

Cow's milk allergy in breast-fed infants: The role of allergen and maternal secretory IgA antibody

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The protective effect of breast-feeding in allergy prophylaxis remains controversial despite many epidemiologic studies. One reason for confusion could be heterogeneity of breast milk with regard to its protective capacity. To clarify this issue, we studied 57 mother-infant pairs where breast-feeding was the sole source of infant nutrition. Family history and infant symptom scores suggestive of allergic disease were noted. The presence and quantity in breast milk of a prominent food allergen (β -lactoglobulin), total IgA, and IgA antibodies to whole cow's milk and casein were measured serially. Eleven infants had symptom scores highly suggestive of allergic disease. The breast milk from mothers of these 11 infants was found to have lower total IgA ($p < 0.01$) and IgA antibodies to both whole cow's milk and casein ($p < 0.001$ and $p < 0.005$, respectively) than milk from mothers whose infants had few or no symptoms. Beta-lactoglobulin was detectable in 45% of breast-milk specimens at concentrations up to 6.4 ng/ml and persisted up to 3 days after maternal dietary milk exclusion but was unrelated to antibody levels or symptom scores. Inadequate quantities of maternal IgA antibodies to food allergens appear to play a permissive role in development of infantile allergic disease in breast-fed infants and are more closely associated with symptoms than parental atopic history. (J ALLERGY CLIN IMMUNOL 77:341-47, 1986.)

Along with the resurgence of breast-feeding in recent years has come a better understanding among physicians and scientists of the many advantages of breast milk with respect to commercial formulas. However, the role of exclusive breast-feeding in protecting infants at risk for atopic disease from the development of sensitization and morbidity secondary to food allergens remains an unsettled question. Many studies have suggested a protective effect of breast-feeding, whereas other studies have failed to document any benefit.¹⁻⁴ Some studies have even found evidence to support an increased risk of sensitization in breast-fed infants, possibly related to entry of maternally ingested food allergens into breast milk and passively transferred to the infant.⁵⁻⁸ A variety of manifestations, such as colic, diarrhea, eczema, and ur-

Abbreviations used

NS: Normal saline
OD: Optical density
BLG: β -Lactoglobulin

ticaria, have been attributed to the presence of particular food allergens in the maternal diet.

One important component of the breast-milk immunologic repertoire is local immunoglobulin A antibody, which is present almost entirely in dimeric or secretory form.⁹ A number of secretory IgA antibodies to common food antigens have been described in breast milk.^{10, 11} Maternal breast-milk IgA antibodies could passively protect the infant after ingestion by decreasing or preventing antigen entry across the infant's gastrointestinal epithelium.¹² Such antibodies also might play a role in excluding potential food allergens from breast milk, for example, by forming immune complexes that could be phagocytosed by breast-milk macrophages.¹³ Such mechanisms are likely to be especially important in the first months of life when the infant's mucosal defense mechanisms are still developing and inefficient in containing foreign antigens to luminal surfaces.¹²

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We have studied the quantitative and temporal relationship of major cow's milk protein allergens and their corresponding IgA antibodies present in breast milk of mothers of exclusively breast-fed infants to the development of allergic manifestations in these infants in the first months of life. We have found that a lack of maternal IgA antibodies to these allergens plays a permissive role in the development of symptoms suggestive of infantile allergy and appears to be a more decisive factor than genetic predisposition to development of atopic disease as assessed by familial history.

METHODS

Subjects

Fifty-seven nursing mother-infant pairs were entered into the study. Age, parity, gestational and atopic history, general health, medication usage, and dietary content of dairy products were recorded for each mother during the initial interview. Term birth without prenatal, perinatal, or postnatal complications and breast milk as an exclusive source of nutrition for the infant were requirements for inclusion in the study. Mothers were carefully questioned about supplemental formula feeds in the nursery in the immediate postpartum period and excluded from the study unless there was reasonable certainty that such feeds had not been given. Infants were dropped from the study at the time of introduction of formula feeds or solid foods, which was decided independently by each family and their physician.

