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# Human α-Lactalbumin and Bovine β-Lactoglobulin Absorption in Premature Infants

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**ABSTRACT. The absorption of α-lactalbumin (ALA) and bovine β-lactoglobulin (BLG) was investigated in 23 healthy preterm infants with gestational ages of 32 to 36 wk. The concentrations of ALA and BLG in serum after a milk feeding were measured at intervals during the first 8 mo of life. We used a time-resolved fluoroimmunoassay to measure the proteins. Measurable amounts of ALA were found on d 7 after birth, and at 1, 2, 3, 5, and 8 mo in 23 of 23, 13 of 18, 13 of 18, six of 17, eight of 16, and five of 13, respectively, of the infants tested; median serum levels of ALA at the respective ages were 120 (range, 19–2598), 16 (range, 0–177), 5 (range, 0–40), 0 (range 0–3), 0.8 (range 0–38), and 0 (range, 0–22) μg/L serum/g ALA given/kg body wt, respectively. The rate of decline in ALA absorption was comparable among the infants. Tests for BLG were begun after the introduction of cow's milk. At 2, 3, 5, and 8 mo of age BLG was detected in two of 7, two of 9, eight of 10, and two of 12, respectively, of the infants tested, where median levels in positive cases were 13, 17, 15, and 3 μg/L serum/g BLG given/kg, respectively. The amounts of absorbed ALA and BLG were $10^{-5}$ to $10^{-3}$ of the oral dose. Serum levels of ALA or BLG did not depend on the gestational age of the infant. Few of the infants had any detectable absorption of either protein shortly after weaning. Thus, systemic absorption of ALA and BLG does occur in preterm infants. Absorption of ALA is significant for a few months after birth but then decreases rapidly. (*Pediatr Res* 35: 344–347, 1994)**

**Abbreviations**

**ALA, human α-lactalbumin**
**BLG, bovine β-lactoglobulin**
**CM, cow's milk**

In several animal species the transfer of maternal antibodies to offspring depends on massive absorption of colostral Ig. This selective transfer of IgG across the epithelium occurs by receptor-mediated endocytosis (1). In the human newborn, a short and transient period exists when the gut may absorb IgA. This period is probably more pronounced in the preterm infant (2–4). However, large milk proteins may penetrate the intestinal wall postnatally (5–8).

The extent to which the gut wall is permeable to isologous protein molecules is regulated by nonspecific factors, such as acidity of the stomach, quality of mucus, efficiency of intestinal proteolysis, and motility of the intestine. Until the 40th wk of gestation, these factors are not fully developed, which can be seen by the greater penetration of isologous protein through the intestinal epithelium in preterm rather than in full-term infants (7, 8). The absorption of heterologous proteins is dependent on local and systemic immune responses (9, 10).

The aim of the present study was to follow up the permeability of the gut in premature infants and to compare it with that of full-term infants studied earlier (11). To gain a better picture of the permeability, we used two different proteins: ALA, which elicits no immune response, and BLG, a major antigen in CM.

## MATERIALS AND METHODS

*Subjects and sampling.* With the informed and written consent of the mothers, 23 healthy premature infants with a median gestational age of 34 wk (range, 32.6–36.6) were recruited into the study on d 3–4. All infants were healthy and free of perinatal infections, and enteral feeding was started on the 1st postnatal d. Two infants received supplemental i.v. infusions, and four infants received part of the feeding as nasogastric boluses via a tube during the 1st days of life. Four infants dropped out after the initial visit because of difficulties in traveling to the hospital. The infants were examined postnatally and at the ages of 1, 2, 3, 5, and 8 mo. They were also seen immediately before the planned weaning and 1 and 2 wk after it. Before weaning, the mothers were advised to use a CM-free diet. Feeding recommendations included fruit, berries, and vegetables from 3 mo and meat, fish, eggs, and cereals from 5 mo. Infants attended well-baby clinics, but any medical care needed was provided by M. K. Not all mothers and infants could 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 the infant's own mother or from a pool of banked breast milk. One hour after the beginning of the feeding, a venous blood sample (2 mL) was drawn and a saliva sample (1 mL) was collected after stimulating salivation with three critic acid crystals. For ethical and empirical reasons, on the basis of studies where the rapid clearance of ALA has been shown (7), we did not take a blood sample before the test meal, but infants had fasted 4 h before. 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 a week.

