Acta Pædiatr 82: 835–8. 1993

Protection against *Campylobacter* diarrhea: role of milk IgA antibodies against bacterial surface antigens

O Torres and JR Cruz

Program of Infectious Diseases, Division of Nutrition and Health, Institute of Nutrition for Central America and Panama (INCAP), Guatemala, Guatemala

Torres O, Cruz JR. Protection against *Campylobacter* diarrhea: role of milk IgA antibodies against bacterial surface antigens. Acta Pædiatr 1993;82:835–8. Stockholm. ISSN 0803-5253

In developing countries, *Campylobacter jejuni* causes diarrhea and dysentery, especially in children less than one year of age. Breast feeding protects against infectious diarrhea, with milk IgA antibody playing a determining role. Therefore, it has been proposed to increase the protective effect of human milk by vaccinating women of child-bearing age. To identify antigens which may induce protective breast-milk IgA, we analyzed 60 strains of *C. jejuni* isolated from asymptomatically- and symptomatically-infected breast-fed children less than 12 months of age. Surface antigens of *C. jejuni*, separated by polyacrylamide gel electrophoresis, were probed with breast milk collected concurrently with the fecal sample from which *C. jejuni* was isolated, and specific IgA was developed by immunoblotting. Our results indicate that milk antibodies against three high molecular weight bacterial surface antigens of 95, 110 and 185 kDa are involved in protection of infants infected with *C. jejuni* ($p = 0.00964$ for one-tailed Fisher's exact test). □ *Breast milk*, Campylobacter jejuni, *diarrhea, IgA, immunoblot*

JR Cruz, Program of Infectious Diseases, Division of Nutrition and Health, INCAP, PO Box 1188, Guatemala City, Guatemala 01011, Central America

Campylobacter jejuni is an important pathogen recognized as a cause of acute and persistent diarrhea and dysentery in children and adults in many parts of the world. In developing countries, children under 12 months of age, especially those who are not breast fed, are at higher risk of *Campylobacter* diarrhea (1).

Several lines of evidence suggest that specific antibodies are an important host defense against *C. jejuni* infection. Severe, recurrent, prolonged and extraintestinal infections with *C. jejuni* have occurred in persons with congenital or acquired hypogammaglobulinemia (2). Where *Campylobacter* is endemic, asymptomatic infections result from the development of specific serum IgA, the levels of which increase with age. Moreover, seroconversion to cell surface antigens has been reported in outbreaks of diarrhea and in studies in volunteers, as well as in patients from developed countries infected with *Campylobacter* (3–5).

Breast feeding protects infants against diarrheal disease through several mechanisms. The most important immunologic component that provides protection at the level of the gastrointestinal tract is the secretory IgA (sIgA). However, breast-fed infants can suffer from diarrheal diseases, especially in poor areas of the world, where children are exposed to a heavy microbiological load associated with poor sanitary conditions (6–8).

A protective role for maternal antibodies against *Campylobacter* sp. was suggested by prospective studies conducted in Central Africa, where *Campylobacter* sp. was isolated from non-diarrheic stools of neonates (9). More recently, Nachamkin et al. (10) reported that breast milk protects against *C. jejuni* diarrhea in the first year of life. Ruiz Palacios et al. (11) reported that human milk consumed by Mexican children with *Campylobacter* diarrhea did not contain secretory IgA antibodies to the glycine acid-extractable antigen of *Campylobacter*, whereas children who ingested breast milk rich in antibodies against that antigen did not have diarrhea. Thus, there appears to be a protective effect of specific antibodies present in human milk against *Campylobacter* diarrhea.

Levels of protective antibodies against infectious diseases can be increased in lactating women by appropriate vaccination of women of child-bearing age. If breast milk provides protection by neutralizing one or more surface antigens or cellular components of *C. jejuni*, vaccination of potential mothers with those antigens can ensure protection of the breast-fed infant and prevent the negative nutritional effect of *Campylobacter* diarrhea.

We studied the specific role that breast feeding plays in protecting against *C. jejuni* diarrhea, by identifying

specific cellular fractions of this bacterium recognized by specific antibodies against them contained in human milk.

Materials and methods

Study population

Sixty-four mother–infant pairs from a poor rural Indian village in Guatemala, Santa María de Jesús (12), who voluntarily agreed to participate in the study, were followed prospectively for 9–12 months after delivery.

