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The Effect of Caloric Supplementation on Selected Milk Protective Factors in Undernourished Guatemalan Mothers

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ABSTRACT. The level and avidity indices of specific antibodies against tetanus toxoid, *Escherichia coli* O6 and a pool of 10 common *E. coli* O antigens, as well as the concentration and daily output of lactoferrin and total secretory IgA (SIgA), were evaluated in the milk of moderately undernourished mothers who were in a random blind design divided into two groups and given different caloric supplementations. Group A received a high caloric supplement (500 kcal/d), and group B received a low caloric supplement (140 kcal/d). Determinations were done using ELISA in various modifications, except for lactoferrin, which was quantified by single radial immunodiffusion. The avidity indices were investigated as an evaluation of the antibody quality. In all the parameters evaluated, the only difference found between the two groups at the end of the supplementation period was in the content of total SIgA, which was lower in group B, both in concentration and daily output. However, the SIgA remained within the normal range. Increases as well as decreases in the levels of specific IgA antibodies occurred within both groups. Avidity was decreased in group B only against one of the antigens tested. We conclude that moderate undernutrition does not impair the levels of milk antibodies, and supplementation does not enhance them but prevents the decrease in the content of total milk SIgA. There is a suggestion that the avidity of certain antibody specificities could be hampered. (*Pediatr Res* 34: 217–221, 1993)

Abbreviations

SIgA, secretory IgA
KSCN, potassium thiocyanate

Several studies have shown the importance of human milk for the overall development in the young infant, not only by providing a well-balanced amount of nutrients, including large quantities of proteins (1, 2), but also by protecting against infections (3–5). SIgA is the main Ig in human milk and one of the major factors responsible for the protective effect (5–7). Lactoferrin is another protein present in human milk that, synergistically with SIgA, has a bacteriostatic effect against several bacterial pathogens *in vitro* (1, 8).

Malnutrition, including protein and protein-energy deficiency, affects the immune response and therefore might aggravate susceptibility to and the outcome of some infections (9, 10). Cell- and antibody-mediated mechanisms (11, 12) as well as macrophage function (13) can be affected. Undernutrition has been shown to impair the SIgA system in children (14, 15), but this effect has not been clearly demonstrated for the SIgA in human milk. Previous studies on the SIgA quantity in breast milk of undernourished mothers have been cross-sectional and regularly included limited numbers of subjects (16). In general, the findings reported indicate that malnourished women are able to produce and transfer milk-specific SIgA antibodies (17–19) and total SIgA (16, 20, 21) to their infants in amounts comparable to those of well-nourished mothers. On the other hand, significantly decreased levels of colostral IgA in undernourished mothers have also been reported (22).

In addition to the investigation of levels of total milk SIgA and specific IgA antibodies, which have thus far been used to evaluate the immunologic quality of human milk, in the present study, we measured avidity, or relative affinity, as an estimation of the functional quality of the antibodies. This term is defined as the summation of forces involved in the multivalent antigen-antibody interaction (23). Data available demonstrate the superiority of high-affinity antibodies over low-affinity ones in certain biologic reactions (24). Here, we report the findings of the first controlled, longitudinal study to explore whether caloric supplementation during lactation affects the formation of milk SIgA, specific milk IgA antibodies, and lactoferrin in the human.

MATERIALS AND METHODS

Subjects. The subjects of the study were undernourished mothers living in the poorest sections of Quetzaltenango, in the western Guatemalan highlands, and its surrounding rural areas. The number of women in this study was 67 of the original 102 (González-Cossio *et al.*, unpublished manuscript). The characteristics of the included and the remaining ones were the same. By mean, the subjects included weighed 41.8 ± 3.8 kg in group A and 43 ± 2.9 kg in group B, were 143.1 ± 5.0 cm tall in group A and 143.6 ± 5.0 cm in group B, and had a calf circumference of 29.5 ± 1.4 cm in group A and 29.5 ± 1.2 cm in group B. Women with a low calf circumference during the last trimester of pregnancy were invited to the study and recruited, after informed consent. Calf circumference was the most sensitive indicator of undernutrition in women from a similar environment (25, 26).

