

# Human $\alpha$ -lactalbumin as a marker of macromolecular absorption

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**SUMMARY**  $\alpha$ -Lactalbumin was purified from human milk and a competitive radioimmunoassay for measuring serum concentrations of human  $\alpha$ -lactalbumin was developed. Human  $\alpha$ -lactalbumin was not detected ( $<5 \mu\text{g/l}$ ) in serum from adult men ( $n=4$ ), non-pregnant women ( $n=6$ ) or in serum from seven of eight formula fed infants.  $\alpha$ -Lactalbumin was found in serum from pregnant women ( $19\text{--}130 \mu\text{g/l}$ ,  $n=4$ ), cord blood ( $22\text{--}72 \mu\text{g/l}$ , median value  $35 \mu\text{g/l}$ ,  $n=9$ ), and from newborn non-fed infants ( $<1$  day old) ( $<5\text{--}50 \mu\text{g/l}$ , median value  $15 \mu\text{g/l}$ ,  $n=11$ ). In breast fed infants the serum concentration of  $\alpha$ -lactalbumin was highest in preterm infants ( $140\text{--}952 \mu\text{g/l}$  serum/l human milk/kg body weight,  $n=4$ ) and decreased in term infants successively with maturity (age 5–30 days: median value  $85 \mu\text{g/l}$  serum/l human milk/kg body weight,  $n=7$ ; age 31–60 days: median value 43,  $n=6$ ; age 61–135 days: median value 12,  $n=6$ ). A human milk feeding to three infants one month of age gave serum peak values of  $\alpha$ -lactalbumin after 30 to 60 minutes. We suggest that human  $\alpha$ -lactalbumin is a suitable marker for investigating macromolecular absorption in physiological and pathological conditions.

It is now generally accepted that antigenic macromolecules can penetrate the small intestinal mucosal membranes in quantities that may be of immunologic importance.<sup>1,2</sup> That this occurs, especially in infants below three months of age has been shown indirectly by higher serum concentrations of antibodies to food antigens.<sup>3</sup> Recent studies<sup>4,5</sup> have also shown higher concentrations of food antigens in the serum of preterm and term infants.

It had been reported that patients with allergic diseases have an increased permeability to macromolecules through the small intestinal mucosa.<sup>6,7</sup>

Heterologic food antigens may start a local intestinal and a systemic immune response that is manifested by the development of circulating antibodies.<sup>8</sup> The use of such proteins in tests for macromolecular absorption is therefore less suitable. The molecules used in most permeability studies are not proteins but – for example, lactulose and polyethylene glycols with relatively low molecular weights.<sup>6,7,9,10</sup> The transfer of these substances

through the gut membranes does not reflect the situation for transfer of food protein.<sup>11</sup>

By using a human protein as a marker of macromolecular absorption, we avoid the drawbacks mentioned above. We have chosen human  $\alpha$ -lactalbumin (MW~14,000), the dominant whey protein of human milk. This protein is not present in the blood except in pregnant and lactating women. This report presents the method and the results from infants with varying maturity and age.

## Methods

### HUMAN MILK

Fresh human milk was obtained from the Milk Bank, Malmö General Hospital, Malmö, Sweden.

### HUMAN SERUM

Serum was obtained from: four adult men; four pregnant women; six non-pregnant, non-lactating women, and from 45 infants. Nine cord blood samples were also obtained. Serum was stored at  $-20^\circ\text{C}$  until analysed.

The infants were divided into the following groups according to different ages and feeding regimen.

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**Group 1**

Eleven healthy term infants ages <1 day. The blood samples were taken before any human milk feeding.

**Group 2**

Eight term infants aged 20–180 days were fed adapted cow's milk based formula only. Blood samples were taken about 60 minutes after the start of a formula feeding.

**Group 3**

Four preterm infants, aged 1–27 days, born at the gestational age of 29–34 weeks and fed human milk. Blood samples were taken about 60 minutes after the start of a human milk feeding.