Field procedures

Every two weeks, specially trained field workers visited the home of the participants under surveillance and collected data on child feeding, morbidity data with particular attention to diarrhea, and routinely collected breast milk and fecal samples from the mother and the child, regardless of their health status (which was also recorded). Additionally, whenever a child had diarrhea the mother brought the child to the INCAP's health clinic where a nurse collected fecal specimens of both mother and child, as well as breast milk. When needed, the child was rehydrated at the clinic.

Milk samples

Breast milk specimens (5–10 ml) were collected with manual pumps, refrigerated and transported to INCAP's headquarters in an ice box on the same day of collection, aliquoted in 1-dracm vials and frozen at −20 C until analysis.

Microbiological analyses

Upon collection, fecal specimens of mothers and infants were inoculated into Cary–Blair transport media which were kept refrigerated. The samples were collected or received, and transported on ice to INCAP's headquarters on the same day, where they were immediately processed to detect *Campylobacter*, *E. coli*, *Shigella*, *Salmonella*, *Yersinia*, *Aeromonas* and *Plesiomonas* by standard methods described previously (13).

Sample size and bacterial strains

Strains of *C. jejuni* were maintained frozen at −70 C. A total of 60 bacterial strains isolated from 33 children, and the corresponding breast-milk samples collected at the time when the fecal specimen was obtained, were analyzed. Thirty-eight of these strains came from fecal samples of children without diarrheal disease and 22 from diarrheal patients. Forty-four strains came from children without other pathogens detected and 16 belonged to samples of children with two or more pathogens.

Study design and statistical analyses

The study was prospective and observational; immunochemical analyses of the bacterial strains were performed with stock cultures of the *C. jejuni* collected from the longitudinal study. Data were analyzed using the one-tailed Fisher's exact test for 2×2 tables, using EpiInfo. It is important to emphasize that statistical analyses included only children who excreted *C. jejuni* as unique pathogen.

Denaturing gel electrophoresis and immunoblots

Polyacrylamide gel electrophoresis (PAGE) was performed in the presence of sodium dodecyl sulfate (SDS), as described by Laemli (14), with a resolving gel of 7.5% and a stacking gel of 4.5% polyacrylamide, using a sandwich gel setting. Surface protein antigens and glycine-extractable antigens of *C. jejuni*, prepared according to McCoy et al. (15), were separated. After electrophoresis, proteins were transferred to nitrocellulose membranes (NC) (Schlyler & Schuell, 0.22 μm) as described by Towbin et al. (16).

The NC membranes containing the blotted antigens were air-dried and blocked with Blotto-Tween (150 mM NaCl, 50 mM Tris.HCl, pH 7.5, 0.02% Tween 20 and 5% non-fat dried milk) at room temperature for 1.5 h. Breast milk diluted 1:10 in Blotto-Tween was added and incubated overnight at 4 C. Then, specific goat anti-human IgA, affinity isolated, conjugated to alkaline phosphatase (Tago Inc, PO Box 4463, 887 Mitten Road, Burlingame, CA 94011), diluted 1:1000 in Blotto-Tween was added and incubated at room temperature for 4 h. The blot was developed using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-indolyl-phosphate (BCIP), as described by Sambrook et al. (17). The immunoblots were analyzed manually, by measuring the Rf of each band, and calculating the molecular weight (MW) from individual calibrating curves plotted for each blot from the molecular weight standards. None of the epidemiological data was known by the technicians who performed the immunoblot analyses.

Results

After SDS-PAGE electrophoresis, surface antigens of *C. jejuni* showed a total of 18–22 bands that were visualized with Coomassie Blue. A pool of breast milk, used as a standard to control the immunoblotting procedure, showed a total of 5–12 bands: the 6 major bands had MWs of 35, 42, 65, 95, 110 and 185 kDa. Depending on the strain tested, other minor bands were observed. Fifty-seven of 60 milk specimens obtained from the mother of the *C. jejuni*-infected child showed