Allergy history and symptom scores

Each mother completed a questionnaire regarding her own dietary and medication history and the incidence of dermatologic, respiratory, gastrointestinal, and behavioral complaints occurring in herself, the infant, and the father. At the time of each breast-milk specimen collection, an infant symptom score questionnaire was completed. Symptoms were corroborated by interview and, if the history was ambiguous with regard to clinical manifestations, by physical examination. Symptoms were assigned point scores in a weighted scale based on generally accepted associations with atopic diseases. Thus, three points each were assigned for one or more episodes of anaphylaxis, wheezing, urticaria, or eczema; two points for recurrent acute otitis media, serous otitis, or chronic rhinitis; and one point for colic, frequent emesis, frequent diarrhea, a single episode of otitis, sinusitis, or frequent upper respiratory tract infections.

Collection of milk specimens

Colostrals and breast-milk specimens were collected in sterile covered glass jars and promptly frozen until further processing. Mothers were requested to cleanse their nipples with soap and water before expressing milk. Although only a small amount of breast milk (15 cc) was required, most donors submitted 1 to 4 ounces, generally expressed from one breast at a single sitting. An average of 3.5 samples were obtained per mother (range, 1 to 16 specimens per

donor, with most donors giving 3 to 4 samples). Most samples were given after 2 weeks of lactation.

Processing of milk specimens

After thawing, breast-milk fatty layer and cellular elements were removed by two consecutive centrifugations at $9000 \times g$ for 20 minutes at 4° C. The resulting milk, which was clear or translucent, was aliquoted into stoppered plastic tubes and stored at -20° C until analyzed.

Total breast-milk IgA was measured by radial immunodiffusion by use of 7S standards (Calbiochem-Behring, La Jolla, Calif.). Although most (about 90%) of IgA in breast milk is secretory (dimeric, 11S), the proportion of secretory to total measurable IgA remains constant throughout lactation, allowing interpretation of longitudinal results with commercially available 7S radial immunodiffusion plates.¹⁴

Breast-milk anti-cow's milk IgA antibodies

ELISA was developed to semiquantitatively measure IgA antibodies in breast milk directed against cow's milk protein allergens. Whole cow's milk diluted 2.5×10^{-4} in NS or casein, 100 mcg/ml (Sigma Chemical Co., St. Louis, Mo.), in NS were incubated in duplicate wells of polyvinylchloride microtiter plates (Dynatech, Alexandria, Va.) at 37° C for 2 hours. Duplicate control wells received only NS. After aspiration the wells were washed five times with wash buffer (phosphate-buffered saline, pH 7.2, 0.1% gelatin, 0.05% Tween 20, and 0.01% merthiolate). In preliminary experiments it was determined that breast-milk protein functioned as an efficient filler, and no exogenous filler step was required. Thawed breast milk specimens diluted 1:10 in NS were then added and incubated at 22° C overnight. After aspiration and washing five times, goat antihuman IgA-peroxidase conjugate (Sigma Chemical Co.) diluted 1:5000 in wash buffer was added and incubated at 22° C for 2 hours. After aspiration and washing, wells were developed for 20 minutes with 0.04% o-phenylenediamine, 0.012% peroxide in phosphate citrate buffer, pH 5.0. The reaction was stopped with 5 N sulfuric acid and absorbance (net OD) was recorded at 490 nm in a microtiter spectrophotometer (Bio-Tek Instruments, Inc., Burlington, Vt.). Absorbance was determined by subtracting average OD for the two control wells from average OD of each pair of antigen-coated wells. A standard curve was generated on each microtiter plate by use of serial tenfold dilutions of a single high-titer breast-milk specimen, and sample results were expressed as ELISA units by interpolation to the standard curve.

