Detection of specific IgE to human milk proteins in sera of atopic infants

Annamaria Cantisani[a], Maria Gabriella Giuffrida[a], Claudio Fabris[b], Enrico Bertino[b], Alessandra Coscia[b], Roberto Oggero[c], Giovanna Monti[c], Paola Stroppiana[c], Amedeo Conti[a,*]

[a] *Centro Studio Alimentazione Animali, CNR, via P. Giuria 7, Torino, Italy*
[b] *Cattedra di Neonatologia dell'Università di Torino, Torino, Italy*
[c] *Dipartimento di Scienze Pediatriche e dell'Adolescenza, Ospedale Infantile Regina Margherita, Torino, Italy*

Received 26 May 1997; revised version received 14 June 1997; accepted 17 June 1997

Abstract Specific IgE (sIgE) for cow's milk proteins (CMP) have been reported to be present in blood sera of exclusively breast-fed infants. The aim of this study was to find whether the presence of sIgE to human milk proteins in the sera of exclusively breast-fed infants could explain the apparent detection of sIgE to CMP in infants that were never previously in contact with cow's milk. sIgE for human milk whey proteins were found in the blood sera of atopic infants, and these sIgE strongly cross-reacted with the corresponding CMP. In none of the sera examined were sIgE to bovine β-lactoglobulin detected.

Key words: Cow's milk allergy; Cross-reactivity; Human milk; β-Lactoglobulin; Mother's diet

1. Introduction

In exclusively breast-fed infants the presence in the blood serum of specific IgE (sIgE) to cow's milk proteins (CMP) has already been demonstrated [1]. The most frequently reported hypothesis to explain the presence of such sIgE in exclusively breast-fed infants are: (i) sensitization to CMP can occur in utero [2,3]; (ii) sensitization to CMP, in particular β-lactoglobulin (β-LG), can occur through ingestion by the mother of cow's milk with the diet and passage of CMP into breast milk [4–8].

However, the true passage of integer bovine β-LG into human milk, at least in healthy subjects, has already been discussed in a previous paper [9]. Moreover, clinical studies on the usefulness of keeping the pregnant and lactating mother's diet free from CMP in preventing cow's milk protein allergy (CMPA) have produced conflicting results, as reviewed by Fälth-Magnusson [10].

Other possible routes for sensitization to CMP in exclusively breast-fed infants have been suggested, including inadvertent neonatal supplementation of cow's milk-based formula, sensitization by inhaling [11] or through contamination of hands or household objects [8].

To explain the sensitization to CMP in exclusively breast-fed infants, a synthesis of sIgE primitively directed against human milk proteins that may cross-react with the CMP has never been considered.

This study shows that sIgE for human milk whey proteins is present in the blood serum of atopic infants, and that these sIgE strongly cross-react with the corresponding CMP.

*Corresponding author. Fax: (39) 11-650-5450.
E-mail: CONTI@ch.unito.it

2. Materials and methods

2.1. Subjects

Six breast-fed infants aged between 1 and 9 months with atopic dermatitis diagnosed as indicated by Hanifin and Rajika [12] were examined.

All infants tested positive for CMP at skin prick test (SPT, Neo Abellò-Madrid-Spain) and sIgE (chemiluminescence Magic Lite SQ-CIBA CORNIG). As control, two healthy infants with negative personal and family history for atopy, and with both SPT and sIgE negative for CMP, were examined. As IgE positive control, an infant with high total IgE level, but without sIgE to milk proteins, was also examined. Individual blood samples were centrifuged (3000 rpm, 10 min) and the sera obtained were divided into aliquots and stored at −20°C until use.

2.2. Specimens

Human milk samples were from two different pools of mature human milk, taken from four mothers on milk-rich diet (> 500 ml/day plus free intake of milk derivatives) and from four mothers on a CMP-free diet. In the latter, care was taken to verify that they completely avoided cow's milk and its derivatives including ready-made food products or mixed foods that may contain small traces of CMP. The samples were taken after the mothers had followed the diet for 2–4 weeks. Breast milk was always collected in the morning, 2 h after last meal ingestion, into sterile monouse tubes using an electric breast pump. Human milk samples were pooled and centrifuged (3000 rpm, 30 min, 20°C). The skimmed milk was then submitted to ultracentrifugation (189 000 × *g*, 120 min, 4°C) to separate casein (pellet) and residual lipids (top) from the clear solution (whey).

Informed consent was obtained from parents of all infants and from donor mothers, after a full explanation of the procedures.

Cow's whey proteins were obtained by ultracentrifugation (189 000 × *g*, 120 min, 4°C) of skimmed milk.

