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Immunoglobulin A antibodies directed against *Campylobacter jejuni* flagellin present in breast-milk

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SUMMARY

We studied the relationship between IgA anti-campylobacter flagellin antibodies in breast milk samples and protection of breastfed infants living in a rural Mexican village from campylobacter infection. There were fewer episodes of campylobacter infection (symptomatic and asymptomatic combined) in infants breastfed with milk containing specific anti-flagellin antibodies (1·2/child/year, 95% CI 0·6–1·8) versus non-breastfed children (3·3/child/year, 95% CI 1·8–4·8; $P < 0·01$). Infants breastfed with milk that was antiflagellin antibody negative by ELISA also had fewer episodes of infection compared with non-breastfed children, but the difference did not reach statistical significance (1·8/child/year, 95% CI 0·7–3·0 versus 3·3/child/year, 95% CI 1·8–4·8, $P > 0·05$). Breastfeeding has a protective effect against campylobacter infection and is associated with the presence of specific antibodies directed against campylobacter flagellin.

INTRODUCTION

The development of vaccines against organisms that are able to cause diarrhoea in infants and children in developing countries has been proposed as a short-term preventative measure. A number of bacterial and viral antigens have been shown to give an immune response in humans and protection against experimental infection.

The question of immunity against *Campylobacter jejuni* infection has been the subject of investigation by a number of groups in recent years. In particular, campylobacter flagellin appears to be a major immunogen during infection [1–3] and antibodies produced in part against flagellin may be associated with subsequent homologous protection [4–7]. Breastfeeding has been shown to provide protection against or modify enteric infection [8–10]. Exclusive breastfeeding during the first 6 months of life seems to be a major factor in protection against campylobacter infection in both Africa and Latin America [6, 11, 12]. The role of

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specific antibodies in breast milk in protection against campylobacter infection is less clearly defined.

In 1985, one of us (A.C.) began a prospective study on diarrhoeal illness in a cohort of 75 rural Mexican infants followed longitudinally during the first 2 years of life [13]. As part of this study, breast milk samples from the infants' mothers were collected for the duration of lactation. The availability of these samples provided a unique opportunity to study the specific role of breast milk secretory IgA anti-campylobacter flagellin antibodies in contributing to the protection of breastfed children against campylobacter infection.

METHODS

Study population and patient selection

The study was conducted in the rural village of Lugar Sobre la Tierra Blanca of the State of Morelos, approximately 180 km southwest of Mexico City. The original cohort consisted of 75 children born consecutively between 15 August, 1985 and 26 January, 1986 and studied longitudinally during the first 2 years of life. The details of this study have been previously published [13]. Only two children in the cohort were not breastfed at birth. Of the remaining 73, 42 were breastfed for at least the first year of life. We chose 35 from these 42 children and mothers at random for the present study. Ten children who were not breastfed or weaned during the first month of life were selected as non-breastfed controls.

Samples

Women were visited daily after birth of their child to detect the beginning of milk secretion. After informed consent was obtained, the women were asked to donate samples of breast milk for the duration of lactation. Samples were obtained daily during the first month and then every fortnight for the duration of lactation. They were obtained in the family home, at the same time of day (plus or minus 1 h), by manual expression into sterile containers after cleaning the nipple area and stored at −70 °C. An average of 15 (range 13–17) milk samples from each of the 35 mothers were assayed for IgA and antibodies against *C. jejuni* flagellin. There were at least two milk samples per month of lactation from each woman who breastfed her child. To avoid selection bias, we chose one of the two samples from each month at random. For colostrum, there were at least seven samples from each mother collected during the first week of lactation. The first sample and two additional samples selected at random were tested. Milk samples were shipped to the US on dry ice, thawed and centrifuged in a microfuge for 15 min at 4 °C to separate the lipid fraction. The liquid portion of the sample was removed, aliquoted and stored at −70 °C.

Analysis of diarrhoeal episodes

A sample of faeces from each child was collected every fortnight from birth and, in the symptomatic infant, within 24 h of the onset of diarrhoea. Campylobacter associated diarrhoea was defined as four or more bowel movements in 24 h with a liquid or semi-liquid consistency and/or the presence of blood or mucus in the stools, as detected by the mother or caregiver of the child and confirmed by the examining physician. Pathogenic organisms obtained from stool cultures taken

within 48 h of initiation of symptoms were considered to be associated with disease. Isolation of *C. jejuni* from the faeces of a child without diarrhoea was considered as the beginning of an asymptomatic infection. Culture of faeces was carried out as previously described [14].

Immunoglobulin A assay

Total IgA in breast milk samples was measured by immunoturbidometric analysis using the Roche Cobas Biocentrifugal Analyzer. The SPQ IgA Test System (Atlantic Antibodies, Scarborough, ME) was used to measure IgA levels. The procedure was followed as described by the manufacturer.