Specificity of antihuman IgA-enzyme conjugate

The specificity of the goat antihuman IgA-peroxidase conjugate used, which did not immunoprecipitate with human proteins in agar gel at the concentration provided, was verified by an inhibition ELISA. Microtiter plate wells were coated with 20 mcg/ml of human-IgA myeloma protein (provided by Dr. Carl Grumet, Stanford University blood

TABLE I. Distribution of infant symptoms

Symptom	High score (n = 11)		Low score (n = 46)	
	n	(%)	n	(%)
Eczema	5	(45)	0	
Hives	3	(27)	0	
Wheezing	2	(18)	0	
Chronic rhinitis	3	(27)	3	(7)
Recurrent otitis	1	(9)	1	(2)
Colic	5	(45)	12	(26)
Sinusitis	3	(27)	0	
Frequent upper respiratory tract infection	2	(18)	0	
Frequent diarrhea	1	(9)	0	
Frequent emesis	0		2	(4)
Otitis media	0		1	(2)

bank) and developed with the conjugate as described above after the conjugate was preincubated for 30 minutes at room temperature with 0.01 to 100 mcg/ml of IgA myeloma, IgG myeloma, polyclonal purified human IgG (Miles Laboratories, Elkhart, Ind.), or IgE myeloma PS (provided by Dr. Lewis Perelmutter, tested to 10 mcg/ml). Preincubation of the conjugate with solubilized myeloma IgA, but not monoclonal or polyclonal IgG or IgE myeloma, inhibited binding to immobilized IgA in a dose-dependent fashion (data not presented).

Detection of bovine antigens in breast milk

Initial efforts to detect cow's milk proteins in breast milk used double-diffusion immunoprecipitation in agar gel. Rabbit antisera to cow's milk proteins were generated by inoculating young, 1 kg white domestic rabbits with either whole cow's milk or purified BLG (Sigma Chemical Co.) by standard immunization methods and schedule.¹⁵ Commercial rabbit antisera to bovine serum albumin (Cappel, Malvern, Pa.) and whey protein (Dako Corp., Santa Barbara, Calif.) were also used. After determining that precipitins that were so detected were probably cross-reacting antigens of human proteins (see Results), an ELISA for detecting presence of BLG, a cow's milk protein without a human counterpart¹⁶ in breast milk, was developed.

First, a monospecific rabbit anti-BLG antiserum was prepared. Serum from hyperimmunized rabbits was passed through a cyanogen bromide-activated Sepharose 4B affinity column (Pharmacia, Piscataway, N. J.) previously saturated with BLG. After elution of unbound rabbit proteins with buffer at neutral pH, rabbit anti-BLG was eluted with low pH buffer. Total protein eluted was monitored by spectrophotometry of collected fractions at 280 nm. Fractions positive by double diffusion and immunoelectrophoresis for immunoglobulin but not other rabbit proteins were combined, lyophilized, and extensively dialyzed against a neutral pH buffer. A portion of the resulting antibody fraction

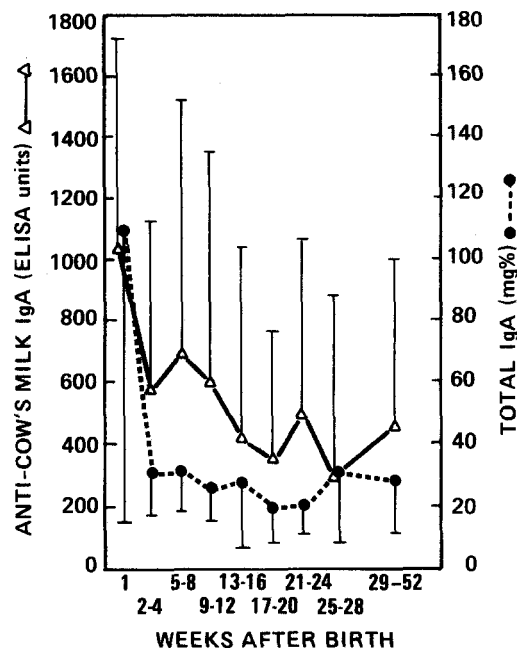


FIG. 1. Total breast-milk IgA (closed circles) and IgA antibodies to cow's milk (open triangles) as a function of lactational duration. Each time point represents mean $\pm$ SD.

was then conjugated to horseradish peroxidase in the presence of glutaraldehyde by the one-step method of Avrameas et al.¹⁷ After an overnight incubation at room temperature, the excess glutaraldehyde was dialyzed against 5 L of buffer with one change of buffer solution. The conjugated antiserum retained monospecific immunoprecipitating activity for BLG at the same dilution as unconjugated antiserum in double-diffusion analysis.

