

The concentration of bovine IgG in human breast milk measured using different methods

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The bovine IgG concentration in human breast milk was measured using three different methods: competition radioimmunoassay (RIA), competition enzyme-linked immunosorbent assay (ELISA) and sandwich ELISA. In 36 samples of human breast milk taken from mothers on diets including cow's milk, the concentration of bovine IgG was significantly higher when measured by either competition RIA or ELISA (mean 324.9 ± 97.4 ng/ml and 396.7 ± 58.7 ng/ml, respectively) than by sandwich ELISA (3.44 ± 0.48 ng/ml). In 7 samples of human breast milk taken from mothers on cow's-milk-free diets of more than one month, the concentration of bovine IgG was still within a detectable range (112.3 ± 41.4 ng/ml) using competition ELISA, whereas it was not detected when using sandwich ELISA, suggesting that competition ELISA might measure some unknown substances in addition to bovine IgG. Gel filtration of pooled human breast milk revealed three large fractions. The concentration of bovine IgG in every fraction was significantly higher using competition compared with sandwich ELISA, suggesting that the difference between these assays does not result from measuring different quantities of bovine IgG, or of fragments of this protein. The time course of the bovine IgG level was different between each human breast milk sample after taking cow's milk. We showed that human breast milk contains bovine IgG at a concentration of approximately 3 ng/ml when analysed using sandwich ELISA. This level is comparable to the quantity of either casein or of β -lactoglobulin in human breast milk. Bovine IgG might be an important allergen in cow's milk allergy due to the quantity in human breast milk. □ *Bovine IgG, cow's milk, enzyme-linked immunosorbent assay, human breast milk, radioimmunoassay*

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Human breast milk contains a number of proteins which are partially derived from substances in the diet of the mother (1–10). For some infants taking breast milk, these proteins in human breast milk can be the allergens of atopic diseases, such as atopic dermatitis or infantile colic. Cow's milk allergy is relatively common among food allergies in infants. Among a number of proteins present in cow's milk, casein, α -lactalbumin and β -lactoglobulin have been shown to be the major allergens (11, 12).

Recently, Clyne & Kulczycki (13) demonstrated that bovine IgG, a protein contained in cow's milk, was present at concentrations of 0.1–8.5 μ g/ml in human breast milk. They also showed that the concentration of bovine IgG in human breast milk from mothers with colicky infants was significantly higher than those without colic. Their findings suggest that bovine IgG may be very important as a causative allergen in cow's milk allergy. According to their report, bovine IgG could be absorbed highly selectively from the maternal intestine and secreted in human breast milk, because bovine IgG is present in cow's milk at a concentration of only 0.6 mg/ml (14). Another possibility may be that the

method used by Clyne & Kulczycki (competition radioimmunoassay (RIA)) measures unknown substances in addition to bovine IgG, resulting in an apparently very high concentration in human breast milk.

In this study, we measured the concentration of bovine IgG in human breast milk using three different methods: competition RIA, competition enzyme-linked immunosorbent assay (ELISA) and sandwich ELISA. As a result, we found that the concentrations of bovine IgG were remarkably different between these assays. To demonstrate the differences between the assay methods, we compared concentrations of bovine IgG in human breast milk taken from mothers on a cow's-milk-free diet (also excluding any milk products).

Materials and methods

Subjects

Human breast milk samples were taken from groups of mothers on different diets. The first group consisted of

36 healthy mothers (1–6 months postpartum) on diets including cow's milk. In 3 of these subjects (3–6 months postpartum), the time course of the bovine IgG level was evaluated using sandwich ELISA. The subjects drank 200 ml of homogenized cow's milk after a 24-h diet strictly free of cow's milk and milk products. Breast milk samples were collected 1, 2, 3, 4, 5, 6, 7, 8 and 12 h after drinking the homogenized milk. The second group consisted of 7 healthy mothers (3–9 months postpartum) placed on a diet completely free of cow's milk and milk products for at least one month. The children of these mothers had atopic dermatitis or other cow's milk allergies with symptoms including diarrhoea and vomiting. We confirmed that these mothers were complying with their diets by interviewing them at two-week intervals and by checking their logs, listing food intake, which they were required to keep. We checked for any cow's milk or cow's milk products, including confectionery such as ice cream or candy made from cow's milk, dairy products, such as butter, cheese and yogurt, and foods and medicines containing casein or lactose which are components of cow's milk and beef.