The women were randomized in a double-blind design into one of two groups, A and B. Starting on the 5th wk postpartum, mothers in group A were given high-caloric cookies (250 kcal/U), while mothers in group B received low-caloric cookies (70 kcal/U); both types of cookies looked identical. The supplement was two cookies per day, Monday to Saturday, delivered to the mothers' homes by community distributors. Supplement consumption was monitored daily by direct observation. The women

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were advised to consume the supplement in addition to their usual diets. The overall difference in caloric intake attributed to the supplement was 269 kcal/d between the two groups. The original protocol was planned to evaluate the effect of supplementation of undernourished lactating women on their milk production (González-Cossio *et al.*, unpublished manuscript).

Milk samples. The samples were obtained at 5, 10, 20, and 25 wk postpartum, *i.e.* 0, 5, 15, and 20 wk after initiation of the supplement. The procedure was as follows: mothers were invited to the study clinic where they spent 28 consecutive h. During this period, the 24-h milk volume was estimated by the test-weighing method, and 2 h after the last feeding, the left breast was totally emptied by extraction with mechanical or battery-operated breast pumps. The milk samples were placed in glass containers, frozen at −20°C and transported to the central laboratory at the Institute for Nutrition of Central America and Panama. The frozen specimens were thawed and homogenized by sonication. A frozen aliquot was taken for this study, defatted, frozen again, and not thawed until its analysis.

In total, 1718 analyses were done on 243 samples, 887 on group A and 831 on group B. The four consecutive samples collected were not always available from each mother. For the studies of the milk antibodies to the *Escherichia coli* O antigen pool, only those who had all the four determinations were selected. The mean number of samples investigated at different times during lactation in group A was 28 (range 16–39) and in group B was 23 (range 15–31). In SIgA and lactoferrin determinations, only the samples at wk 5 and 25 were investigated.

Antigens. To evaluate the effects of undernutrition on milk antibodies, we selected two heat-extracted lipopolisaccharide antigens (27), from *E. coli*, O6 and a pool of O1, O2, O4, O6, O7, O8, O18, O22, O25, and O75 (abbreviated as "10 pool") and the tetanus toxoid, protein antigen, (kindly provided by Dr. I Heron at the State Serum Institute, Copenhagen, Denmark).

Determination of levels and avidity indices of IgA antibodies. The levels of specific IgA to the above antigens were measured using the ELISA (28). Quantifications were done in 96-well Dynatech Immunolon II plates (Alexandria, VA) coated with 100 μL of the antigen in each well. The *E. coli* antigens were diluted before coating in PBS, pH 7.2, to a concentration corresponding to about 2×10^9 bacteria/mL; the tetanus antigen was diluted to approximately 6 μg/mL. The plates were left overnight at room temperature in a moist chamber, then washed three times with PBS + 0.05% Tween 20 (Merck Darmstadt, Germany). The samples to be analyzed were thawed and diluted 2-fold, beginning with 1:8. One hundred mL of each of the dilutions were added in duplicate wells. Readings were done at 405 nm in a Titertek Multiscan MCC/340 (Flow Labs, Ayrshire, Scotland), usually after 50 min, but all the OD were determined by extrapolating to 100 min. The reading of the samples was expressed in percentage of a human milk standard pool.

The relative affinity, or antibody avidity index determinations were done by a modification of the ELISA, using KSCN for the elution of antibodies (29). The samples were administered in duplicate wells at the optimal dilution found at the titer determinations. KSCN diluted in PBS-Tween solution was added and incubated at seven different molarities, ranging from 0.25 to 4 M. The plates were read at 100 min. A standard milk sample was always run in each plate. The molarity of KSCN required to elute 50% of the antibodies was taken as the measure of relative antibody affinity, or avidity index (30).

Quantification of SIgA and lactoferrin. The method used was a modification of the ELISA assay described for quantification of SIgA (28, 31), where soft polyvinyl chloride plates (Dynatech) were directly coated with anti-F(ab')$_2$ α-chain, (Dakopatts, Copenhagen, Denmark). The conjugate was an antisecretory component (Seward Laboratories, London, UK), linked to alkaline phosphatase (Boehringer-Mannheim; Mannheim, Germany). Samples were read against a known standard (human milk pool, with a concentration of 3.0 g/L). The normal levels reported for this protein are between 0.5 and 1.0 g/L (1, 32).

The single radial immunodiffusion test (33) was used with rabbit antihuman lactoferrin (Dakopatts AS, Copenhagen, Denmark). A human lactoferrin standard with a concentration of 10 g/L (Serva, Feinbiochemica, Heidelberg, Germany) was used to plot the reference curve for each plate. The normal concentration reported in milk for healthy mothers is 1 to 3 g/L (1, 32). The determinations for SIgA and lactoferrin were done only on the milk samples at wk 5 and 25.