Serum was stored at −20°C. Breast milk was stored at −20°C and before analysis defatted 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 BSA (Wilfrid Smith Ltd., Middlesex, UK) in PBS to give a final BSA concentration of 0.3% and centrifuged at 17 300 × *g*; supernatants were stored at −70°C.

*Quantitation of ALA and BLG.* A sensitive solid-phase double-sandwich time-resolved fluoroimmunoassay was used to measure

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ALA and BLG (12, 13). Commercial ALA (Sigma Chemical Co., St. Louis, MO) and BLG (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- to 2-mo 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 cyanogen bromide-activated Sepharose 4B column (Pharmacia). These specific antibodies were used at a concentration of 5 μg/mL for coating polystyrene microtiter strips (Labsystems, Helsinki, Finland). The strips were incubated for 24 h at 20°C, washed, and saturated with 1% BSA for 1 h at 37°C. The isolated antibodies were also labeled with europium labeling reagent (Wallac, Turku, Finland) (14). Samples were diluted (1:100–1:2), added to the coated strips, incubated for 1 h at 37°C, and washed, and the europium-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 an Arcus 1230 fluorometer (Wallac). Serum levels are expressed in relationship to the amount of milk protein given per meal as micrograms of ALA or BLG per liter of serum per gram of ALA or BLG given per kilogram of body weight. All samples were analyzed in duplicate. The sensitivity of the assay was 0.25 μg/L for ALA and 1.0 μg/L for BLG. The limit of sensitivity was defined as twice the mean of 12 blanks.

*Other measurements.* Total IgA levels were measured by immunoturbidometry (15), and CM and BLG antibodies were measured with an ELISA method (16).

*Statistical methods.* We compared differences between the groups with the Mann-Whitney U test because of the skewed distribution of the data and with the Fisher's exact test for quantitative variables. Results are given as two-tailed $p$ values.

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

## RESULTS

The mean birth weight was 2346 g (range, 1500–2970). The mean duration of exclusive breast-feeding (no CM formula, no solid foods) was 2.1 mo (range, 0–6.4). Formulas based on CM were introduced at a mean age of 2.9 mo (range, 0–8.1). Solid foods (not containing CM) were introduced at a mean age of 3.7 mo (range, 2.0–6.4). Some mothers continued to breast-feed their infants after the introduction of CM formula; the mean age for partial breast-feeding was 4.2 mo (range, 0–9). None of the infants manifested any atopic disease or had gastroenteritis during the follow-up period.

*Absorption of ALA.* ALA was detected in all samples taken on d 7 after birth. The absorbed amounts varied widely between the infants, from 19 to 2598 μg/L serum/g ALA given/kg. The median value for ALA absorption fell significantly during the 1st few months, from 120 μg/L serum/g ALA given/kg at the age of 7 d to 16 μg/L serum/g ALA given/kg at 1 mo and 5 μg/L serum/g ALA given/kg at 2 mo. At 3, 5, and 8 mo, most values were below the level of detectability (Fig. 1). The decline in absorption of ALA was comparable among the infants. Absorption of ALA did not depend on the gestational age of the infant (Fig. 2).

The amount of absorbed ALA varied widely; it was $10^{-5}$ to $10^{-3}$ of the oral dose, assuming that ALA diffuses to twice the blood volume. The amount diminished from birth to 8 mo; median amounts absorbed at 1, 2, and 3 mo were 42%, 26%, and 21%, respectively, of median values of d 7.