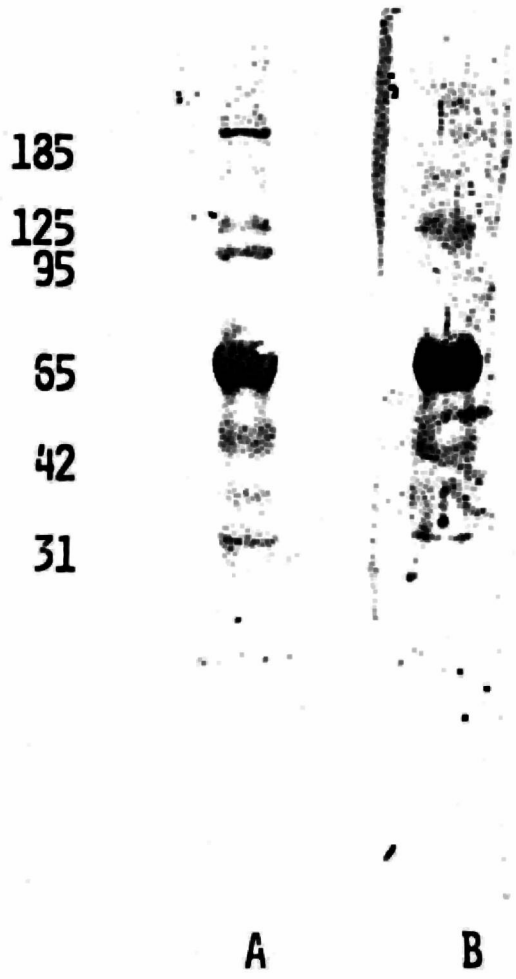

Fig. 1. A: Immunoblot showing the three bands associated with protection by breast milk: 95, 110 and 185 kDa. B: Immunoblot from a child with *Campylobacter* diarrhea, not showing the 95, 110 or 185 kDa bands.

one or more bands, distributed in a pattern similar to the pool, with the most common bands migrating at 35, 42, 65, 95, 110, 155 and 185 kDa (Fig. 1). Only 3 of 57 strains did not show any band recognized by the milk of the mothers. The most commonly observed band was a strongly reactive one, of relative molecular mass (Mr) of 65 000 ± 6000, found in 38 of 57 strains. For the glycine extracted antigens, the 31 kDa protein was found in 14 of 20 strains tested.

Immunoblots performed with breast milk obtained from 17 mothers of children who were excreting *C. jejuni* (as the only pathogen) but were not suffering from diarrhea, showed three major bands that were not present in blots developed with specimens collected from mothers whose children had *C. jejuni* diarrhea (and no other pathogens) ($p = 0.00964$, one-tailed Fisher's exact test). The Mrs of these bands were 95 000, 110 000 and 185 000. The presence of any of these single bands individually was not protective ($p > 0.05$, one-tailed Fisher's exact test) but a combination of the 95- and 110-kDa bands ($p = 0.025694$, one-tailed Fisher's exact test) or of the 95-, 110- and 185-kDa bands ($p = 0.00964$, one-tailed Fisher's exact test) was associated with the absence of diarrhea (Table 1).

The presence of the 65-kDa band, which corresponds to flagellin, was not associated with protection against the symptoms of diarrhea ($p = 0.1189737$).

Proteins of 42 and 31 kDa were detected in surface antigens and in glycine extractions, respectively. No difference in the group of children with and without diarrhea was observed concerning these bands.

Table 1. Surface antigens detected in immunoblots of *C. jejuni* with breast milk in children who had no other pathogen detected.

	Health status			
Mr[a] (10^{-3})	Diarrhea	Healthy	Convalescence	Total
35 ± 3	1[b]	11	4	16
42 ± 4	2	8	6	16
65 ± 6	5	25	8	38
95 ± 9	0	15	8	23
110 ± 5	0	2	4	6
155 ± 15	2	9	5	16
185 ± 18	0	13	3	16

[a] Relative molecular mass. [b] Indicates number of isolates presenting that band.

Discussion

Several studies have identified three proteins of *Campylobacter* with antigenically cross-reactive epitopes, initially thought to be potential vaccine candidates: flagellin (66 kDa), the outer membrane protein (OM, 42 kDa) and the glycine-extractable protein (31 kDa). Flagellin, the immuno-dominant antigen of *C. jejuni*, is not a direct candidate for use in a monovalent vaccine, because there are numerous serotypes of *Campylobacter* flagella and these are subject to both phase and antigenic variation; there is, however, a strong area of research trying to overcome these constraints (18).