Statistical analysis. The treatment effect within each group, *i.e.* a central tendency value different from zero was evaluated using the Wilcoxon signed rank test. The evaluation was performed of the individual differences, *i.e.* the value at the last determination (25 wk postpartum) subtracted with the value for the first determination (5 wk postpartum) for each of the groups. The same approach was used to evaluate the effect of supplementation between other weeks (wk 10 to 5, 20 to 5, 20 to 10, 25 to 10, and 25 to 20) (34).

To test a difference between the two supplemented groups (A and B), the Mann-Whitney U test was used. This method was applied to the individual differences (for wk 25 and 5 postpartum, as explained above) of one group against the other. Only two-tailed tests were used (34).

The groups were compared per individual differences. Still, medians are provided in the tables to show the weight of the balance in the groups at different times.

RESULTS

Lactoferrin concentration and daily output. The concentration of lactoferrin in milk did not differ between the two groups of women, either at the beginning or at the end ($p > 0.05$) of the supplementation period, but decreased significantly at wk 25 ($p < 0.05$; Table 1). Both groups of mothers also had similar daily outputs of lactoferrin at wk 5 and 25 and, despite the decrease observed in lactoferrin concentration during the study period, the daily output remained unchanged (Table 1).

SIgA concentration and daily output. The concentrations of

Table 1. *Concentration of lactoferrin and total secretory IgA in g/L measured at wk 5 and 25 postpartum and statistical analysis of effect of supplementation**

			Wk of lactation					Supplementation effect	
			5			25		Within group‡	Between groups§
Protein	Group	*n*	Mean	SD	*p*†	Mean	SD	(*p*)	(*p*)
Lactoferrin									
g/L	A	31	1.37	0.86	NS	0.98	0.83	<0.05	NS
	B	25	1.46	0.65		1.07	0.48	<0.05	
g/24 h	A	31	0.95	0.68	NS	0.77	0.59	NS	NS
	B	25	0.94	0.39		0.84	0.38	NS	
SIgA									
g/L	A	30	0.93	0.34	NS	0.92	0.51	NS	<0.05
	B	25	1.00	0.35		0.75	0.33	<0.05	
g/24 h	A	30	0.63	0.26	NS	0.73	0.46	NS	<0.05
	B	25	0.66	0.25		0.57	0.26	NS	

* The 24-h output of the proteins is also shown. A, high-calorie—supplemented group; B, low-calorie—supplemented group.

† Value for the two groups at the initiation of the supplementation.

‡ The effect of the diet within each group is the individual difference during the 20 wk of supplementation, *i.e.* from wk 5 to 25 postpartum (Wilcoxon signed-rank test), with an expected central location value of zero.

§ The effect of the diet compared between the two groups (Mann-Whitney U test): two-sample central location test. The significance value *p* is given for both statistical analyses.

SIgA in groups A and B were comparable ($p > 0.05$) at the initiation of the study. In group A, the concentration did not vary between wk 5 and 25, but group B showed a significant decrease at wk 25 ($p > 0.05$, Table 1). At the end of the supplementation period, group B showed a significantly lower value than group A (Table 1).

The daily SIgA output did not differ between groups A and B at the initiation of the supplementation period ($p > 0.05$) but became significantly lower in group B at wk 25 ($p < 0.05$), although it remained within the normal range.

Antibody levels. The levels of milk IgA antibodies against *E. coli* O6 showed fluctuations between the various sampling weeks. The p values for the fluctuations along the weeks are not shown, only those in wk 5 and 25 (Table 2), *i.e.* at the onset and at the end of the supplementation period. The median values and ranges for all the weeks are shown in Table 3. For the high calorie supplemented group (group A), there was a significant increase from wks 10 and 20 to wk 25. However, no effect of the supplementation, measured as the difference between wk 5 and 25, was found within this group (Table 2, $p > 0.05$). The low calorie–supplemented group (group B), also showed a significant increase in the antibody levels between wk 10 and 20, 10 and 25, and 20 and 25 (all p values <0.05, data not shown), as well as between wk 5 and 25 (Table 2). There was no significant difference in the levels of *E. coli* anti-O6 between the two groups at any of the times tested postpartum (Table 2).