2.3. Sodium dodecylsulfate–polyacrylamide gel electrophoresis

Milk samples were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on Phast Gel-Gradient 8–25 gels (Pharmacia) as described by Laemmli [13].

2.4. Western blotting

Western blotting was carried out onto ProBlott membranes (Applied Biosystems) using a 25 mM Tris, 192 mM glycine, pH 8.3, transfer buffer. Complete transfer was achieved at 20 V, 25 mA, 1 W, 5 Vh, 15°C, for 1 h.

2.5. Immunostaining

The blotted membranes were incubated overnight at room temperature with individual serum diluted 1:10 with Tris-buffered saline (TBS). A second incubation was then done with an anti-human IgE, (ε-chain specific) peroxidase conjugate (Sigma) diluted 1:1000 with TBS, polyclonal antibody. Color was developed in the presence of H_2O_2 with 3-3′-diaminobenzidine as substrate. All the washing steps used TBS containing 0.2% Tween 20.

2.6. Cross-inhibition

A pool of sera of atopic infants sensitized to CMP was divided into aliquots and pre-incubated overnight at room temperature with 100, 250 and 1000 μl of either denatured bovine milk whey or human milk whey proteins. The proteins were denatured by boiling the whey sam-

PII S0014-5793(97)00828-4

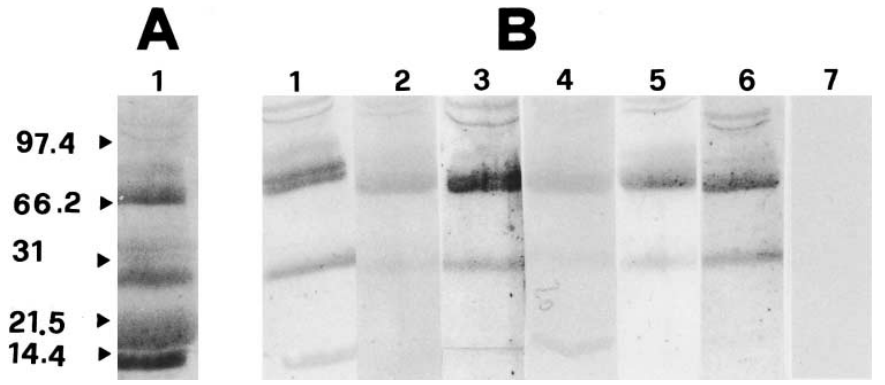

Fig. 1. SDS-PAGE and Western blotting of cow's milk (whey) proteins. Molecular size standards (in kDa) are indicated at the left. A: Lane 1: Coomassie Blue staining of cow's whey proteins after SDS-PAGE and Western blotting. B: Immunostaining of human IgE specific to cow's milk (whey) proteins. Lanes 1–6: cow's milk protein that had bound to IgE from six individual atopic infants. Lane 7: serum from a healthy infant with negative personal and family history for atopy and with STP and sIgE to CMP negative.

ples for 5 min. in the presence of 1% SDS and 2% β-mercaptoethanol. The pre-incubated sera were then incubated with human (H) and bovine (B) milk whey samples after SDS-PAGE and Western blotting, following the protocol given above for immunoblotting.

2.7. Sequencing

Sequence determinations were carried out after SDS-PAGE and Western blotting on ProBlott membranes, on a gas-phase Applied Biosystems Model 470A Protein Sequencer.

3. Results

The immunostaining of sIgE to CMP for each of the six atopic infants under investigation is in Fig. 1. The six patterns are rather similar, with only small quantitative differences. Bovine serum albumin, β-casein, lactoferrin and α-lactalbumin, in that order, were recognized as the most reactive CMP. None of the subjects showed sIgE to bovine β-LG.

Fig. 2A shows the SDS-PAGE Coomassie Blue staining of human milk samples from mothers on bovine milk-rich and milk-free diets. No significant differences are visible between the two patterns. Fig. 2B shows the immunostaining of sIgE of each infant serum reacting against human milk protein from the bovine milk-rich diet and milk-free diet pools. For each subject the IgE reactivity towards the human milk proteins in the milk-rich diet pool was almost identical to that towards those in milk-free diet pool. By comparison with the SDS-PAGE Coomassie Blue staining of the human milk proteins shown in Fig. 2A, human lactoferrin was identified as the most reactive protein against the serum IgE of each subject, followed by serum albumin, β-casein and α-lactalbumin in that order.

Fig. 3 shows the result of competitive inhibition of IgE binding. Inhibition of IgE binding to CMP (Fig. 3A) or to human milk protein (Fig. 3B) appears to be correlated to the amount of denatured bovine or human whey added in the pool of human sera (dose-dependent inhibition). No inhibition of IgE binding to either human or bovine milk proteins was seen when incubation was done with either native (not denatured) bovine or human whey.

