

Human α -lactalbumin and bovine β -lactoglobulin absorption in infants

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We investigated gut permeability to human α -lactalbumin (ALA) and bovine β -lactoglobulin (BLG) in 20 infants from birth to 8 months or until weaning, before which they were on a strictly cow's-milk-free diet. We measured the proteins with a sensitive, solid-phase, double-sandwich immunofluorometric assay. Median (range) levels of serum ALA on days 3–4 after birth, and at 1 and 2 months of age were 31 (12–225), 6 (0–55), and 2 (0–16) $\mu\text{g/l}$ serum per g ALA given per kg body weight, respectively. At 3, 5, and 8 months of age, only trace amounts of ALA were found. One week after weaning, serum BLG was found in 5/13 infants (38%) and at 2 weeks in 3/14 infants (21%), with median concentrations of 7 and 4 $\mu\text{g/l}$ serum per g BLG given per kg body weight, respectively. No ALA could be detected in any of these samples. In absorption of ALA, the four infants who had allergic symptoms did not differ from those without symptoms. Thus, systemic absorption of ALA and BLG does occur in infants. Absorption of ALA is greatest after birth, when 3×10^{-4} (median) of the given antigens are absorbed, but absorption decreases rapidly. The gut may often be transiently permeable to BLG when cow's-milk-based formula is started.

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The intestinal wall of the young infant is permeable to macromolecules of food, including both isologous and foreign proteins (2, 12, 15, 17, 20, 21). The amounts involved are large enough to provoke food allergy and induce the appearance of antibodies of the different immunoglobulin classes. Both these responses are features of early infancy; levels of antibodies against food antigens later decline, and the food allergies mainly resolve during childhood (5). Food antigens are also secreted in breast milk (1), and these may influence the immune response even in an infant that is exclusively breast-fed (18).

The permeability of the intestinal epithelium to proteins depends on both nonspecific factors and the specific immune response. Nonspecific factors, such as efficiency of intestinal proteolysis, acidity of the stomach, motility of the intestine, and quality of mucus, regulate the extent to which the gut wall is permeable to macromolecules. These factors are not fully developed at birth. Proteins in infant formulas and other foods provoke specific immune responses, which prevent the penetration of antigenically active components of the food and so produce oral tolerance (6).

The aim of the present study was to assess gut

permeability in 20 infants during the first 8 months of life and the possible effects of allergy, immunoglobulins – especially IgA in secretions – and breast-milk β -lactoglobulin (BLG) on permeability. We investigated gut wall permeability to two common food proteins, human α -lactalbumin (ALA), a major protein component of breast milk which elicits no immune response, and bovine BLG, an abundant and highly antigenic protein in cow's milk (CM) whey (22).

Material and methods

With the informed, written consent of the parents, 20 full-term newborn infants, with birth weights appropriate for gestational age, were recruited into the study on days 3–4. All infants were healthy and free of perinatal infections, and enteral feeding was started on the first postnatal day. One of the authors (M.K.) examined the infants postnatally and at 1, 2, 3, 5, and 8 months of age. They were also seen immediately before the planned weaning, and 1 and 2 weeks after it. Before weaning, the mothers were advised to adhere strictly to a CM-free diet. Fruit, including berries, and vegetables were introduced at

3 months, and meat, fish, eggs, and cereals at 5 months. Infants attended well-baby clinics, but any medical care needed during the study was provided by M.K. Not all the mothers and infants were able to make every planned visit.

At each visit the infant was given a measured dose of breast milk and, after weaning, a meal consisting of an equal amount of adapted formula and previously frozen breast milk either from its own mother or from a pool of banked breast milk. For 4 h before the test meal, the infants had fasted. One hour after the beginning of feeding, a venous blood sample (2 ml) was drawn, and a saliva sample (1 ml) was collected with a soft, plastic pasteur pipette after stimulation of salivation with three citric acid crystals. The mothers brought an expressed breast-milk sample and a sample of the child's feces. If the child had an acute infection, the visit was postponed for 1 week.

Any atopic manifestations were recorded; criteria for atopic dermatitis were set according to Hanifin (9).

Serum was stored at -20°C . Breast milk was stored at -20°C and defatted before analysis by centrifugation at 10 000 g. Fecal samples were lyophilized, eluted with PBS, and centrifuged at 10 000 g; the supernatants were stored at -70°C . Saliva was mixed with 0.5% bovine serum albumin (BSA) (Wilfrid Smith, Middlesex, UK) in PBS, and centrifuged at 17 300 g; supernatants were stored at -70°C .