ELISA

We used ELISA to measure specific IgA antibodies against the flagellin protein of *Campylobacter jejuni* [2]. The antigen was prepared from *C. jejuni* strain IN1 (heat labile serotype 7) as previously described [1]. Briefly, microwells were coated with 150 μl of stock antigen (10 μg/ml). Antibodies were detected using alkaline phosphatase conjugated goat anti-human IgA (alpha chain specific) (Cappel Laboratories). The breastmilk IgA concentration was adjusted to 6·7 μg/ml and tested by ELISA for activity against campylobacter flagellin as previously described [2]. Each assay was performed in duplicate using test wells (containing antigen) and control wells (containing no antigen). After blanking the wells, the optical density (o.d.) was measured using an ELISA plate reader (Dynatech ELISA Reader MR500) and the final o.d. calculated by subtracting the average control well reading from the test well readings. The o.d. values from each set of mothers' milk were averaged; a value of $\geqslant 0{\cdot}100$ was considered to have detectable antibody (> 3 S.D. above background).

Statistical analysis

One-way analysis of variance (ANOVA) was used to assess statistical differences in episodes of infection between groups with InSTAT v2.04 (GraphPad Software, Inc., San Diego, CA).

RESULTS

The average IgA content for each set of milk samples tested was 69·2 mg/dl (range 31·8–236·4). Only three of the sets had means above 100 mg/dl. IgA levels were higher during the first 3–4 weeks of lactation and levelled off during the remaining 11 months of breastfeeding (Fig. 1).

Twenty-four mothers had breast milk antibodies against flagellin (mean o.d. $\geqslant 0{\cdot}100$) and 11 mothers had no detectable antibody activity (mean o.d. $< 0{\cdot}100$). In most mothers studied, antibody activity, when detected, persisted throughout the year of breastfeeding. Similarly, if antibody was not detected in the initial samples, antibody activity was not detected during the first year.

There were significantly fewer episodes of all campylobacter infections (symptomatic and asymptomatic) in infants whose mothers had antibodies against campylobacter flagellin in breast milk compared with non-breastfed controls (Table 1). Of the children breastfed milk containing antibody, there were fewer diarrhoeal and asymptomatic episodes compared with non-breastfed controls, but statistical significance was only attained in the asymptomatic group.

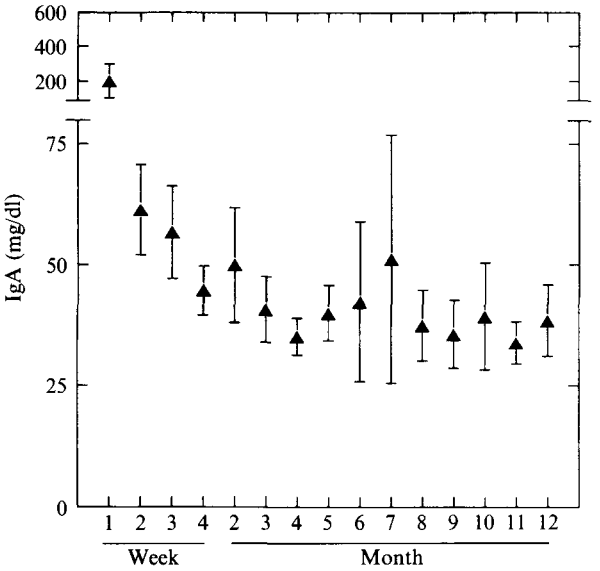

Fig. 1. Immunoglobulin A concentrations in breast-milk during the first year of breastfeeding in the 35 mothers of the study cohort. Each data point represents the mean IgA concentration for the cohort of samples studied. Error bars represent 95% confidence intervals.

Table 1. *Breast milk IgA anti-campylobacter flagellin antibodies and protection of infants against campylobacter infection in the rural village of Lugar Sobre la Tierra Blanca, 1985–7*

		Episodes of campylobacter infection per child per year (±95% confidence interval)		
	No. infants	Diarrhoea	No. diarrhoea	All episodes
Breastfed				
Antibody present	24	0·3 (0–0·7)	0·8* (0·4–1·3)	1·2† (0·6–1·8)
Antibody not detected	11	0·4 (0–0·7)	1·4 (0·6–2·3)	1·8‡ (0·7–3·0)
Non-breastfed controls	10	1·0 (0·2–1·8)	2·3 (1·2–3·4)	3·3 (1·8–4·8)

* $P < 0·05$ compared with non-breastfed controls.
† $P < 0·001$ compared with non-breastfed controls.
‡ NS, $P > 0·05$ compared with non-breastfed controls.

To a lesser degree, infants fed breast milk with no detectable anti-flagellin antibodies also had fewer diarrhoeal and asymptomatic infections than non-breastfed controls, but the numbers were too small to attain statistical significance. A correlation between increasing breast milk antibody level and fewer episodes of campylobacter infection was not observed.