**Group 4**

Nineteen term breast fed infants aged 5–135 days with varying diagnoses (acute respiratory and urinary infections, minor feeding problems, diaper dermatitis). The blood samples were taken about 60 minutes after the start of a human milk feeding.

**Group 5**

Three term, breast fed infants aged 22, 29, and 37 days were followed with repeated blood sampling before and after a human milk feeding.

This study was approved by the Ethical Committee, University of Lund.

**PURIFICATION OF HUMAN  $\alpha$ -LACTALBUMIN**

A modification of the method described by Forsum *et al*<sup>12</sup> was used. Fresh human milk (250 ml) was defatted by centrifugation at 38 000 g during 60 minutes. Casein was precipitated by adjusting pH of

the milk to 4.6 by 1 M HCl at 26°C. The milk was kept at this temperature during two hours and then casein was separated from the whey by centrifugation at 38 000 g during 60 minutes. The whey fraction was concentrated in Spectrapor about 10 times and then dialysed over night in 0.02 M Tris HCl buffer, pH 7.4, 0.15 M NaCl. Gel filtration was then done through an ACA 54 gel (LKB Products, France) (column K16/100) with the same buffer. Fractions containing  $\alpha$ -lactalbumin (from the 2nd peak) were pooled and concentrated in Spectrapor. The crude  $\alpha$ -lactalbumin thus obtained was purified further on a Sephadex G50 column (Pharmacia Chemicals, Uppsala, Sweden) in 0.02 M Tris HCl, pH 7.0, 0.15 M NaCl. Fractions containing  $\alpha$ -lactalbumin were pooled and concentrated in Spectrapor. Finally a preparative electrophoresis in 0.8% agarose gel with a barbital-sodium-barbital buffer pH 8.6, 0.002 M calcium-lactate was done. The anodal part 5 mm from the slit of the agarose gel contained pure  $\alpha$ -lactalbumin which was de-salted and lyophilised. Sodiumdodecylsulphate-polyacrylamide gel electrophoresis<sup>13</sup> of the purified  $\alpha$ -lactalbumin showed one single and distinct band and a crossed immunoelectrophoresis against antibodies to whole human milk showed one single peak (Fig. 1a).

**IMMUNISATION PROCEDURE**

One hundred micrograms of purified human  $\alpha$ -lactalbumin (in 0.05 ml 0.9% saline) were mixed with an equal amount of Freund's complete adjuvant and injected intracutaneously, subscapularly into adult rabbits. Booster shots were given with the same solution after two, four, and six weeks. Ten

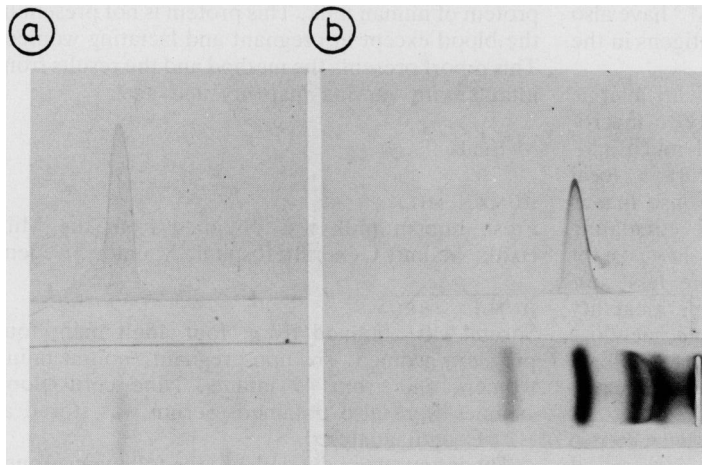


Fig. 1 Crossed immunoelectrophoresis (a) Purified  $\alpha$ -lactalbumin against antibodies to whole human milk. (b) Human milk against antibodies to  $\alpha$ -lactalbumin.

days after the fourth injection serum samples were collected and IgG fractions were isolated with the aid of caprylic acid.<sup>14</sup> Thereafter immunisation was done at four week intervals and serum samples were collected 10 days after each immunisation. Fig. 1b shows that the obtained antibodies against human  $\alpha$ -lactalbumin formed one single precipitate in crossed immunoelectrophoresis against human milk.