Collection and processing of breast milk samples

Breast milk samples were collected in sterile tubes using manual expression. The samples were promptly centrifuged at 14 000 g for 1 h at 4 °C. The clear or translucent layer after removal of the fatty layer and cellular elements was stored at –20 °C until analysis.

Measurement of bovine IgG in human breast milk

Three methods were utilized for the measurement of bovine IgG in human breast milk. All the tests were performed in duplicate. The bovine IgG was purchased from Cappel Camp, West Chester, USA and was diluted with phosphate-buffered saline (PBS) from 0.2 ng/ml to 20 µg/ml for the standard curve of each method. The concentration of bovine IgG in human breast milk was read from the standard curve for each method. Bovine IgG (Cappel, West Chester, USA) showed a single peak at molecular weight (MW) 160 kD on high-performance liquid chromatography (HPLC).

Competition RIA. Polystyrene tubes (13 × 100 mm) (Becton Dickinson, Oxnard, CA) were coated with 1 ml of rabbit anti-bovine IgG (IgG fraction) (Cappel, West Chester, USA) (1:500 dilution) in 0.01 mol/l Tris buffer (pH 9.0) by incubation overnight at 25 °C. Bovine IgG (Cappel, West Chester, USA) was labelled with ¹²⁵I by the chloramine-T method (15) with 0.2 mCi of carrier-free ¹²⁵I and 10 µg of chloramine-T per 80 µg of protein. Percent binding was 61.23% and specific activity was 9.3 × 10⁵ cpm/µg. Test mixtures which consisted of samples (100 µl) or a series of the standard (100 µl) and ¹²⁵I-labelled bovine IgG (100 µl) were added to the antiserum-coated polystyrene tubes and incubated for 3

h at 37 °C. The contents of the tubes were aspirated and the tubes were washed and radioactivity was counted using a gammacounter (Auto Well System, JDC-755, Aloka). Using this method, intra-assay and inter-assay coefficients of variation are 3.8% and 1.2%, respectively.

Competition ELISA. A 96-well polystyrene, microtitre plate (Corning, New York, USA) was coated with rabbit anti-bovine IgG (IgG fraction) (Cappel, West Chester, USA) (1:500 dilution) in 0.1 M carbonate buffer, pH 9.6, 100 µl/well overnight at 25 °C and washed four times with PBS with Tween 20 (PBS-T). To minimize non-specific binding, 2% human serum albumin (HSA) was added and incubated for 3 h at 37 °C. Test samples and a series of the standard were added to the plate at a volume of 100 µl per well. Horse-radish peroxidase (HRP) conjugated bovine IgG (1:200 dilution), which was made by the maleimido method (16), was added to the wells without prior washing and incubated for 3 h at 37 °C. The excess was removed by washing. Finally, enzyme substrate (O-phenylenediamine/H₂O₂) was added giving a colour change proportional to the concentration of the antigen and stopped with 4N H₂SO₄. The optical density (OD) at 490 nm was recorded by Immuno Reader NJ-2000 (Jasco, Tokyo, Japan). Using this method, intra-assay and inter-assay coefficients of variation are 6.7% and 2.6%, respectively.

Sandwich ELISA. Test samples and a series of the standard were added to the same microtitre plates (as with competition ELISA) which were coated with anti-bovine IgG at a volume of 100 µl per well. After incubation for 2 h at 37 °C, these plates were washed four times with PBS-T. HRP conjugated rabbit anti-bovine IgG (EY LABS INC, San Mateo, USA) (1:2000 dilution) was then added and incubated on the plate for 1 h at 37 °C. After washing with PBS-T, the enzyme substrate was added and the OD at 490 nm was recorded. Using this method, intra-assay and inter-assay coefficients of variation were 4.6% and 0.8%, respectively.