A sensitive, solid-phase, double-sandwich immunofluorometric assay (IFMA) was used to measure ALA and BLG (14, 24).

Commercial ALA (minimum 90% by electrophoresis, Sigma Chemical, St Louis, MO, USA) and BLG (purified by electrophoresis and showing only β_1 and β_2 components; BDH Ltd, Dorset, UK) were purified on a Sephadex G-75 column (Pharmacia LKB Biotechnology, Uppsala, Sweden). The eluted monomers of ALA and BLG were tested with 12% SDS-PAGE, in which both showed a single band. Rabbits were immunized with 1 mg of the purified protein mixed with Freund's complete adjuvant and boosted three times at 1–2-month intervals with the same amount in Freund's incomplete adjuvant. They were bled 10 d after the last immunization. Antibodies were separated from the IgG fraction by affinity chromatography on an ALA- or BLG-coupled CNBr-activated Sepharose 4B column (Pharmacia) (30). These specific antibodies were used at a concentration of 5 $\mu\text{g/l}$ to coat polystyrene microtiter strips (Microstrip[®], Labsystems, Helsinki, Finland).

The strips were incubated for 24 h at $+20^{\circ}\text{C}$, washed, and saturated with 1% BSA for 1 h at 37°C . At this stage, the strips could be stored for several weeks at $+4^{\circ}\text{C}$ without loss of assay sensitivity. The isolated antibodies were also labeled with eu-

ropium (Eu) labeling reagent (Wallac, Turku, Finland) (10). The samples were diluted (1:100–1:2), added to the coated strips, incubated for 1 h at 37°C , and washed, and the Eu-labeled antibody was then added. After incubation for 1 h at 37°C , the plates were washed, a solution that liberated the europium was added (Enhancement solution, Wallac), and the fluorescence was measured in a 1230 Arcus fluorometer (Wallac).

Serum levels were expressed in relation to the amount of milk protein given per meal as $\mu\text{g ALA}$ or $\text{BLG/l serum per g ALA}$ or $\text{BLG given per kg body weight}$. All samples were analyzed in duplicate. The sensitivity of the assay was 0.25 $\mu\text{g/l}$ for ALA and 1.0 $\mu\text{g/l}$ for BLG. The limit of sensitivity was defined as twice the mean of 12 blanks. The range for ALA measurements was 0.25–25 $\mu\text{g/l}$; for BLG, 1.0–20 $\mu\text{g/l}$. The coefficient of variation within assays varied from 3% to 10%, and between assays from 3% to 24%.

IgA levels were measured by immunoturbidometry.

We also measured serum IgG and IgA antibodies against CM (CMAb) and BLG (BLGAb), using an ELISA method (28).

Statistical methods

We compared differences between the groups with the Mann-Whitney U test because of the skewed distribution of the data. Statistical analyses were performed with Statview II (Abacus Concepts, Berkeley, CA, USA) on an Apple Macintosh computer (Apple Computers, Cupertino, CA, USA).

Ethics

The study was approved by the Ethics Committee of the Children's Hospital, University of Helsinki, Finland.

Results

The median (range) duration of exclusive breast feeding was 3.0 months (0–5.1), solid foods were introduced at a median age of 3.0 months (2.6–5.1), and formulas based on CM were introduced at a median age of 5.7 months (0–11).

Absorption of α -lactalbumin

ALA was detected in all samples taken on days 3–4, although the variation was wide, from 12 to 225 $\mu\text{g/l serum per g ALA given per kg body weight}$. During the first 2 months, the median value for ALA absorption fell significantly from 32 $\mu\text{g/l serum per g of ALA given per kg}$ on days 3–4 to 6 $\mu\text{g/l}$ at

1 month, and then further to 2 µg/l at 2 months. The median values for absorption at 1 and 2 months were 19% and 5%, respectively, of the median value on days 3–4. In the samples from most infants, very little, if any, ALA could be detected at 3, 5, and 8 months of age (Fig. 1). The amount of ALA absorbed was 10⁻⁵–10⁻⁴ of the oral dose, assuming that ALA diffuses to twice the blood volume.

At weaning, 1 week after the introduction of CM, no ALA could be detected in the 10 samples available. Again, 2 weeks after weaning, no ALA was detected in the 12 samples tested.

