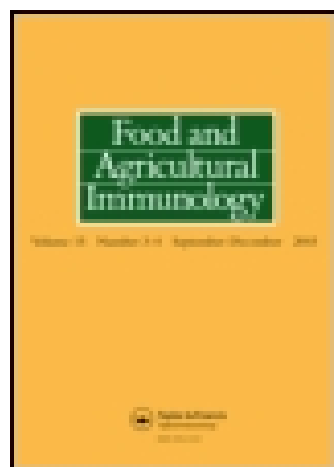




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Bovine β -lactoglobulin in hypoallergenic and ordinary infant formulas measured by an indirect competitive ELISA using monoclonal and polyclonal antibodies

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Bovine β -Lactoglobulin in Hypoallergenic and Ordinary Infant Formulas Measured by an Indirect Competitive ELISA Using Monoclonal and Polyclonal Antibodies

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Whey protein concentrates of cows' milk are used widely in dairy products including infant formulas. A major component is the potent allergen bovine β -lactoglobulin (BLG). Two easy and sensitive substrate-amplified indirect competitive ELISAs for BLG, one using a rabbit polyclonal antibody (PAb) K97 raised against heat-treated BLG, and another using a monoclonal antibody (MAb) 61B4 specific to the epitope Thr¹²⁵-Lys¹³⁵ are described. Furthermore, a new whey protein test kit specific for BLG was tested. A new video-based digital image processing system was used to read, evaluate and calculate data from the microtitre plates. The detection limit was 0.08 ng ml⁻¹ for the PAb assay, which was sufficient for the use of this assay as a test method for 'milk-free' products. The detection limit was 3.2 ng ml⁻¹ for the MAb assay and 144 ng ml⁻¹ for the commercially available kit. Thirteen commercially available infant formulas on the Danish market, including four hypoallergenic products and cows' milk, were tested. The BLG content varied from 0.2 to 727 μ g ml⁻¹, quantified with the PAb assay. The hypoallergenic infant formula with the lowest amount of BLG was Nutramigen containing about 0.1% residual BLG compared with the ordinary infant formula Nidina 1. The knowledge of residual BLG content in hypoallergenic infant formulas is important for a more qualified nutritional recommendation to parents with children genetically susceptible to allergies and for quality assurance of infant formulas claimed to be hypoallergenic.

Keywords: Cows' milk allergy, infant formulas, hypoallergenic, β -lactoglobulin, ELISA, monoclonal antibody, polyclonal antibody, video digital image processing

INTRODUCTION

Cows' milk allergy (CMA) occurs with a variable incidence of 1.9–5.2% among infants in Sweden, the US and Denmark (Jakobsson & Lindberg, 1979; Bock, 1987; Høst *et al.*, 1988). Childhood CMA is supposed to be linked to genetic susceptibility, an immature gastrointestinal tract, a rudimentary immune system and to environmental factors (Ferguson, 1984; Høst *et al.*, 1988; Savilathi & Kuitunen, 1992). An important environmental factor seems to be early exposure to cows' milk proteins, especially if they are given to the child in the first months after birth (Stintzing & Zetterström, 1979; Høst *et al.*, 1988; Savilathi & Kuitunen, 1992).

A major cows' milk allergen is bovine β -lactoglobulin (BLG) (Savilathi & Kuitunen, 1992; Brodard *et al.*, 1993), which is a low molecular weight protein of 18.3 kDa, existing in different forms varying only by a few amino acids (Brunner, 1977). A fraction of BLG exists as a dimer with a molecular weight of 36.6 kDa at pH 3–7 held together by non-covalent forces (McKenzie, 1971; Lyster, 1972). Since BLG is a non-human protein (Hambræus *et al.*, 1976) and the gastrointestinal tract is immature in newborn infants (Ferguson, 1984; O'Hagen *et al.*, 1987), small amounts of immunogenic BLG will be able to stimulate both the mucosal and the systemic immune system. Furthermore, BLG is heat-stable up to about 70°C (Kaminogawa *et al.*, 1987), and relatively resistant to proteolytic hydrolysis (Okamoto *et al.*, 1991) and thus still immunogenic when entering the gastrointestinal tract.

Most infant formulas are based on cows' milk. For both prophylactic purposes and the nourishment of allergic children, several hypoallergenic infant formulas are available. These are based on soya protein, cows' milk whey or casein, more or less proteolytically hydrolyzed and/or heat-denatured to diminish the content of potent allergens (Wahn, 1993). The difference between pre-treatment and fragmentation procedures during production and the heat treatment during preparation and gastrointestinal digestion have not been investigated. A pronounced prophylactic effect is surprising, but postulated and supported by several studies (Chandra *et al.*, 1989).