#### RADIOIMMUNOASSAY

The same method as described for quantifying bovine  $\beta$ -lactoglobulin<sup>15</sup> was used for measuring serum concentration of human  $\alpha$ -lactalbumin. <sup>125</sup>I-labelled purified  $\alpha$ -lactalbumin was mixed in tubes with the serum sample or standard solution and rabbit-anti- $\alpha$ -lactalbumin and incubated for 24 hours. After addition of normal rabbit serum and goat anti-rabbit serum another incubation of 24 hours was done. The tubes were then centrifuged, supernatants decanted and the radioactivity of the precipitates measured in a gammacounter (LKB Wallac 1282 Compugamma). Each test run included duplicates of a dilution series of the purified human  $\alpha$ -lactalbumin (1.563–800  $\mu$ g/l. All samples were run in duplicate.

Results were expressed as  $\mu$ g  $\alpha$ -lactalbumin/l serum in serum samples from adults, cord blood,

formula fed infants, infants in group I. In infants fed human milk (groups III, IV, V) results were expressed as  $\mu$ g  $\alpha$ -lactalbumin/l serum/l human milk given/kg body weight.

#### Results

Human  $\alpha$ -lactalbumin in serum could be detected down to 5  $\mu$ g/l. Day-to-day variation in repeated determinations of the same samples was within  $\pm 3\%$ . Studies on the effect of storing at  $-20^\circ\text{C}$  showed a decrease of the  $\alpha$ -lactalbumin concentration by about 10% after four months.

The specificity of the method was studied by adding purified human  $\alpha$ -lactalbumin both to a male adult serum and to the dilution buffer. The resulting curves ran in parallel indicating no unspecific influence by the serum (Fig. 2). Moreover, dilution of a serum sample containing  $\alpha$ -lactalbumin (from a pregnant woman) gave a curve that ran in parallel with the standard curve of  $\alpha$ -lactalbumin (Fig. 3).

#### SERUM SAMPLES

In four male adults and six non-pregnant and non-lactating women no detectable  $\alpha$ -lactalbumin was found (Fig. 4). The figure also illustrates the results in four pregnant women (19, 98, 110, 130  $\mu$ g/l,

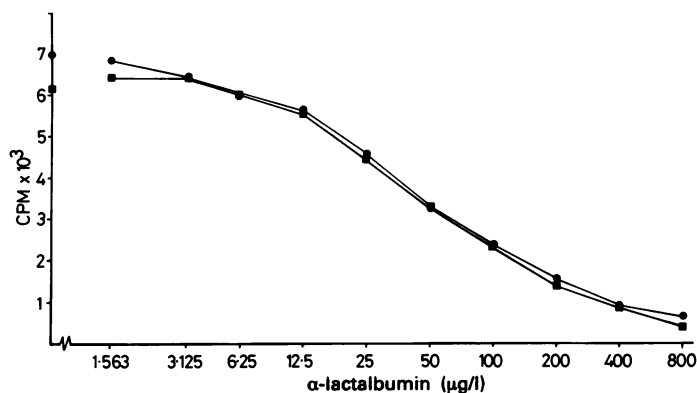


Fig. 2 Radioimmunoassay of human  $\alpha$ -lactalbumin. Comparison between standard dilution curves of  $\alpha$ -lactalbumin in the dilution buffer  $\blacksquare$ — $\blacksquare$  and in human serum  $\bullet$ — $\bullet$ .

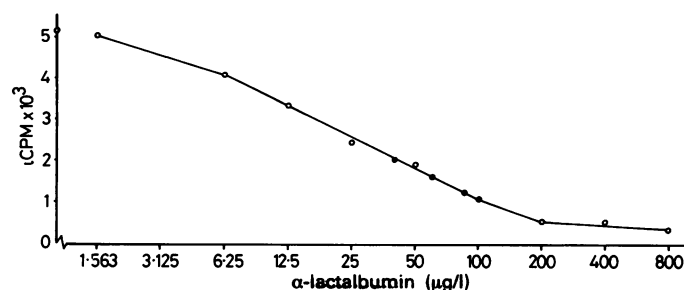


Fig. 3 Radioimmunoassay of human  $\alpha$ -lactalbumin. dilution curve for human  $\alpha$ -lactalbumin in serum  $\bullet$ — $\bullet$  compared with a standard dilution series of purified  $\alpha$ -lactalbumin  $\circ$ — $\circ$ .