Absorption of β-lactoglobulin

Analyses for absorption of BLG could be made only after the introduction of CM formula. Median (no. of infants where BLG was detected/no. of observations) values at 0, 1, 2, 3, 5, and 8 months were as follows: 25 (1/1), 0 (0/1), 6.5 (2/3), 2.9 (2/3), 0 (1/5), and 0 (3/9) µg/l serum per g BLG given per kg. No association was observable between the amounts of BLG and ALA absorbed. When the infants were 2 and 3 months old, the proportion of samples in which BLG could be measured was higher than it was later. The proportions of infants in whom absorption of ALA or BLG was measurable were similar after the age of 3 months. The sensitivity of the measurement was greater for ALA, but this did not affect the results (Fig. 2). At weaning, 1 week after the introduction of CM-based formula, BLG was found in 5/13 infants tested (38%). The median concentration was 7 µg/l serum per g BLG given per kg. Two weeks after weaning, BLG was found in 3/14 infants (21%); the median concentration was 4 µg/l

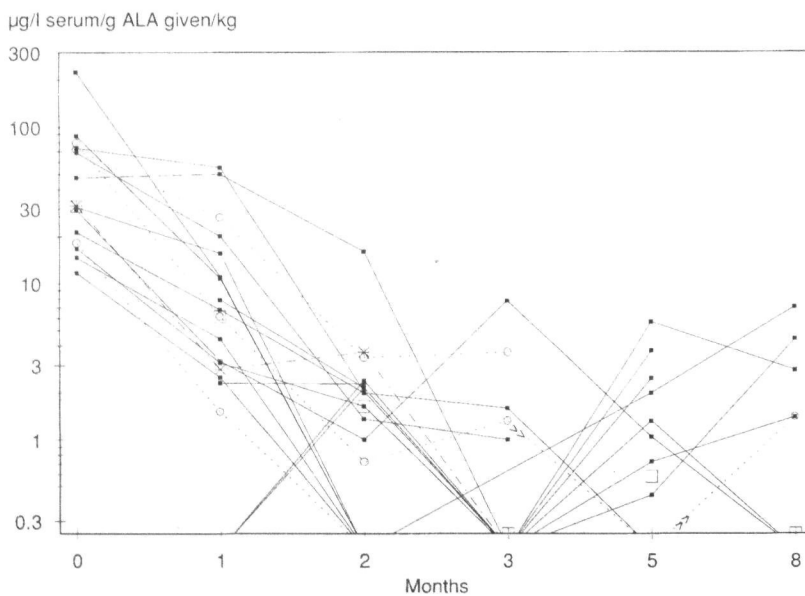


Fig. 1. Serum ALA in 20 healthy infants during first 8 months of life. Dotted lines designate atopic infants, ○ = atopic dermatitis; * = cow's milk allergy. □ = median value. Detection limit was 0.25 µg/l. Only one infant with atopic eczema had detectable ALA after onset of symptoms; > > = after onset of symptoms.

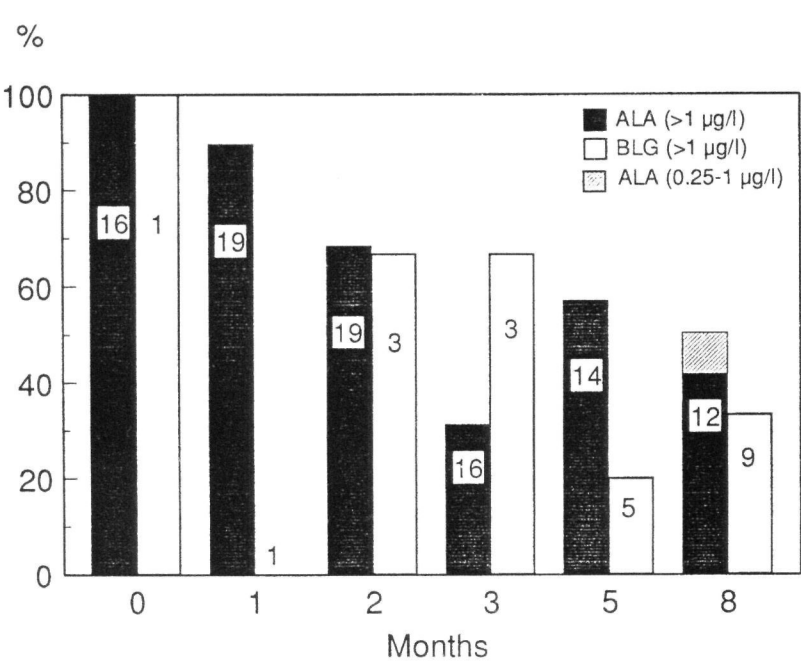


Fig. 2. Percentages of infants in whom ALA and BLG absorption was detected. Number of observations on each column. The proportion of infants with ALA absorption was determined by sensitivity of test (>0.25 µg/l) and sensitivity of measurement for BLG (>1 µg/l).

serum per g BLG given per kg (Fig. 3). The amount of absorbed BLG was 10⁻⁵ of the oral dose.