The presence of BLG in breast milk was determined by a sandwich ELISA assay. Anti-BLG was diluted in carbonate buffer, pH 9.6, and then incubated in triplicate microtiter wells at 37° C for 2 hours with control wells receiving buffer only. After aspiration and washing, wells were incubated with filler at 37° C for 4 hours, followed by addition of breast-milk specimens 1:10 in NS and overnight incubation at 22° C. A standard curve with purified BLG at concentrations of 1 pg to 1 mg/ml was added to each plate. After aspiration and washing, rabbit anti-BLG-peroxidase conjugate 1:500 in wash buffer was added and incubated at 22° C for 2 hours. After a final aspiration and wash step, breast milk or BLG sandwiched between anti-BLG and anti-BLG-peroxidase was developed as described above. The concentration of BLG in breast milk was obtained by interpolation to the standard curve on each plate.

Statistical analysis

Chi-square 2 by 2 contingency tables or Fisher's exact test was used for most comparisons. To compare means between groups, the nonparametric Wilcoxon test was used.¹⁸

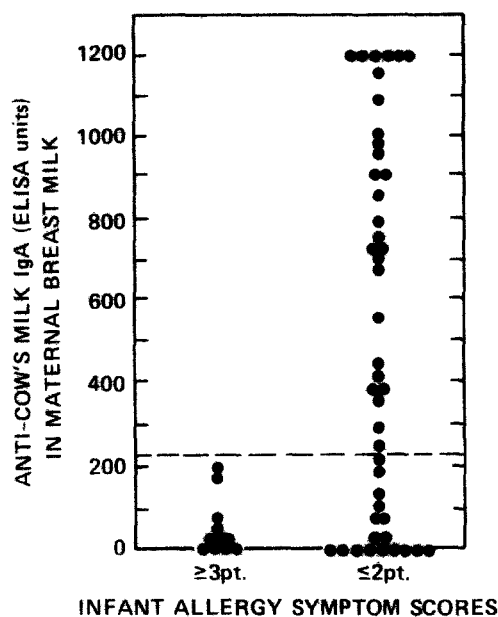


FIG. 2. Breast-milk IgA antibody levels (in ELISA units) in infant groups of high and low symptom scores. Each point represents average antibody content of multiple samples from an individual mother's breast milk (mean 3.5). All 11 of the high-score symptom group received milk with antibody levels below the median value (220 ELISA units, dashed line) versus 17 of 46 in the low-score symptom group (chi-square = 13.7; $p < 0.001$).

RESULTS

Fifty-seven nursing mothers completed questionnaires before introduction of any foods other than breast milk. Twenty-nine infants (51%) were reported to have had at least a single occurrence of a symptom suggestive of a food-related intolerance. Forty-six infants had symptom scores of 2 or less (low-score group), and 11 infants had scores of 3 or more (high-score group). Colic was by far the most commonly reported complaint, accounting for 12 infants with low symptom scores (26%) and five infants with high scores (45%). The distribution of symptoms is illustrated in Table I.

There was no correlation between infant symptom scores and history of atopic disease in either or both parents. A family history of allergy in one or both parents was present in 29/46 (63%) of the low-score group and 8/11 (73%) of the high-score group. There was also no correlation between maternal atopic disease and average total immunoglobulin A content of maternal breast milk. However, elevated infant symptom scores were associated with average total breast-milk IgA levels <20 mg/dl (chi-square = 8.51, $p < 0.01$).