When sera from healthy subjects and from a subject with high total serum IgE not specific for milk proteins were used as first antibody solution in the immunoblotting experiments, no sIgE to either CMP or human milk proteins were detected.

When cow's milk was suspended from the maternal diet and, at the same time, environmental precautionary measures were introduced, clinical observations revealed a marked regression of clinical symptoms in all infants. Subsequent direct challenge by administering 10 ml of cow's milk to each infant was in all cases highly positive.

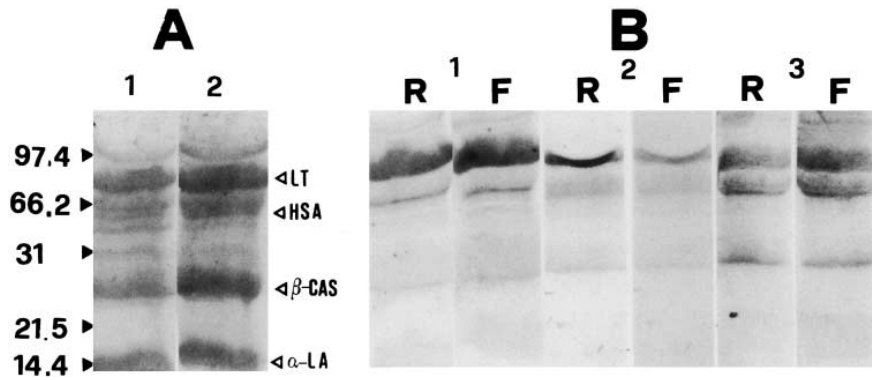

Fig. 2. SDS-PAGE and Western blotting of human milk whey proteins. Molecular size standards (in kDa) are indicated at the left. A: Lane 1: Coomassie Blue staining of human whey proteins from mothers on strictly controlled cow's milk-free diet. Lane 2: Coomassie Blue staining of human whey proteins from mothers on cow's milk-rich diet. Bands in lane 2 labeled with LT, HSA, β-CAS, and α-LA were identified by N-terminal sequence analysis as human lactoferrin, serum albumin, β-casein and α-lactalbumin, respectively. B: Lanes 1–3: human milk samples from mothers on cow's milk-rich diet (R) and strictly controlled cow's milk-free diet (F) were incubated with the sera three atopic infants corresponding to Lanes 1, 3, 6 in Fig. 1, after SDS-PAGE and Western blotting.

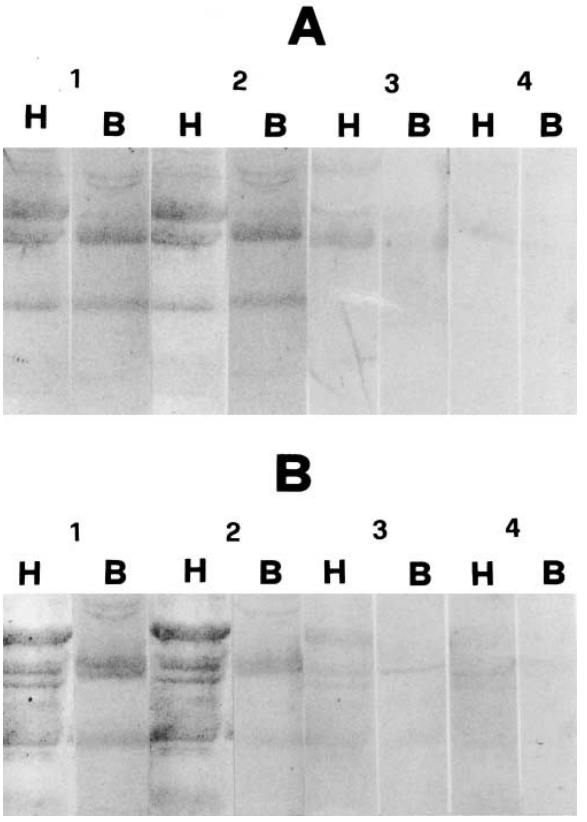

Fig. 3. Competitive inhibition of IgE binding. A: Pool of sera of atopic infants sensitized to CMP was divided into aliquots and pre-incubated overnight at room temperature with denaturated cow's milk proteins. The pre-incubated sera were then incubated with human (H) and bovine (B) milk whey samples after SDS-PAGE and Western blotting, following the protocol given above for immunoblotting (Fig. 1). Lane 1: Pre-incubation with dilution buffer (TBS) only; Lanes 2–4: Pre-incubation with 100, 250 and 1000 μl of denaturated cow's whey, respectively. B: The same as in (A), but pre-incubation of atopic sera was with human whey. Lane 1: Pre-incubation with dilution buffer (TBS) only. Lanes 2–4: Pre-incubation with 100, 250 and 1000 μl of denaturated human whey, respectively.