In group A, the anti-*E. coli* 10 pool levels showed significant decreases between wk 5 and 10 and between wk 5 and 20; in group B, a decrease in antibody levels was found only between wk 5 and 10.

For the tetanus antibody levels, the initial values were comparable in the two groups. There was a significant increase in the levels in group A from wk 5 to 25 (Table 2). A significant increase in the levels was also observed for wk 10 to 20 and 10 to 25. In group B, a significant increase was only observed between wk 10 to 25 (p values not shown).

No significant difference was obtained between the two groups for any of the antigens investigated (Table 2).

Table 2. *Statistical analysis of effect of supplementation within each group and between groups A and B for IgA-specific levels and avidities**

Antigen	Antibody	Group	n	Supplementation effect: Within group† (p)	Supplementation effect: Between groups‡ (p)
E. coli O6	Level	A	31	NS	NS
		B	27	<0.05	
	Avidity	A	30	NS	NS
		B	24	NS	
E. coli 10 pool	Level	A	16	NS	NS
		B	15	NS	
	Avidity	A	16	NS	NS
		B	15	<0.05	
Tetanus	Level	A	29	<0.05	NS
		B	25	NS	
	Avidity	A	26	NS	NS
		B	19	NS	

* The values taken into account are the differences between the values at wk 5 and 25 (*i.e.* at the onset and at the end of the supplementation period).

† The effect of the diet within each group is the individual difference during the 20 wk of supplementation, *i.e.* at wk 5 and 25 postpartum (Wilcoxon signed-rank test), with an expected central location value of zero.

‡ The effect of the diet on the two groups is analyzed comparing individual differences at wk 5 and 25 of lactation (Mann-Whitney U test): two-sample central location test.

Antibody avidities. The avidity of the antibodies to the three antigens studied showed no significant effect relating to the two different supplementation levels, but there was a tendency to an increase with time, especially for group A. In group B, the avidity indices for the tetanus antibodies also tended to increase, but those against the *E. coli* 10 pool became significantly lower at wk 25 compared with wk 5 ($p < 0.05$, Table 2). A decrease was also observed from wk 10 to 20 ($p < 0.05$). The avidity indices for the IgA anti-*E. coli* O6 showed no significant differences between the various weeks of sampling and between the groups A and B (Table 2). Median values and ranges are shown in Table 3.

DISCUSSION

The milk lactoferrin levels between the two groups of undernourished mothers were comparable to the ones reported previously, including the decrease during the first 12 wk of lactation (32, 35). Decreased SIgA concentration and daily SIgA output were the only findings associated with low- compared with the high-caloric supplementation treatment, although in both groups the women showed SIgA levels within the normal range (1, 32). The values of SIgA reported here are comparable to those found among Guatemalan rural women at 1 mo of lactation (36). In the latter study, the mean concentrations observed at 3 mo of lactation tended to be lower than those at 1 mo, but the tendency did not attain statistical significance, possibly because of small sample size. It is clear that, in the present study, the group of women who received a low-caloric supplement had a significantly lower quantity of SIgA after 25 wk of lactation. This indicates that caloric supplementation of undernourished lactating mothers prevents a deterioration of the milk SIgA output.

We noted two significant differences in the antibody levels. Group A showed what might be regarded as a positive treatment effect on the antibody production against the tetanus toxoid. The milk antibodies against this antigen most likely originate from memory cells, stemming mainly from previous vaccination, although a study in India has suggested that "natural" intestinal exposure to tetanus may induce antibody responses (37). The other difference was observed in group B, in which a large increase in the antibody production against *E. coli* O6 occurred at the end of the supplementation period. This effect is not clearly associated with nutritional treatment but may be related to reinfection of *E. coli* O6. A selective exposure to the O6 antigen in group B, however, is unlikely because the mothers of both groups lived in the same geographic area. Independent of the triggering factor, it was possible for the undernourished mothers with the low-calorie supplementation to increase their levels of antibodies in spite of their deficient nutritional status.

During the supplementation period, there were significant fluctuations in some of the specific antibody levels. The fluctuations were noted for antibodies to all three antigens studied and may be due to several factors beyond the nutritional situation, including varying antigen exposure, possibly adding polyclonal stimulation by O antigens, variations in milk volume, the state of hydration of the mother, or possibly effects of intercurrent infections. Antibody levels showed no significant differences between the two supplemented groups. A similar pattern of fluctuation of milk IgA antibodies was observed also in earlier studies (36).