Total breast-milk IgA levels were highest in colos-

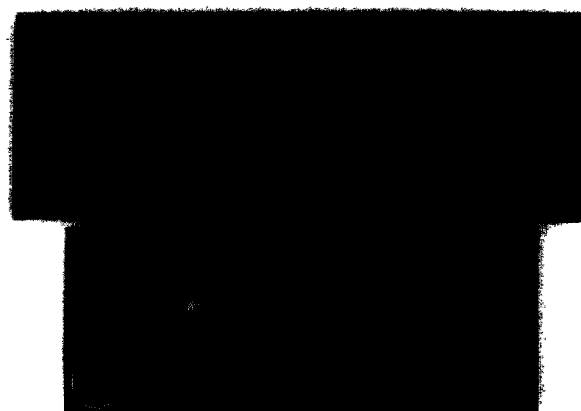


FIG. 3. A, Double-diffusion analysis (top) of rabbit anti-bovine whey (center well, left) and antiserum (center well, right) reacting with (clockwise from top) whole cow's milk, breast milk from a subject on a cow's milk-containing diet, and breast milk from the same subject on a cow's milk-free diet for 1 day, 2 days, 3 days, and 6 days. Precipitins remain unchanged at 1:16 titer for both antisera despite dietary changes. Precipitins were found in breast milk of all 39 individuals tested (120 samples). B, Monospecificity of adsorbed rabbit anti-BLG (bottom). Center wells contain anti-BLG, 10 mg/ml. Antigens (clockwise from top) are, left, purified BLG, 10 mg/ml; BLG, 1 mg/ml; cow's milk, 1:10; casein, lactalbumin, and bovine serum albumin; right, six breast-milk specimens. No precipitins with anti-BLG were found in any breast-milk samples.

trum, fell rapidly during a 1 to 2-week period of transition, and reached stable 7S levels of 20 to 30 mg/dl in breast milk obtained at several weeks to more than 1 year postpartum with little interindividual variation (Fig. 1). In contrast, breast-milk IgA antibody levels to whole cow's milk and casein followed over time demonstrated a much greater variance with some individuals' antibody levels resembling the total IgA pattern and others demonstrating relatively unchanging levels (whether high or low), undetectable levels, increasing levels, or widely fluctuating levels at intervals as little as 1 week. There was thus no discernible relation between total and specific IgA in individual samples. No relationship was found between breast-milk antibody levels and maternal dietary milk intake, since all but one mother ingested at least a pint of cow's milk or its equivalent in dairy products per day during lactation. Furthermore, withholding milk products in two mothers and subsequent milk challenge in one had no effect on breast-milk antibody levels.

Mean breast-milk IgA antibody levels to whole cow's milk and casein were similar in atopic and non-atopic groups of nursing mothers. In contrast, breast-milk IgA antibody levels to whole cow's milk and

TABLE II. Distribution of milk samples by lactational duration between 2 and 36 weeks postpartum in 32 donors whose infants were exclusively breast-fed for 6 months and examined at 1 year

	Weeks postpartum								
	2 to 4	5 to 8	9 to 12	13 to 16	17 to 20	21 to 24	25 to 28	29 to 32	33 to 36
S × Sc									
<3	2	0	4	8	6	12	4	5	3
≥3	0	1	4	3	2	1	5	1	3

S × Sc = infant symptom score.

casein were significantly lower in mothers of infants with high symptom scores than in mothers of infants with low scores (Fig. 2). Anticow's milk IgA levels were below the median (approximately 200 ELISA units) in all 11 of the high-score group versus 17 of 45 in the low-score group (chi-square = 13.7, $p < 0.001$). Similarly, anticasein IgA levels were below the median in 10 of the high-score group versus 17 of the low-score group (chi-square = 9.6, $p < 0.005$). Although samples from mothers of the high-score symptom group were obtained on an average of 4 weeks later than those from the low-score symptom group (21 weeks versus 17 weeks), this difference was not statistically significant. Moreover, when breast-milk samples from both infant groups were matched for lactational duration, the same decrease in antibody levels in mothers of infants of the high-score symptom group was found.

To verify further the apparent relationship of lower milk-antibody levels to higher scores in symptoms, we reanalyzed results after (1) excluding all donors and specimens obtained earlier than 2 weeks or later than 36 weeks postpartum, (2) including only infants exclusively breast-fed at least 6 months whose symptoms appeared during breast-feeding, and (3) including only those with follow-up including examination at 1 year of age. This reanalysis reduced the study population to 32 mother-infant pairs; 10 high-score and 22 low-score infants remained in this group. The distribution of milk samples as a function of lactational duration was similar in the high- and low-score groups (Table II). Breast-milk anticow's milk IgA antibodies remained lower in the high-score symptom group than in the low-score symptom group. (<100 ELISA units in 8/10 of the former versus 6/22 of the latter, $p = 0.02$ by Fisher's exact test).