The major OM protein and the 31 000 dalton cross-reactive protein found with glycine extraction are not good vaccine candidates because most of their cross-reactive epitopes are buried in the OM or in the inner membrane, respectively (19–21). In our study, breast-milk antibodies directed against these proteins were not associated with protection against diarrhea. Most of the immunoblots showed a strongly reactive band of approximately 62–65 kDa, where flagellin migrates, in both healthy and ill children. Our results agree with those of Nachamkin & Yang, who found no difference in the levels of intestinal IgA directed to flagellin in healthy and sick individuals (22).

Our findings suggest that breast milk rich in antibodies to two (95 and 110 kDa) or three (95, 110 and 185 kDa) bands of high molecular weight protects children against *C. jejuni* diarrhea. Data for single bands come from small sample sizes, so non-significance may reflect lack of statistical power. The protective action of each individual band may be through coordinate inactivation of the two or three high molecular weight antigens, which may need to interact for *C. jejuni* to be pathogenic. These bands might represent the 92.5-kDa, strain-specific OMP, associated with human isolates and identified by polyclonal antibodies raised in rabbits by Logan & Trust (19), or adhesins (23), high molecular weight LPS (24) or other traits associated with pathogenicity and not described to date. Nachamkin & Yang

(22) measured intestinal IgA anti-*Campylobacter* flagellin by an enzyme immunoassay in patients with and without *Campylobacter* infections. The level of fecal IgA anti-flagellin antibody was not significantly higher in patients than in controls, with only 12 of 29 patients (41.4%) with a *Campylobacter* infection showing detectable levels of specific anti-flagellin IgA antibodies. We observed IgA antibodies against flagellin in most of the patients studied; however, breast-milk samples of mothers of sick children also showed strong signals at the 65-kDa band.

Wu et al. (25) investigated the specific antigens of *C. jejuni* recognized by antibodies present in serum, urine and intestinal lavage, in infected and parenterally immunized mice by immunoblot analysis. Two antigens of 62 000 and 43 000 were predominantly detected by the three fluids. Urinary IgA detected predominantly the 43-kDa protein, while intestinal lavage IgA recognized mainly the 62-kDa protein. Urinary IgA also detected many other minor bands with Mrs ranging from 18 000–97 000; our findings are similar to those that Wu et al. reported for urinary IgA.

In conclusion, our findings indicate that breast milk rich in IgA that recognizes two (95 and 110 kDa) or three (95, 110 and 185 kDa) bands of high molecular weight protects children against diarrhea caused by *C. jejuni*. These results should be complemented with further studies that include quantitative determinations of specific IgA. Although we followed 64 mother–child pairs for 9–12 months (a statistically adequate sample number), only strains of 33 children came from children without any other pathogens, thus limiting the power of our conclusions. There is a definite need for re-evaluating the role of flagellin in the protection afforded by human milk. Our study identified three antigens involved in protection against *Campylobacter* diarrhea, which must be characterized in detail in order to evaluate them as vaccine candidates.

Acknowledgements.—This work was supported by the Nestlé Foundation and The Swedish Agency for Research Cooperation with Developing Countries (SAREC). The authors thank the technical assistance of T Véliz, F Cano, A Díaz, L Rodríguez, G González, F Leiva and J Hernández; M Castillo and N Coj for field procedures and J Matute for statistical analyses.