Recovery test for bovine IgG

The recovery rate for bovine IgG in each of the methods was compared by adding a known concentration of bovine IgG (20, 200, 2000 ng/ml, Cappel, West Chester, USA) into human breast milk, in which the concentration of bovine IgG had already been measured using the same method.

Gel filtration of human breast milk on Sephadex G-50

The samples of human breast milk were fractionated on the basis of molecular weight by gel filtration. A sample

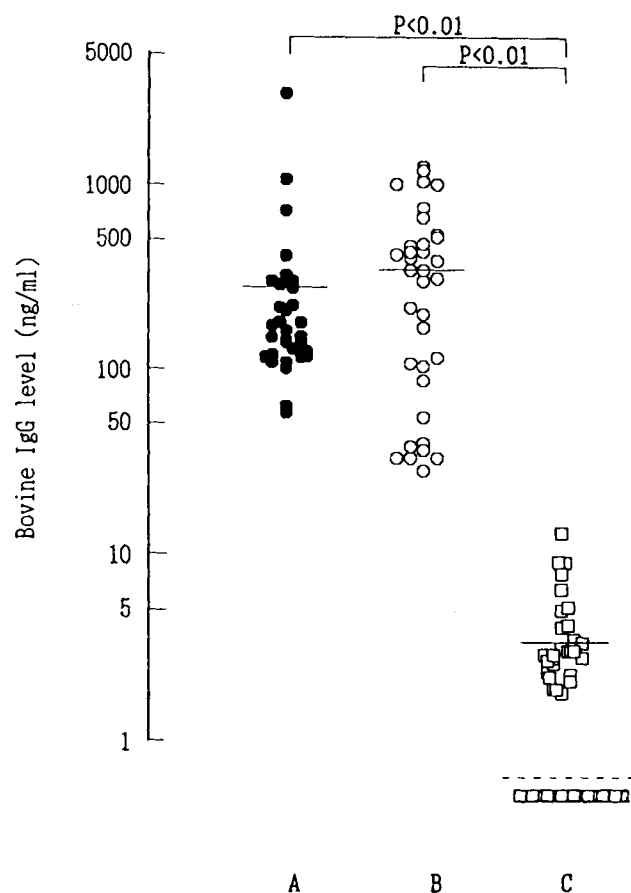


Fig. 1. Concentrations of bovine IgG (ng/ml) in 36 human breast milk samples measured using three different methods. These samples were obtained from 36 mothers on diets including cow's milk. A (●): measured using competition RIA (mean 324.9 ± 97.4 ng/ml); B (○): measured using competition ELISA (mean 396.7 ± 58.7 ng/ml); C (□): measured using sandwich ELISA (mean 3.44 ± 0.48 ng/ml).

of 10 ml was applied to a Sephadex G-50 (Pharmacia LKB, Uppsala, Sweden) fine column (2×100 cm) and eluted with distilled water at room temperature. The rate was 20 ml/h. The eluted fluid was collected in a volume of 1.5 ml per tube and the OD at 280 nm was recorded. The column was calibrated with a molecular weight marker (Biolad, Richmond, CA).

Statistical analysis

Data are reported as mean $\pm$ SE. Non-parametric analysis of variance (Friedman two-way analysis of variance) was used to determine the significant variance between groups. If a significant variance was found, a Wilcoxon's matched-paired signed ranks test was performed to assess the significant difference between individual groups. A p value less than 0.05 was considered significant.