One week after the introduction of CM, ALA could be detected in only 1 of 13 samples tested, and 2 wk after weaning ALA could be detected in 2 of 14 samples tested. The infants with detectable absorption of ALA were weaned at the ages of 0.2, 0.4, and 0.7 mo, and the median age for weaning of those with undetectable levels was 3.3 mo.

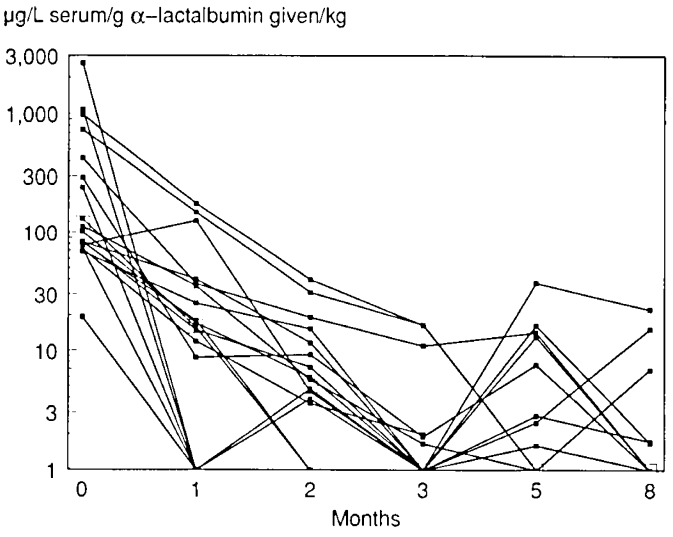


Fig. 1. Serum ALA in all 23 infants during the first 8 mo of life. □, median value.

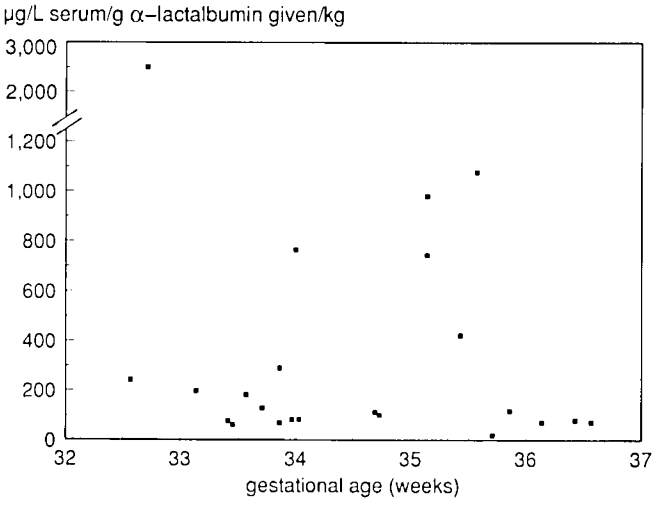


Fig. 2. Serum ALA 7 d after birth plotted against the gestation age of the infant.

Table 1. *Median serum concentrations of human α-lactalbumin (μg/L serum/g α-lactalbumin given/kg body wt) in preterm (gestational age 32–36 wk) and term infants*

| Age | Preterm | Full term* | Mann-Whitney U test ($p$ value) |
|---|---|---|---|
| 7 d ($n$) | 120 (23) | 31 (16) | 0.0001 |
| 1 mo ($n$) | 16 (18) | 6 (19) | 0.20 |
| 2 mo ($n$) | 5 (18) | 1.6 (19) | 0.01 |

*Absorption of ALA in premature infants compared with term infants.* Serum levels of ALA in premature infants were three to four times that in term infants (11) (Table 1).

*Absorption of BLG.* Analyses for absorption of BLG could be made only after the introduction of CM formula. Of the four infants given CM at 1 mo, none had any detectable BLG in their sera. At 2, 3, 5, and 8 mo, BLG was detected in two of seven, two of nine, eight of 10, and two of 12, respectively, of the infants tested, where median values of positive cases were 13, 17, 15, and 3 μg/L serum/g BLG given/kg, respectively. The

gestational age of the infant did not affect BLG absorption (data not shown). The amount of absorbed BLG was less than $10^{-3}$ of the oral dose throughout the study.