The antibody avidity indices showed more stable and constant results during the supplementation period than the antibody levels. We found no correlation between the antibody levels and the avidity indices of those antibodies. The only significant difference in avidity was a decrease in group B against the *E. coli* 10 pool antigens. We consider the probability of exposure to these antigens to be high, as they represent a mixture of 10 common bacterial serotypes. The avidity index for antibodies against this antigen pool was lower in both groups (Table 3) than the levels reported by Roberton *et al.* (30) in Pakistani (2.5 M)

Table 3. *Median levels, avidity index values, and ranges of IgA antibodies in milk samples against E. coli O6, E. coli 10 pool, and tetanus toxoid antigens for the different weeks evaluated**

			Wk of lactation											
			5			10			20			25		
Antigen	Antibody	Group	*n*	Median	Range	*n*	Median	Range	*n*	Median	Range	*n*	Median	Range
E. coli O6	Level	A	39	20.9	5.5–47.6	34	15.3	4.3–69.9	30	17.3	5.5–103.8	31	24.2	4.4–65.5
		B	31	20.2	5.3–81.8	26	14.6	4.0–60.0	25	27.2	5.3–61.0	27	33.5	8.7–99.3
	Avidity	A	38	1.6	0.4–3.3	30	1.6	0.6–3.6	29	1.6	0.3–4.0	31	1.5	0.5–4.0
		B	27	1.2	0.5–2.5	24	1.4	0.7–3.5	24	1.3	0.4–2.6	27	1.1	0.6–2.6
E. coli 10 pool	Level	A	18	44.6	15.2–82.5	18	46.0	15.5–69.7	16	43.0	17.4–81.5	16	43.0	14.7–80.0
		B	17	48.0	13.0–96.4	17	41.9	16.1–83.5	15	30.2	15.5–77.7	15	34.2	14.6–87.7
	Avidity	A	18	2.0	0.8–3.3	18	1.9	1.0–2.8	16	2.2	1.3–4.0	16	2.0	1.0–3.1
		B	17	1.8	0.9–2.6	17	2.0	0.7–3.0	15	1.3	0.6–2.6	15	1.4	0.5–3.4
Tetanus	Level	A	38	44.3	25.1–106.0	33	38.1	16.9–106.5	26	51.9	19.1–97.8	29	46.9	21.6–103.3
		B	29	49.5	25.6–95.6	23	48.1	27.8–86.6	22	49.8	27.4–86.7	26	54.0	33.3–99.6
	Avidity	A	35	1.6	0.5–4.0	31	1.3	0.4–4.0	27	1.7	0.6–4.0	27	1.4	0.7–4.0
		B	26	1.4	0.6–3.0	22	1.2	0.5–3.3	21	1.7	0.5–3.3	22	1.8	0.5–4.0

* A, high-calorie–supplemented group; B, low-calorie–supplemented group. The level of IgA-specific antibodies is expressed in percentage of a human milk standard pool; the avidity index values are expressed as the molarity of KSCN required to elute 50% of the antibodies.

and Swedish (3.1 M) mothers. The exact reasons for the lower avidity among the Guatemalan mothers are unknown. On the other hand, the median avidity results (Table 3) for anti-*E. coli* O6 were comparable to the ones obtained from well-nourished Costa Rican (1.3 M) and Swedish (1.4 M) mothers in a recent study (38). The basis for the observed fluctuations between the two groups in the avidity indices for the antibodies tested remain unclear to us, although it may relate to geographic differences in exposure to the *E. coli* serotypes used in the 10 pool. We feel that it is important to evaluate the effect of antigenic stimulation during lactation for a better understanding of the relationships between milk antibody levels and their avidities.

In conclusion, we found that SIgA was higher in the high-calorie–supplemented group. We were not able to show an improvement in the antibody response to specific antigens. The avidity of milk antibodies and their expression were not improved with the caloric supplement, but supplementation prevented the decrease in the content of total SIgA in milk. The fact that specific antibody levels were not hampered and could even increase in spite of the undernutrition may be especially important, because it has been shown that protection in the breast-fed infant against cholera (5), ETEC (7), and *Campylobacter* (4) is related to the specific SIgA antibody levels in the mother's milk. The relevance of milk-mediated protection may be illustrated by the fact that breast-feeding shows a striking protection against the two infections that are among the most common causes of death in early life in a poor community: diarrhea and neonatal septicemia (3, 39).

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