother may be important, as suggested by studies of oral immunization of lactating women (24, 25).

These observations should be taken into consideration in planning maternal vaccination strategies to increase specific breast milk antibody levels.

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