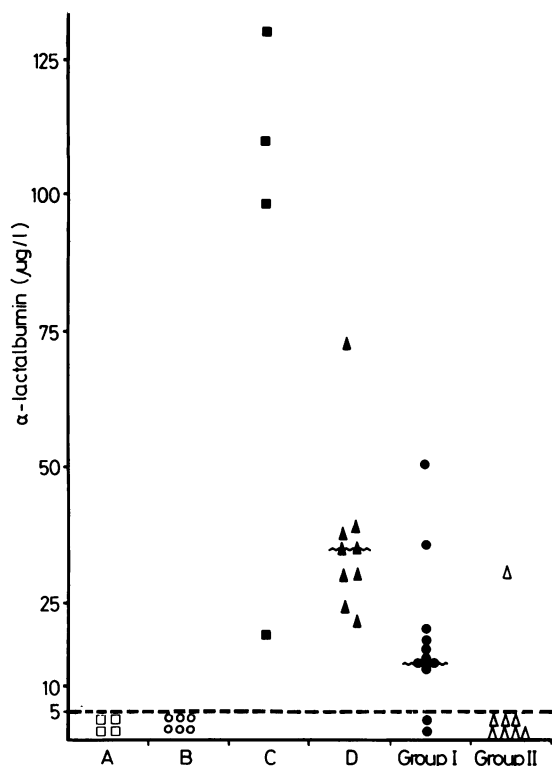


Fig. 4 Content of human  $\alpha$ -lactalbumin in serum ( $\mu\text{g}$   $\alpha$ -lactalbumin/l serum)

A ( $\square$ )=4 male adults  
 B ( $\circ$ )=6 non-pregnant women  
 C ( $\blacksquare$ )=4 pregnant women  
 D ( $\blacktriangle$ )=9 cord blood samples.  
 ( $\bullet$ )=Group I, 11 term infants aged <1 day  
 ( $\triangle$ )=Group II, 8 cow's milk formula fed infants  
 ~ =median values

weeks of gestation: 20, 20, 30, and 33 respectively). Nine cord blood samples were analysed (Fig. 4). Median value was 35  $\mu\text{g/l}$  with range 22–72  $\mu\text{g/l}$ .

Figure 4 also illustrates the results in 11 healthy term infants aged <1 day, blood samples taken before they had got any human milk. Median value was 15  $\mu\text{g/l}$  with range <5–50  $\mu\text{g/l}$ .

In seven of eight serum samples from cow's milk formula fed infants there were no measurable amounts of  $\alpha$ -lactalbumin (Fig. 4). One infant had 29  $\mu\text{g/l}$ .

Serum from four preterm infants, aged 1–27 days, born with gestational age 29–34 weeks showed 952, 238, 190, 140  $\mu\text{g/l}$  serum/l human milk/kg body weight (Fig. 5).

Nineteen breast fed term infants were divided into three different groups according to age (Fig. 5).