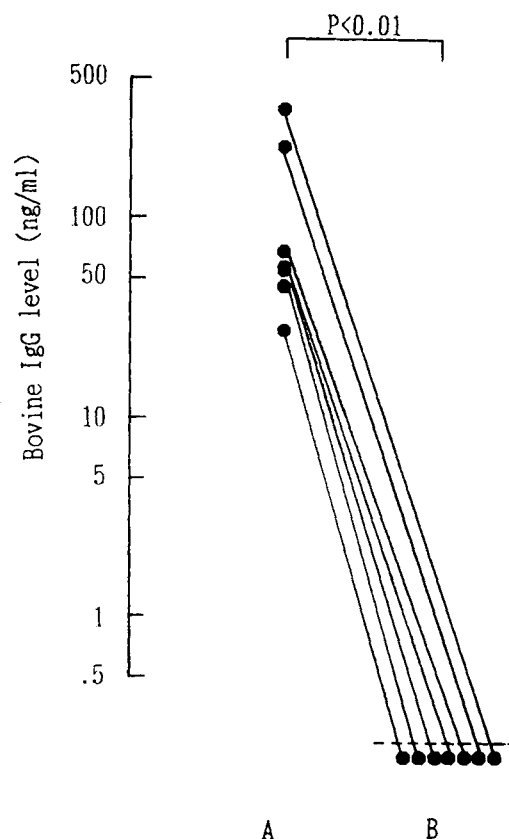


Fig. 2. Concentrations of bovine IgG in seven human breast milk samples measured using two different methods. These samples were obtained from seven mothers on strict cow's-milk-free diets. A: measured using competition ELISA; B: measured using sandwich ELISA.

Results

Concentration of bovine IgG in human breast milk

Using competition RIA, bovine IgG was detected (detection limit = 20 ng/ml) in all 36 samples from mothers on diets including cow's milk, ranging from 58 to 3133 ng/ml (324.9 ± 97.4 ng/ml, mean $\pm$ SE). Using competition ELISA, it was detected (detection limit = 2 ng/ml) in the same samples, ranging from 33 to 1220 ng/ml (396.7 ± 58.7 ng/ml). Using sandwich ELISA, bovine IgG was detected (detection limit = 0.2 ng/ml) in 25 of the 36 samples from mothers on diets including cow's milk, ranging from 1.0 to 13.0 ng/ml (3.44 ± 0.48 ng/ml) (Fig. 1).

Using competition ELISA, bovine IgG was detected in all of the 7 samples from mothers on cow's-milk-free diets, ranging from 28 to 363 ng/ml (112.3 ± 41.4 ng/ml). In none of the samples was bovine IgG detected using sandwich ELISA (Fig. 2).

Time course of bovine IgG levels in human breast milk

As shown in Fig. 3, the time courses of the bovine IgG

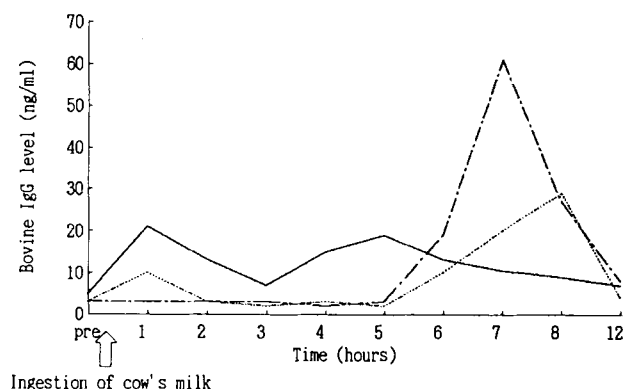


Fig. 3. Time courses for appearance of the bovine IgG levels in human breast milk measured using sandwich ELISA. Human breast milk samples were obtained from three mothers up to 12 h after ingestion of cow's milk followed by more than 24 h on cow's-milk-free diets.

levels were different for human breast milk from three mothers. Two of the samples showed a biphasic increase, whereas the other showed a single peak at around 6 h after ingestion. In this case, the peak value reached as high as 60 ng/ml (Fig. 3).

Recovery test of bovine IgG

With all three methods, the recovery rates of bovine IgG when added to human breast milk in known concentrations were approximately 100%.