Factors possibly affecting absorption of ALA

According to the absorption of ALA at 0, 1, and 2 months, values for the five infants of the uppermost and of the lowermost quartiles were tested for factors possibly affecting absorption of ALA (Table 1).

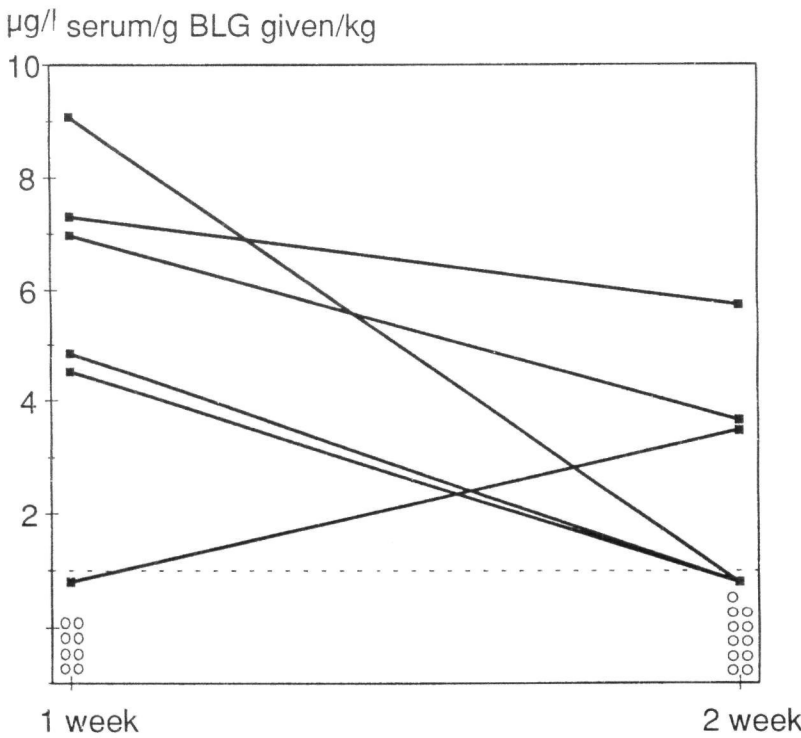


Fig. 3. Serum BLG 1 and 2 weeks after introduction of cow's milk. Open circles denote BLG values below detection limit (horizontal dotted line) (1 µg/l).

Table 1. Factors possibly affecting absorption of α -lactalbumin

	Age in months	Absorption of α -lactalbumin		P*
		Lowermost quartile (n=5)	Uppermost quartile (n=5)	
Number of infants with BLG in their mothers' milk	0	0	0	nt
	1	0	1	nt
	2	1	0	nt
Median sIgA in breast milk (g/l)	0	58(5)	55(4)	0.8
	1	23(5)	19(5)	0.6
	2	14(5)	16(5)	0.7
Median sIgA in saliva (mg/l)	0	66(4)	20(5)	0.01
	1	60(5)	54(5)	0.3
	2	33(5)	38(5)	0.3
Median sIgA in feces (g/l)	0	5.3(3)	1.4(4)	0.03
	1	2.5(5)	2.4(5)	0.8
	2	3.2(5)	2.9(5)	0.6
Number of infants with family member with atopy	0	1	2	nt
	1	1	1	nt
	2	1	2	nt

* P value for Mann-Whitney U test; nt=not tested.

Figures in parenthesis denote number of samples analyzed.

BLG in breast milk. BLG could be detected at a low median level (5 μ g/l) in the milk of only seven mothers and in only 15 (13%) of the 115 samples tested. In the infants of these mothers, absorption of ALA did not differ from that of the other infants at the ages of 0, 1, and 2 months. The mother's CM intake did not affect the BLG level of her breast milk.

Total IgA in the breast milk of mothers of infants in the uppermost and lowermost quartiles was similar on days 3–4, and at 1 and 2 months postpartum.