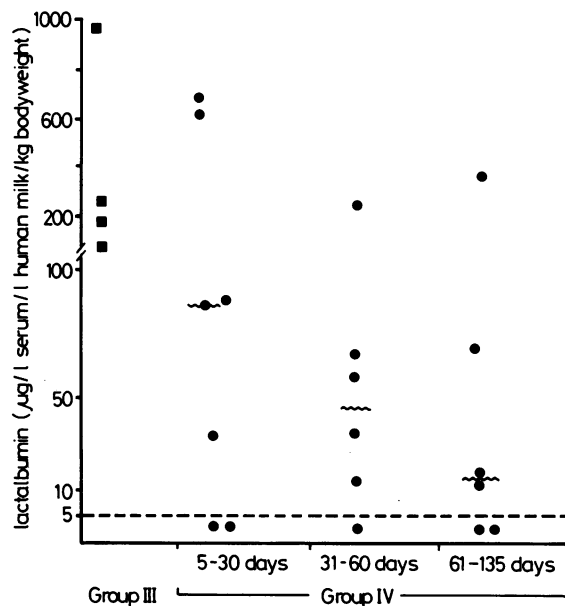


Fig. 5 Content of human  $\alpha$ -lactalbumin in serum ( $\mu\text{g}$   $\alpha$ -lactalbumin/l serum/l human milk/kg body weight). Group III ( $\blacksquare$ )=4 preterm breastfed infants Group IV ( $\bullet$ )=19 term breastfed infants of different ages ~ =median value

Seven infants aged 5–30 days had a median value of 85  $\mu\text{g/l}$  serum/l human milk/kg body weight with range <5–673. Six infants aged 31–60 days had a median value of 43  $\mu\text{g/l}$  serum/l human milk/kg body weight with range <5–220. Six infants aged >60 days had a median value of 12  $\mu\text{g/l}$  serum/l human milk/kg body weight with range <5–274.

Figure 6 shows the results in three breast fed term infants followed with repeated blood samples before and after a human milk feeding. Maximal concentrations of  $\alpha$ -lactalbumin were reached 30–60 minutes after the start of the meal.

## Discussion

A reliable radioimmunological method had been developed to measure content of human  $\alpha$ -lactalbumin in serum samples. In all studies published to date on absorption of macromolecules, serum concentrations of heterologous food proteins have been measured.<sup>5,8</sup> As described by Danneus *et al*<sup>8</sup> such phenomena as local intestinal and systemic immune responses must be considered when evaluating the results. In this respect the method described here is advantageous.

When using polyethylene glycol absorption test in

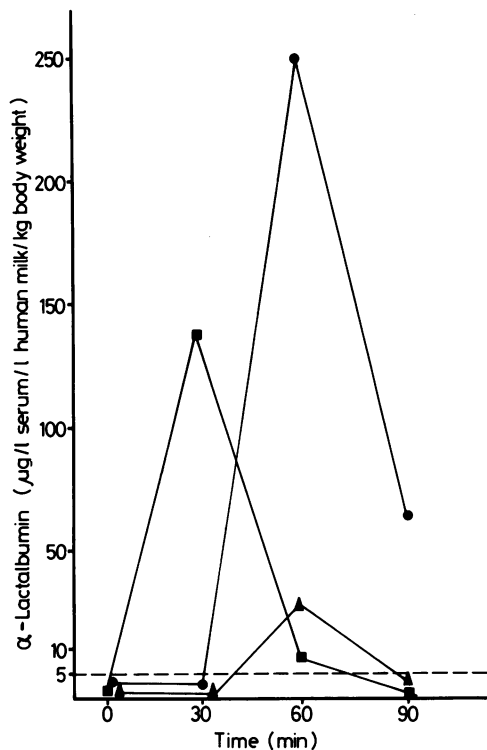


Fig. 6 Content of human  $\alpha$ -lactalbumin in serum ( $\mu\text{g}$   $\alpha$ -lactalbumin/l serum/l human milk/kg body weight) in three infants (Group V) in relation to a human milk feed starting at time 0 minutes.

■—■ = healthy infant aged 22 days  
 ●—● = infant aged 29 days. Had a diaper dermatitis  
 ▲—▲ = healthy infant aged 37 days

the study of gut permeability it should be borne in mind that these molecules might cross the intestinal mucosa at different rates because of size regardless of whether the mucosa is permeable or not to protein.<sup>11</sup>

Neither male serum samples nor those from non-pregnant women showed measurable amounts of  $\alpha$ -lactalbumin. No  $\alpha$ -lactalbumin could be expected to exist in serum from infants fed a cow's milk formula (age 21–187 days). In seven of eight infants no measurable amounts were found, but one infant had a concentration of 29  $\mu\text{g}/\text{l}$ , for which we have no explanation. This infant was a girl aged 21 days, staying in hospital because of feeding problems. Her records do not show whether she may have been fed human milk, but of course this could be a possible explanation.