DISCUSSION

This study showed that breastfeeding provided protection against campylobacter infection (symptomatic and asymptomatic combined) and was associated

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with the presence of specific anti-flagellin antibodies in breastmilk. Non-breastfed infants had nearly three times as many infections as infants breastfed with milk containing specific antibody. There were fewer infections in infants fed breastmilk containing no detectable antibody compared with non-breastfed controls but these differences were not statistically significant. It is possible that a protective level of antibody, albeit undetectable by ELISA, was present in the milk and accounted for the lower number of campylobacter infections. Of the 80 episodes of campylobacter infection in all groups examined, only 34 (26%) were associated with diarrhoea. Because of the relatively high proportion of asymptomatic infections and low number of episodes within each subgroup, analysis of a larger series would help to verify trends observed in our study.

Only a few studies have shown a correlation between specific breastmilk antibodies and protection against infection. In a prospective study by Ruiz-Palacios and colleagues [12], breastfed children less than 6 months of age remained free of diarrhoea for a longer time than non-breastfed infants. The number of campylobacter infections per child-year was greater in non-breastfed infants up to 6 months of aged. It was also suggested that breast milk antibodies against campylobacter had a protective role, but the specificity of breast milk antibodies was less well defined, since the antigenic preparations used by these authors were highly heterogeneous. Their glycine-extracted campylobacter surface antigens probably included also a high concentration of flagellin, making their results comparable to ours. A case-control study by Megraud and colleagues [11] suggested a protective role for breastfeeding against campylobacter infection, but the role of specific antibodies in breastmilk was not examined.

The levels of IgA present in breastmilk measured in our study were highest during the first month and remained steady, although at a lower level, during the remaining year of lactation. These results were consistent with studies by Hennart and colleagues [15] and Chang [16] showing a decrease in milk IgA content during the course of lactation. Breastmilk IgA anti-campylobacter flagellin antibodies were recently measured in a study by Renom and colleagues [17] who examined IgA and IgG antibody responses in breast-milk samples from women of French and African origin. Antibodies of the IgA isotype directed against campylobacter flagellin were detected in most samples and antibody activity was highest in colostrum and then dropped in later milk samples. IgG anti-flagellin was detected in only 5 of 120 African milk samples and in none of the French samples [17]. Our studies confirm and extend these observations by showing that for individual women, specific antibodies against flagellin remain fairly constant during the first year of life. We further correlated the presence of breastmilk antibodies with protection, factors not addressed in the study by Renom and colleagues [17]. Antibodies to other enteropathogens such as shigella [18], *Escherichia coli* [19] and *Vibrio cholerae* [8] have also been demonstrated in human milk samples and may account for the protective effect of breast-feeding against these diseases as well [8, 9].

The present study shows that campylobacter flagellin is an important immunogen during human infection. The lack of specific antibodies to flagellin in 11 of 35 women has three possible explanations. First, these 11 women had never come in contact with campylobacter strains. Given the high frequency with which

children are infected with *C. jejuni* during the first 2 years of life in the rural community studied, this is not likely. Second, anti-flagellin antibodies present in breastmilk did not cross-react with flagellar antigens used in this study. We have previously studied the cross-reactivity of IgA antibodies to flagellin proteins [2] and have not observed significant loss of sensitivity using antigen derived from a single strain. Third, it is possible that these 11 women do not have intestinal receptors to surface antigens of *C. jejuni* and are resistant to campylobacter infection. Studies in animals have indicated that the presence of specific receptors to surface antigens of enterobacteria in the intestine, such as the K88 fimbriae of enterotoxigenic *E. coli*, is genetically determined as a dominant autosomal trait (20). When K88 resistant sows are mated with K88 sensitive males mortality due to diarrhoea in their offsprings is very high. This increased mortality is due to the lack of specific antibodies in breast milk against K88 fimbriae in the resistant sows (21). Lack of specific antibodies against these fimbriae in breast milk is a direct reflection of the lack of receptors to these antigens in the intestine of resistant animals. Although genetic studies of this type in humans are not possible, differences in the rate of infection and diarrhoea in human groups living in areas of high risk, sometimes even within the same family, could be explained by this type of genetic factor.

A further point arising from the present findings is the possibility that antibodies in breastmilk might interfere with bacterial surface antigens used as oral vaccines in small infants. A solution to this problem might be the use of campylobacter flagellin as an oral vaccine in pregnant women to increase the concentration of specific antibodies against it in breast milk. Although breast milk, and especially colostrum, have been extensively used to prevent diarrhoea in the newborns of different animals [22], and studies in small infants have clearly indicated the benefits of this therapy in severe infections [23], the possibility of vaccination through breastfeeding has not been given a field test in humans. This approach would encourage the continuation of breastfeeding during the first 6 months of life and protect children against malnutrition. The use of bacterial antigens as oral vaccines in adults, rather than small infants, would be preferable since their long-term immunological effects in infants are unknown.

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