References

1. Cover T, Blaser M. The pathobiology of *Campylobacter* infections in humans. Annu Rev Med 1989;40:269–85
2. Johnson R, Nolan C, Wang S, Shelton W, Blaser M. Persistent *Campylobacter jejuni* infection in an immunocompromised patient. Ann Intern Med 1984;100:832–4
3. Figueroa G, Galeno H, Troncoso M, Toledo S, Soto V. Prospective study of *Campylobacter jejuni* infection in Chilean infants evaluated by culture and serology. J Clin Microbiol 1989;27:1040–4
4. Taylor D, Echeverria P, Pitarangsi C, Seriwatana J, Bodhidatta L, Blaser M. Influence of strain characteristics and immunity on the epidemiology of *C. jejuni* infections in Thailand. J Clin Microbiol 1988;26:863–8
5. Black R, Levine M, Brown K, Clements ML, Hughes T, Blaser M. Experimental *Campylobacter jejuni* infection in humans. J Infect Dis 1988;157:472–9
6. Glass R, Svennerholm A, Stoll B, Khan M, Hossain B, Huq I, Holmgren J. Protection against cholera in breast-fed children by antibodies in breastmilk. N Engl J Med 1983;308:1389–92
7. Cruz JR, Carlsson B, Hofvander Y, Holmes T, Hanson L. Studies of human milk: II Concentration of antibodies against *Salmonella* and *Shigella* in milk of women from different populations and the daily intake in their breast-fed infants. Acta Paediatr Scand 1985;74:338–41
8. Cruz JR, Gil L, Cano F, Cáceres P, Pareja G. Breast-milk anti-*Escherichia coli* heat labile toxin IgA antibodies protect against toxin-induced infantile diarrhea. Acta Paediatr Scand 1988;77:658–62
9. Martin P, Mathiot J, Ipero J, Kirimat M, George A, Georges-Courbot M. Immune response to *C. jejuni* and *E. coli* in a cohort of children from birth to two years of age. Infect Immun 1989;57:242–6
10. Nachamkin I, Fisher S, Yang X-O, Benitez O, Cravioto A. Protective effect of breastmilk directed against *C. jejuni* during the first year of life. Annual Meeting of the American Society of Microbiology 1990: Abstract B-50:35
11. Ruiz-Palacios G, Calva J, Pickering L, López-Vidal Y, Volkow P, Pezzarossi H, West S. Protection of breast-fed infants against *Campylobacter* diarrhea by antibodies in human milk. J Pediatr 1990;116:707–13
12. Cruz JR, Bartlett A, Méndez H, Sibrián R. Epidemiology of persistent diarrhea among Guatemalan rural children. Acta Paediatr 1992;81(Suppl. 381):22–6
13. Cruz JR, Cano F, Cáceres P, Chew F, Pareja G. Infection and diarrhea caused by *Cryptosporidium* sp. among Guatemalan infants. J Clin Microbiol 1988;26:88–91
14. Laemli U. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5
15. McCoy E, Doyle D, Burda K, Corbeil L, Winter A. Superficial antigens of *Campylobacter (Vibrio) fetus*: characterization of an antiphagocytic component. Infect Immun 1975;11:517–25
16. Towbin H, Stachelin T, Gordon J. Electrophoresis transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979;76:4350–4
17. Sambrook J, Fritsch E, Maniatis T. Molecular Cloning: A Laboratory Manual, 2nd Edn. Cold Spring Harbor, USA: Cold Spring Harbor Laboratory Press, 1989:18.60–71
18. Logan S, Trust T. Molecular identification of surface protein antigens of *Campylobacter jejuni*. Infect Immun 1983;42:675–82
19. Logan S, Trust T. Outer membrane characteristics of *Campylobacter jejuni*. Infect Immun 1982;38:898–909
20. Blaser M, Hopkins J, Berka R, Vasil M, Wang W. Identification and characterization of *Campylobacter jejuni* outer membrane proteins. Infect Immun 1983;42:276–84
21. Dubreuil J, Kostrzynska M, Logan S, Harris L, Austin J, Trust T. Purification, characterization and localization of a protein antigen shared by thermophylic campylobacters. J Clin Microbiol 1990;28:1321–8
22. Nachamkin I, Yang X-H. Local immune responses to the *Campylobacter* flagellin in acute *Campylobacter* gastro-intestinal infection. J Clin Microbiol 1992;30:509–11
23. McSweegan E, Walker R. Identification and characterization of two *Campylobacter jejuni* adhesins for cellular and mucous substrates. Infect Immun 1986;53:141–8
24. Preston, Penner. Structural and antigenic properties of LPS from serotype reference strains of *C. jejuni*. Infect Immun 1987;55:1806–12
25. Wu S-J, Pacheco N, Oprandy J, Rollwagen F. Identification of *C. jejuni* and *E. coli* antigen with mucosal and systemic antibodies. Infect Immun 1991;59:2555–9

Received Sep. 21, 1992. Accepted Dec. 28, 1992