IgA in saliva and feces. Infants with high absorption of ALA on days 3–4 had a significantly lower amount of total IgA in saliva and feces. At 1 and 2 months, no statistically significant differences were found.

Absorption of ALA in infants with atopy or with a family history of atopy. Four infants had atopic symptoms during the first year of life (Table 2). CM allergy was verified in patient 4 by open challenge. In absorption of ALA, these infants did not differ from the non-atopic infants, nor was there any change in ALA absorption at the time of appearance of the symptoms (Fig. 1). Of the 20 infants, 11 (55%) had a family member with atopic manifestations. Median values for absorption of ALA at the ages of 0, 1, and 2 months in the infants with a family history of atopy and those without such a history were 31 and 39, 7 and 3, and 0.7 and 1.9 μ g/l serum per g ALA given per kg, respectively; the differences were not statistically significant.

Factors possibly affecting absorption of BLG

We assigned the infants to two groups according to positive and negative serum BLG levels at 5 and 8 months, and analyzed factors possibly affecting BLG absorption (Table 3).

Serum IgG and IgA antibody levels against CM and BLG. Serum CMAb and BLGAb (data not shown) levels at 5 and 8 months were analyzed in these groups, but no statistically significant differences were found.

Breast-milk total IgA and IgA antibody levels against CM. Infants with measurable absorption of BLG had a mother with significantly lower total IgA in her breast milk at 2 months ($P=0.02$). No other significant differences were found.

Salivary and fecal IgA levels. Total IgA levels in saliva and feces at 5 and 8 months were compared, but no significant differences were found.

Table 2. Characterization of infants with atopy

	Patient 1	Patient 2	Patient 3	Patient 4
Family member with atopy	Father: allergic rhinitis	Mother: AD*	Mother: allergic rhinitis and AD* Father: allergic rhinoconjunctivitis	Brother: CMA**
Exclusive breast feeding (months)	3.1	3.5	3.3	5.1
Symptoms	Erythema and papules on face; erythema on body; dry, coarse skin. Skin flushing from cereals	Erythema and papules on face and hands; dry skin	Erythema and papules on face, hands, and legs; dry skin	Facial urticaria after CM and CM formula
Age in months at onset of symptoms	6	5	8	8
Total IgE at onset of symptoms (iu/l)	3	33	17	130

* Atopic dermatitis; ** cow's milk allergy.

Table 3. Factors possibly affecting absorption of β -lactoglobulin

	Months	β -lactoglobulin absorption at 5 and 8 months		<i>P</i> *
		High (<i>n</i> =3)	Low (<i>n</i> =6)	
Median serum IgG CMAb (% of positive reference)	5	232(2)	1307(6)	0.7
	8	272(3)	180(5)	0.9
Median serum IgA CMAb (% of positive reference)	5	76(2)	169(6)	0.7
	8	67(3)	48(5)	0.7
Median sIgA in breast milk (g/l)	0	58(3)	66(3)	0.5
	1	27(3)	19(5)	0.2
	2	23(3)	16(5)	0.02
Median IgA CMAb in breast milk (% of positive reference)	0	243(3)	36(3)	0.5
	1	53(3)	91(4)	0.2
	2	50(3)	65(5)	0.5
Median sIgA in saliva (mg/l)	5	27(3)	28(6)	0.4
	8	33(2)	23(6)	0.5
Median sIgA in feces (g/l)	5	2.8(3)	0.8(6)	0.4
	8	0.6(3)	0.7(6)	0.8

* *P* value for Mann-Whitney U test; CMAb=cow's milk antibodies. Figures in parenthesis denote number of samples analyzed.

Discussion

Gut closure has been thought to occur in human infants before birth (21). However, we detected measurable amounts of milk proteins in the sera of infants in the first months of life. At 2 months of age, absorption of ALA had fallen to less than 10% of the level on days 3–4, and, later, little or no absorption of ALA could be detected. The amount of intact protein absorbed was small, 10^{-5} – 10^{-4} of the oral dose. Our results agree with those of Jakobsson et al. (12). Data on absorption of heterologous food antigens is scanty. Müller et al. (15) detected BLG and casein in sera of term infants during the first 10 days after birth, but Robertson et al. (21) found only trace amounts of BLG in one of 13 term infants. In the present study, tests for absorption of BLG were made only after the introduction of CM. Absorption of BLG could be found at 0, 2, 3, 5, and 8 months, but before 5 months the sample size was small. The mean absorptions of BLG and ALA were comparable, but there was no association between the amounts of the two proteins absorbed by individual infants.