In contrast to earlier studies indicating that cow's milk proteins are present in sufficient amounts to yield specific precipitin reactions in double-immunodiffusion reactions,^{19, 20} we observed that precipitins present in breast milk with antisera to whey and bovine albumin represent cross-reacting antigens present in

human milk and bovine analogues (Fig. 3). We therefore developed an ELISA for detection of BLG, a protein not present in human milk.¹⁶ BLG in test samples was quantified by interpolation to a standard curve generated with serially diluted BLG on each plate. The interassay coefficient of variation was 10% by use of BLG concentrations between 100 pg/ml and 1 ng/ml on standard dilution curves (Fig. 4). The sensitivity of the assay, defined as average background absorbance plus 2 SD, was ~100 pg/ml. The specificity of the assay was demonstrated by the use of two heterologous antigens (casein and ovalbumin) that did not increase nonspecific binding of BLG at concentrations up to 1 µg/ml.

BLG was found in 24/54 breast-milk specimens (45%) at concentrations up to 6.4 ng/ml, with levels >200 pg/ml in 24% and >1 ng/ml in five samples. The presence or quantity of BLG was unrelated to breast-milk antibody levels, infantile symptom scores, or maternal atopic history.

The persistence of BLG in breast milk after cessation of cow's milk ingestion was evaluated in a woman who had a low level (9 ELISA units) of anticow's milk IgA antibody. Her infant had colic that resolved within 24 hours after maternal milk intake was stopped and did not recur while she remained on a milk-free diet. BLG in breast milk was detected at 600 pg/ml after she was again placed on an unrestricted diet, persisted for 3 days after removal of dietary milk, and then was undetectable (<100 pg/ml) while she remained on the milk-free diet (Fig. 5).

DISCUSSION

Prior studies have established the presence of breast-milk IgA antibodies to food proteins, but their clinical significance has not been assessed. McClelland and McDonald²¹ found colostral antibodies to casein in 13 of 25 women with a passive hemagglutination test. Hanson et al.¹⁰ by use of an enzyme-linked immunosorbent assay, found breast-milk IgA antibodies to both casein and BLG in all 20 sam-

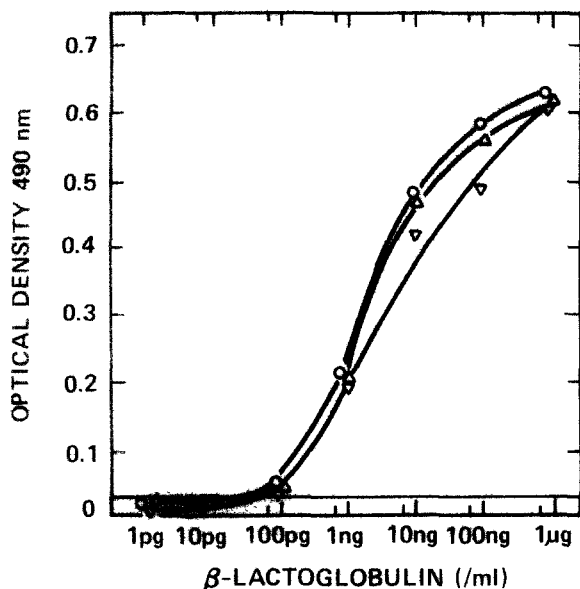


FIG. 4. Interassay standard curves on three microtiter plates for BLG quantitation. Increasing concentrations of purified BLG are captured on immunoadsorbed monospecific anti-BLG and developed with anti-BLG-peroxidase conjugate. Coefficient of variation for all points between 100 pg and 1 μ g/ml of BLG was 9.7%.

ples studied. Our methodology, which is similar to that of Hanson et al., detected breast-milk IgA to casein or whole cow's milk in 84% of donors. By comparing maternal breast-milk IgA antibody levels to symptoms in exclusively breast-fed infants, we found that infants with likely atopic disease (high symptom scores) received milk with lower levels of antibodies to cow's milk protein than infants without clinical manifestations (Fig. 2). This finding may explain, at least in part, the confusing and controversial data regarding the efficacy of breast-feeding in preventing the expression of atopic disease in infancy.