Concentration of bovine IgG in gel-filtrated samples

Gel filtration of pooled human breast milk from three mothers on diets including cow's milk produced three principal fractions. The first, second and third fraction were eluted at about MW 300–20 kD, MW 20–10 kD and MW 5 kD >, respectively (Fig. 4).

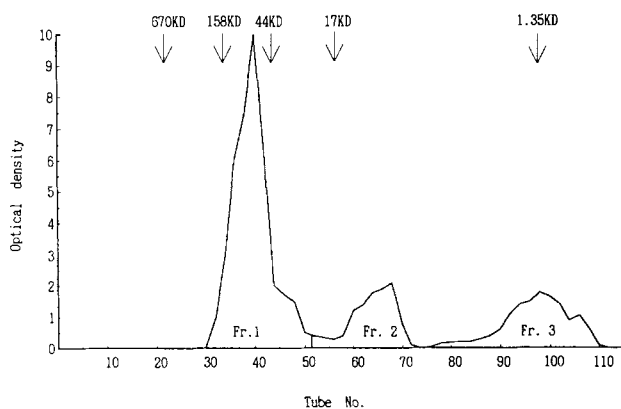


Fig. 4. Gel filtration curve of pooled human breast milk on Sephadex G-50. Fr. 1: MW 300–20 kD; Fr. 2: MW 20–10 kD; Fr. 3: MW 5 kD >. ↓ = Molecular weight marker.

Gel filtration of pooled human breast milk from four mothers on cow's-milk-free diets also produced three principal fractions. In samples from mothers on diets including cow's milk, the concentration of bovine IgG in the first, second and third fractions were 229.5, 14.8 and 459.6 ng/ml using competition ELISA and 15.5, 4.0 and 7.2 ng/ml using sandwich ELISA, respectively.

In samples from mothers on cow's-milk-free diets, the concentration of bovine IgG in the first, second and third fractions were 75.5, 22.0 and 131.1 ng/ml using competition ELISA, respectively. Using sandwich ELISA, the concentration of bovine IgG in every fraction was below the detectable limit.

Discussion

We have shown that the concentration of bovine IgG in human breast milk was unexpectedly different depending on the method of measurement. When measured using competition ELISA, the concentration of bovine IgG was still high in human breast milk even from mothers on cow's-milk-free diets, suggesting that some unknown substances in addition to bovine IgG may also be measured using this method. Conversely, in samples measured using sandwich ELISA, bovine IgG was below the detectable limit, suggesting that this method accurately measures bovine IgG. We do not know the reason why the concentration of bovine IgG was so high when measured using competition ELISA. It is possible that our assay system is not reliable when measuring bovine IgG. However, this is unlikely, because the recovery rate when adding known concentrations of bovine IgG was accurate. It is also possible that competition ELISA could detect another size of bovine IgG which could not be measured using sandwich ELISA, including fragments of this protein. However, this is also unlikely, because competition ELISA measures significantly higher concentrations of bovine IgG in every fraction of human breast milk compared with sandwich ELISA. As mentioned above, some substances which are present in human breast milk and cross react with bovine IgG might also be measured in addition to bovine IgG itself when the competition method was employed. Further study is required to reveal the reason for this difference.

Using sandwich ELISA, the mean value of bovine IgG was approximately 3 ng/ml in human breast milk from mothers on diets including cow's milk. The concentration of bovine IgG in human breast milk is comparable with that of β -lactoglobulin (approximately 0.1–20 ng/ml) (6–10) or casein (1.5–17 ng/ml) (10), which are present in cow's milk at concentrations (2.4 mg/ml or 23 mg/ml, respectively) greater than bovine IgG (0.6 mg/ml) (14).

The time course of the bovine IgG levels was shown to be different between each sample of human breast milk after ingesting cow's milk. Furthermore, the peak level

reached approximately 60 ng/ml at 6 h after ingestion by one of the mothers. This result suggests that we cannot simply compare the bovine IgG levels in human breast milk without considering the period after the last ingestion of cow's milk.

Our results suggest that bovine IgG may also be important as the causative allergen for cow's milk allergy in view of its concentration in human breast milk.

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