Serum from pregnant women showed high amounts of  $\alpha$ -lactalbumin (highest values during late

pregnancy). In cord blood the median value was 35  $\mu\text{g}/\text{l}$ . In serum from infants 0–24 hours old before receiving breast milk the value was 15  $\mu\text{g}/\text{l}$ . These amounts of  $\alpha$ -lactalbumin must be of maternal origin, passing from mother to infant *via* placental circulation.

Gastrointestinal absorption of antigenically intact proteins has been shown to be increased in the neonatal period of several mammalian species. The calf and piglet have a gut which is freely permeable to macromolecular proteins during the first few days post partum.<sup>11 16</sup> Also in human infants a potential for increased absorption in early life exists.<sup>2</sup>

In this study human  $\alpha$ -lactalbumin could be detected in serum from 18 of the 23 breast fed infants indicating that this protein is absorbed antigenically intact through the intestinal mucosa. The four preterm infants had higher serum concentrations than the term infants of the same age since birth. This is in agreement with the results of Robertson *et al*<sup>5</sup> who measured serum concentrations of bovine  $\beta$ -lactoglobulin in preterm and term neonates.

Serum concentrations of  $\alpha$ -lactalbumin in the breast fed term infants aged 5–135 days decreased as age increased. Thus, a significant absorption of this macromolecule occurs in term infants even up to three to four months after birth. These results agree with those of Eastham *et al*<sup>3</sup> who found higher concentrations of serum antibodies to food antigens during the first three months. Our results are in contrast with those of Robertson *et al*<sup>5</sup> who could not detect any  $\beta$ -lactoglobulin in serum from term neonates and they concluded that gut closure occurs gradually with fetal maturation and is normally complete by birth in the term neonate. This discrepant result may be caused by methodological differences and by the different proteins studied.

The concentration of human  $\alpha$ -lactalbumin in the blood may be the result of several functions such as the proteolytic capacity in the gut, the transfer of the antigenic protein from the gut lumen to the blood, and the clearance of the protein from the blood *via* the kidneys and perhaps also *via* the gut.

From a physiological point of view it is tempting to speculate that the phenomenon of an increased absorption of macromolecules in neonates is a naturally occurring event leading to a state of systemic tolerance to proteins in the normal food.<sup>17</sup> An increased absorption of macromolecules acting as antigens may, however, be associated with a variety of diseases<sup>18</sup> such as gastrointestinal allergy, inflammatory bowel disease, coeliac disease, chronic hepatitis, and autoimmune diseases.

In a study by Kilshaw *et al*<sup>19</sup> a method was developed to measure absorption of macromolecules

in calves by measuring serum concentrations of bovine  $\beta$ -lactoglobulin. Significant increases in serum concentrations were shown 30–60 minutes after a cow's milk feeding. It is of interest to note that similar results were found in this study with maximal serum concentrations of human  $\alpha$ -lactalbumin 30–60 minutes after a human milk feeding, in the three infants studied.

Moreover Kilshaw *et al*<sup>19</sup> showed that the absorption of this, for calves, homologous protein increased during challenge with soy flour in a group of calves sensitised to soy protein. Analogous to this the method described here might be useful in diagnosing – for example, cow's milk allergy. In this disorder absorption of human  $\alpha$ -lactalbumin may be increased immediately after a cow's milk challenge.

We suggest that monitoring serum concentrations of human  $\alpha$ -lactalbumin before and after a human milk feeding can be used as a test for macromolecular absorption in immature and mature infants and in various pathological conditions such as food allergy, infantile colic, coeliac disease, gastroenteritis, and inflammatory bowel disease. Studies elucidating these questions are in progress.

In conclusion, human  $\alpha$ -lactalbumin is considered a suitable marker for investigation of macromolecular absorption in physiological and pathological conditions.

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