In the gastrointestinal tract, secretory IgA prevents the mucosal penetration and systemic absorption of foreign antigens.¹² IgA in breast milk is believed to be produced by local plasmacytes derived from B cells that have migrated from the gastrointestinal tract submucosa.¹³ A deficiency of IgA at one mucosal site, e.g., in breast milk, may thus reflect a more widespread condition affecting other areas, such as the gut. Maternal deficiency of IgA in the intestinal tract with systemic absorption of food proteins could result in their entry into breast milk and sensitization of the infant.²³⁻²⁵ It is not known if such an enteromammary defect or other mechanisms underlie the deficiencies in food-specific breast-milk IgA antibodies that we observed in some of our study subjects.

Our findings further suggest that nursing mothers

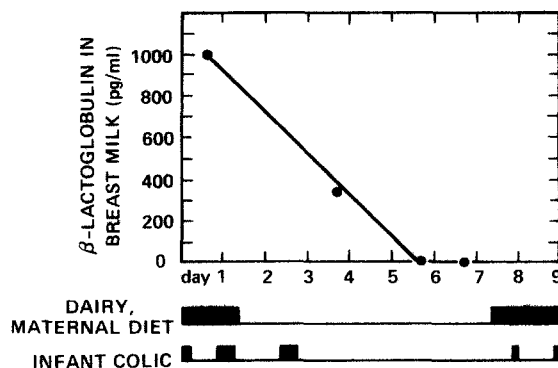


FIG. 5. Persistence of BLG in breast milk for at least 3 days after reintroduction of cow's milk into maternal diet. Note correlation of infantile colic with maternal milk intake and presence of BLG in ingested breast milk.

with low levels or absence of breast-milk IgA antibodies to major allergens represent a high-risk group for whom appropriate dietary restriction and environmental allergen avoidance measures might prove valuable in preventing infantile allergy. If mucosal immunity is linked at different sites by common antigen-processing and antibody-producing mechanisms, measurement of such antibodies in easily obtainable secretions such as saliva could provide useful information before parturition. Obviously, such speculation requires further study.

The presence of an important cow milk's allergen (BLG) in breast milk was unrelated to the level of anticow's milk antibody. Technical difficulties prevented us from directly measuring breast-milk anti-BLG IgA antibody, but the presence of BLG in nearly half the samples tested indicates that exposure of breast-fed infants to food antigens ingested by the mother is a common occurrence. We do not know as yet whether BLG or other food proteins present in breast milk are in free form or complexed to local antibody. Our findings on the prevalence and quantity of BLG penetration into breast milk are in close accord with those recently reported for other food proteins by Kilshaw and Cant.²⁶

It is well-known that other factors play a significant role in the development of infantile allergic disease. The most extensively studied of these is genetic predisposition to atopy. We found that parental history of atopic disease was less important a factor with respect to infant symptoms than maternal breast-milk IgA antibody level. Objective determinants of the infantile genetic risk factor, such as cord or early life infant serum blood total IgE level, are more specific for atopic disease and probably provide stronger predictive information.²⁷⁻²⁹ We are prospectively investigating this possibility by correlating infantile allergic

symptoms with IgE levels and objective assessment of sensitization as determined by serum IgE antibodies to common allergens. Recently, the appearance of food-specific IgE antibodies in serum of exclusively breast-fed infants before the onset of clinical allergic disease has been reported.³⁰ Finally, it is important to note that other environmental factors such as early viral infections undoubtedly also partly determine the onset and nature of allergic disease in infancy.^{31, 32} Our conception of the various risk factors postulates a permissive, or necessary but insufficient, role of inadequate maternal passive antibody protection in the development of infantile allergic disease caused by food allergens.

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