One week after the introduction of CM, 2 of 14 infants had detectable BLG absorption, and 2 wk after weaning two of 12 infants had detectable absorption of BLG. The concentrations were 5, 62, 27, and 15 μg/L serum/g BLG given/kg, the three highest values from infants weaned early, at the ages of 1.2 and 2 mo, and the infants with no detectable BLG were weaned at a median age of 2.4 mo.

*Absorption of ALA versus absorption of BLG.* After the introduction of CM in the infants' diet, we made an age-matched comparison of absorption of ALA and BLG. At the ages of 2, 3, 5 and 8 mo, the mean absorption values of positive cases of ALA and BLG were comparable: 12.4 ($n$ = 13) *versus* 13.3 ($n$ = 2), 8.3 ($n$ = 6) *versus* 16.5 ($n$ = 2), 12.0 ($n$ = 8) *versus* 13.9 ($n$ = 8), and 9.6 ($n$ = 5) *versus* 3.1 ($n$ = 2) μg/L serum/g ALA or BLG given/kg, respectively.

*Factors possibly affecting absorption of ALA.* The infants breast-fed exclusively were assigned to two groups according to the absorption of ALA on d 7 and at 1 and 2 months: at the respective ages, the 11, 7, and 5 infants of the upper and of the lower half were tested for factors possibly influencing absorption of ALA. Data for analyses on d 7 are shown in Table 2.

The mothers' breast-milk IgA content on d 7 was significantly lower in infants with absorption of ALA in the upper half, $p$ = 0.01. At 1 and 2 mo no differences were observed between the two groups.

IgA contents in saliva and feces in the upper and lower halves were similar on d 7 and at 1 and 2 mo.

*BLG in breast milk.* BLG could be detected in the milk of 14 of the 16 mothers tested and in 27 (37%) of the 72 samples tested during the whole lactation period; the median level was 10 μg/L. The variation in BLG concentration was considerable both between mothers and between different samples from the same mother. All the mothers drank CM, the median value being 500 mL/d (range, 50–1000 mL). The mother's CM intake did not influence the breast-milk BLG level. Five mothers had an atopic disease, and they all had detectable BLG in their breast milk. Of the 11 mothers without allergy, nine had detectable BLG in their breast milk. No difference in breast-milk BLG level was observed between mothers of infants with high and low absorption of ALA at 0, 1, and 2 mo.

Median values of CM antibodies and BLG antibodies of IgA and IgG classes are shown in Table 3.

Table 2. *Factors possibly affecting absorption of human α-lactalbumin in breast-fed infants**

| | Human α-lactalbumin absorption on d 7 | | |
|---|---|---|---|
| | High ($n$ = 11) | Low ($n$ = 11) | Statistical value |
| Median IgA in breast milk (g/L) on d 7 | 25 (8) | 49 (6) | $z$ = 2.46† $p$ = 0.01† |
| Median IgA in feces (g/L) on d 7 | 3.6 (8) | 4.2 (10) | $z$ = 0.62† $p$ = 0.5† |
| No. of infants with β-lactoglobulin in breast milk >10 μg/L at any age | 6 | 5 | $p$ = 1.0‡ |
| No. of infants with positive history of atopy in parents or siblings | 2 | 6 | $p$ = 0.2‡ |

* Numbers in parentheses indicate number of infants in each category.
† Mann-Whitney U test: $z$ corrected for ties and two-tailed probability.
‡ Fisher's exact test, two-tailed probability.