At weaning, the infant ingests a large amount of highly antigenic foreign protein which could induce an inflammatory response in the gut mucosa. Some absorption of the newly introduced CM protein could be detected both 1 and 2 weeks after the start of formula feeding. Enhanced systemic uptake of a bystander protein has been shown in rats during local intestinal anaphylaxis (3, 29), but we could not detect absorption of the bystander protein ALA. Absorption of a foreign protein into the circulation is

sufficient to elicit an immunologic response, recognizable from the rising CMAb titers. The significance of this absorption of proteins for the later development of allergic and gastrointestinal diseases is not yet known. Only two studies have used intact proteins to measure the permeability of the intestine in children with allergic diseases (11, 13). They show increased absorption of antigenic protein after positive challenge tests, while during elimination of the offending antigen no passage of protein was observed. We found no differences in permeability to ALA in the first months of life between infants with and without atopic manifestations, or between infants with and without a family member with atopy.

The introduction of the highly sensitive, rapid, and nonradioactive, time-resolved immunofluorometric assay marked an advance in immunologic methods (25). The detection limit in our assay is 0.25–1 μ g/l, as compared with 5 μ g/l reported by Jakobsson et al. (12), who used a competitive RIA. The sensitivity of solid-phase IFMA methods is reported to be 10 times that of RIAs with the same antibodies (26).

Antibodies in the sera affect the sensitivity of conventional RIAs. Paganelli & Levinsky (19) described a two-site, solid-phase RIA for measuring BLG that detected the antigen whether in free or in complex form, and especially if present in excess. Our IFMA method is a similar two-site, solid-phase method with europium as label. Differences in sensitivity may also be due to the affinities of the antibodies used.

Bovine BLG in breast milk may cause allergic reactions (8). Weaver et al. (33) have shown that CM-fed guinea pigs have a persistently higher intestinal permeability to sugar, and the enhanced uptake of a bystander protein shown in rats during local intestinal anaphylaxis (3, 29) made us speculate that BLG in breast milk might affect the absorption of ALA in breast-fed infants. BLG was found in only 13% of breast-milk samples, and we did not find more BLG in milk from mothers of infants with high levels of ALA absorption. The measurable levels were low, with a median of 5 μ g/l, in contrast to the results of Axelsson et al. (1), who found BLG in 93/232 (40%) samples and concentrations of 5–800 μ g/l, with most of the positive values above 15 μ g/l. Stuart et al. (27), on the other hand, detected BLG in 5/28 (18%) mothers. The variation may be due to methodologic differences and particularly to the cross-reaction that may occur between human β -casein and BLG (16). Antibodies produced against bovine BLG may react with human β -casein. Axelsson et al. (1) used a competitive RIA in which the antibodies were not purified by affinity chromatography. Stuart et al. (27) used an ELISA method, but did not describe the nature of the antibody. We used a solid-phase IFMA in which we immunized rabbits with BLG monomers purified on a Sephadex

G-75 column, and we purified the antisera by affinity chromatography.

The anti-inflammatory properties of breast milk may affect gastrointestinal permeability. In addition, secretory IgA (sIgA) in the mucus coat binds to foreign antigens, preventing their migration to the epithelial membrane surface, and interferes with pinocytosis by the enterocytes (31). Complexing of food antigens with sIgA also promotes their proteolysis by luminal enzymes (32). Cunningham-Rundles et al. (4) showed that persons with IgA deficiency absorb protein in larger amounts. The values for salivary and fecal IgA were used as estimates of the child's own ability to secrete this immunoglobulin. This ability may be an important factor in contributing to the exclusion of foreign antigens (31). The specific IgG antibody response in the serum is also associated with the levels of sIgA in the gut (7). We therefore looked for an association between these factors and gut permeability. In the two subgroups divided on the basis of ALA absorption on days 3–4, IgA levels were significantly higher in the saliva and feces of the infants who absorbed less ALA. Furthermore, at 2 months, IgA levels were significantly higher in the breast milk of the mothers whose infants absorbed less BLG. We have reported that mothers whose infants become allergic to CM have a lower total concentration of IgA in breast milk than mothers whose infants do not show CM allergy (23). Our findings suggest that IgA in breast milk and secretions may reduce the penetration of protein through the gut mucosa.

We conclude that in full-term infants the gut epithelium is permeable to both human and foreign protein molecules during the first months after birth. The consequences of this absorption for the development of allergic diseases still remain to be determined.

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