4. Discussion

The dose-dependent inhibition of sIgE to CMP binding to human milk proteins shown by denatured bovine whey proteins and vice versa leads to the hypothesis of the presence of common epitopes (cross-reactivity) between bovine and human milk proteins. Lack of inhibition by native bovine and human whey proteins suggests that such epitopes are probably linear (continuous) and should lie in the internal part of the molecules. The rather high degree of homology between the primary structures of human milk proteins and the corresponding bovine proteins (serum albumin, identity 76.6%; α-lactalbumin, identity 73.9%; lactoferrin, identity 69.5%; β-casein, identity 56.5%), is congruent with a possible cross-reactivity towards specific antibodies, since IgE antibodies from birch profilin-allergic individuals have been reported to cross-react with human profilin where the identity between the two proteins is only 34% [14].

Although the reactivity of the IgE of each subject towards the human milk proteins from the totally bovine milk-free diet pool could be explained as a cross-reaction between sIgE to CMP in subjects previously sensitized in utero and human milk proteins (but this sensitization should have occurred in six out of six subjects!), the constant absence of sIgE to β-LG, the only bovine protein absent in human milk, leads rather to hypothesize the presence of sIgE primitively directed against human milk proteins and cross-reacting with the homologous CMP.

In high-risk atopic infants, if adverse reactions to CMP are mediated by the sIgE primitively directed against human milk proteins, this could either occur at the first direct ingestion of CMP or because of the passage of small peptides of CMP in breast milk after the mother's ingestion of CMP with the diet. This might provide a rational explanation for Ghisolfi et al. [15] observation that CMPA, type I, appears at the first introduction of CMP mainly in infants who were previously exclusively breast fed.

The regression of clinical symptoms seen during the period when the infants were exclusively breast fed and were subjected to protective maternal dietary regimen and environmental measures, together with the strong positive reaction to direct challenge with CMP, suggest that IgE circulating in the serum of the controlled atopic infants, able to cross-react in vitro with both CMP and human milk proteins, have no clinical significance against human milk, but only against cow's milk.

We are at present engaged in some studies aimed at further clarifying the clinical significance of the cross-reactivity between human milk proteins and CMP, including one that will examine pairs comprising mother and breast-fed atopic infant.

References

[1] Hattevig, G., Kjellman, B., Sigurs, N., Grodzinsky, E., Hed, J. and Björstén, B. (1990) J. Allergy Clin. Immunol. 85, 108–115.
[2] Jacobsen, H.P., Halken, S., Høst, A. and Bender, L. (1995) Pediatr. Allergy Immunol. 6, (8) 98–102.
[3] Høst, A., Husby, S., Gjesing, B., Larsen, J.M. and Løwenstein, H. (1992) Allergy 47, 218–229.
[4] Axelsson, I., Jakobsson, I., Lindberg, T. and Benediktsson, B. (1986) Acta Paediatr. Scand. 75, 702–707.
[5] Björksten, B. and Kjellman, N.-I.M. (1990) Clin Exp Allergy 20, (3) 3–8.
[6] Harmatz, P.R. and Bloch, K.J. (1988) Ann. Allergy 61, 21–24.
[7] Barau, E. and Dupont, C. (1994) Allergy 49, 295–298.
[8] Kilshaw, P.J. and Cant, A.J. (1984) Int. Arch. Appl. Immunol. 75, 8–15.
[9] Bertino, E., Prandi, G.M., Fabris, C., Cavaletto, M., Di Martino, S., Cardaropoli, S., Calderone, V. and Conti, A. (1996) Acta Paediatr. 85, 543–549.
[10] Fälth-Magnusson, K. (1994) Pediatr. Allergy Immunol. 5, (1) 29–32.
[11] Høst, A. and Halken, S. (1990) Allergy 45, 587–596.
[12] Hanifin, J.M. and Rajika, G. (1980) Acta Dermatol. Venereol. 92, 44–47.
[13] Laemmli, U.K. (1970) Nature 227, 680–685.
[14] Valenta, R., Duchêne, M., Pettenburger, K., Sillaber, C., Valent, P., Bettelheim, P., Breitenbach, M., Rumpold, H., Kraft, D. and Scheiner, O. (1991) Science 253, 557–560.
[15] Ghisolfi, J., Olives, J.P., Le Tallec, C., Cohen, J. and Ser, N. (1995) Arch. Pédiatr. 2, 526–531.