Table 3. *Median serum CM antibodies and BLG antibodies of IgA and IgG classes in all infants given as percentage of positive reference*

| | CM antibodies | | BLG antibodies | |
|---|---|---|---|---|
| Mo | IgA | IgG | IgA | IgG |
| 0 | 0.1 | 0.4 | 0.1 | 0.1 |
| 1 | 0.1 | 0.6 | 0.1 | 0.1 |
| 2 | 0.1 | 0.6 | 0.1 | 0.1 |
| 3 | 0.3 | 58 | 0.1 | 0.1 |
| 5 | 23 | 64 | 0.1 | 6.2 |
| 8 | 60 | 342 | 4.0 | 49 |

## DISCUSSION

In premature infants, less than 1/1000 of the amount of isologous protein given was absorbed during the 1st wk of life. Absorption fell rapidly to about 1/10 during the first months, to an undetectable level in most infants at 3 mo. Absorption of ALA in preterm infants was 3- to 4-fold that of full-term infants, but the duration of higher absorption in the two groups was comparable (11). Boehm *et al.* (8) reported mean ALA concentrations of 3900 in prematures with gestational ages of 34–36 wk, at a postnatal age of 7 d, compared with 930 in our study, when reported as μg/L serum/L milk given/kg. Differences in methods may explain the discrepancy; we used a solid-phase time-resolved fluoroimmunoassay with affinity-purified antibodies, whereas Jakobsson *et al.* (17) used a competitive RIA with antibodies that were not affinity purified. The proportion of protein absorbed, $10^{-5}$ to $10^{-3}$ of the oral dose, is in accordance with earlier studies (6, 7), being twice that of the term infants (11). This may be due to the immaturity of the intestine in premature infants (18). The level of absorption of ALA was consistent in the same infant: those with high absorption 7 d after birth tended to have high absorption at 1, 2, and 3 mo. Boehm *et al.* (8) found high absorption of ALA in premature infants less than 33 wk of gestation and a decline in absorption with increasing maturity. We could not detect any relationship between gestational age and absorption of ALA, but infants in our study were born after 32 wk of gestation.

When an infant ingests a large amount of highly antigenic CM proteins at weaning, we speculated that this would induce an inflammatory response in the gut mucosa as in hypersensitivity reactions (19). Secretion of IgA in the mucosa is induced to inhibit absorption of foreign antigens and to protect the host from harmful effects (20). Specific mucosal antibodies appear within 2 wk of exposure to new antigens (21). Our study was designed to detect the effects of these events on the permeation of BLG and ALA. Only small effects were seen. Immediately after weaning, only a few of the preterm infants tested were found to have absorbed ALA or BLG, suggesting that the introduction of a CM formula does not render the intestinal mucosa leaky. However, at weaning, more than half the infants tested were eating solid foods and thus had been exposed to large amounts of foreign antigens. They had been given solid foods 1.7 mo (median) earlier than CM. Small rises in the absorption of ALA from 3 to 5 mo were observed in most infants with detectable ALA. This rise in absorption of ALA and the increasing proportion of infants in whom BLG was detected from 3 mo to 5 mo may have been related to the introduction of a variety of dietary antigens after the age of 3 mo.

The quantities of antigenically intact protein absorbed are nutritionally insignificant. However, they are sufficient to elicit the production of antibodies (22). Rising titers to CM and BLG could be detected in all the infants after the introduction of CM.

BLG was transferred to breast milk throughout the lactation period, which is consistent with the findings of Axelsson *et al.* (23), but we found BLG in fewer mothers and in lower concentrations. A possible explanation is a cross-reaction between human β-casein and BLG (24), which is more likely to occur when

the antibodies in the test have not been purified by affinity chromatography. BLG in breast milk did not enhance the absorption of ALA in infants, indicating that the small amounts of BLG in breast milk does not induce a functional impairment of the intestinal mucosa.

In the two subgroups divided by ALA absorption on d 7, IgA levels were significantly higher in the breast milk of those mothers whose infants absorbed less ALA. We have reported that mothers whose infants became allergic to CM had a lower total concentration of IgA in their breast milk than mothers of infants who had no symptoms of atopy (25). These findings suggest that IgA in breast milk may reduce the penetration of protein through the mucosa with its antiinflammatory properties and inhibit the development of food allergy.

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