Several studies support the hypothesis that allergenicity correlates with immunogenicity (Atassi, 1980; Asselin *et al.*, 1988). Xenogeneic polyclonal antibodies (PABs) have been raised against BLG (Machtinger & Moss, 1986; Mäkinen-Kiljunen & Palosuo, 1992; Haque & Pruett, 1993), and epitope-specific monoclonal antibodies (MAbs) have been described (Granato & Piguët, 1986; Kaminogawa *et al.*, 1987). In order to get a better clinically related estimate of the intestinal allergenic challenge, the authors and others (Mäkinen-Kiljunen & Palosuo, 1992; Haque & Pruett, 1993) preferred to use PAB against BLG in the measurement of antigenic BLG content in foodstuffs. In previously published methods for quantification of BLG in infant formulas and other foodstuffs, rabbit antisera (Mäkinen-Kiljunen & Palosuo, 1992) or mouse antisera (Haque & Pruett, 1993) were used. Kaminogawa *et al.* (1987) produced several mouse IgG monoclonal BLG antibodies, of which 61B4 expressed specific reaction to the epitope Thr¹²⁵–Lys¹³⁵ (Kaminogawa *et al.*, 1989), which was one of four possible antigenic sites on BLG (Otani & Hosono, 1987; Takahashi *et al.*, 1990). Granato and Piguët (1986) produced a mouse monoclonal IgE antibody against BLG, but the epitope specificity was not described. However, a polyclonal BLG-specific IgE class antibody from patients with CMA would theoretically be the best detector in an ELISA for quantification of the allergenic BLG content.

The aim of this study was to develop two quantitative methods for estimation of residual antigenic BLG in hypoallergenic and ordinary infant formulas and in samples of milk. Three indirect competitive ELISAs—one assay with a MAb (Kaminogawa *et al.*, 1987), another with a PAB and a third with a commercially available whey protein test kit—were compared using samples of 13 infant formulas and cows' milk available on the Danish market. The absolute and relative differences in the BLG contents were determined and discussed.

MATERIALS AND METHODS

Standard and Samples

Thirteen infant formulas were prepared according to the manufacturers' instructions, except that magnetic stirring was carried out for approximately 15 min at room temperature. The suspensions were centrifugally filtered using a filter unit (UFC3 LTK 25, Millipore, Bedford, MA, USA) and frozen at -18°C until analysis. The liquid samples were directly centrifugally filtered and frozen at -18°C until analysis.

The ordinary infant formulas analyzed were: Nidina 1, Nan 1, Nan 2, (Nestlé Danmark A/S, Copenhagen, Denmark), Babymin A, Babymin B (Milupa A/S, Hørsholm, Denmark), Allomin, Allomin with lactulose, Allomin with rice flour (Beauvais Industry A/S, Tåstrup, Denmark) and Babymælk (MD-foods, Viby, Denmark). The following hypoallergenic infant formulas were analyzed: Nutramigen (Mead-Johnson, Nijmegen, The Netherlands), Profylac (ALK, Hørsholm, Denmark), Nan HA and Alfaré (Nestlé Danmark A/S, Denmark). Whole cows' milk was obtained from a local dairy (Kløver mælk, Amba, Fredericia, Denmark). Only one single batch of each type of infant formula and whole milk was tested. Nidina 1 served as the internal reference in all tests.

Purified BLG (L-0130, Sigma Chemical Co., St Louis, MO, USA) was used for immunization of rabbits, as antigen for the coating of plates and as the standard. All other chemicals were of analytical grade.

Antibodies

A rabbit PAb K97 was raised against heat-treated BLG in our laboratory. Heat denaturation was performed at 80°C for 60 min in Eppendorf tubes containing BLG in water (5 mg ml^{-1}) placed in a shaking Termomixer (type 5436, Eppendorf, Hamburg, Germany). The heat-treated BLG was used immediately as immunogen. The immunizations were carried out by multiple intra-dermal injections in the neck of a New Zealand White female rabbit with 1 ml BLG solution mixed with 1 ml Freund's incomplete adjuvant (Harboe & Ingild, 1983). The seventeenth bleed, taken 12 months after the first immunization, was used throughout this study having gained a high titer and a good antibody avidity. A mouse MAb IgG (61B4) reacting with the epitope Thr¹²⁵-Lys¹³⁵ on BLG was kindly provided by Dr A Ametani, Tokyo, Japan (Kaminogawa *et al.*, 1987). The antibody was purified from ascites by Protein A affinity chromatography (A. Ametani, 1991, personal communication).

ELISA

Two indirect competitive ELISAs have been established in 96-well Maxisorb F microtitre plates (Nunc, Roskilde, Denmark). The plates were coated with $100\text{ }\mu\text{l well}^{-1}$ of either $1\text{ }\mu\text{g ml}^{-1}$ BLG for the MAb assay or 5 ng ml^{-1} BLG for the PAb assay dissolved in carbonate-bicarbonate buffer, pH 9.5 (C-3041, Sigma, USA). Tris-buffered saline (TBS), pH 7.5, to which was added 0.05% (v/v) Tween 20 (TBS/Tw) was used as diluent and as washing solution. The plates were coated for 20 h at 4°C and washed five times with TBS/Tw on an ELISA microplate washer (#AM 60(4), Dynatech, Alexandria, VI, USA). Blocking was performed with a solution of 1% (w/v) polyvinyl pyrrolidone (PVP-40; Sigma, USA) and 1% (v/v) Tween 20 in TBS ($350\text{ }\mu\text{l well}^{-1}$) for 2 h at room temperature. Finally, the plates were washed five times, emptied, tapped dry, sealed with adhesive tape and stored at -18°C until used.

A standard stock solution of BLG was prepared in water at a concentration of 1 mg ml^{-1} , aliquoted into Eppendorf tubes and frozen at -18°C . Two-fold serial dilutions from $1.56\text{--}100\text{ ng ml}^{-1}$ (MAb assay) or five-fold from $0.064\text{--}10\text{ }000\text{ ng ml}^{-1}$ (PAb assay) were prepared for the standard curves. The internal reference Nidina-1 was diluted 10^4 -fold. Samples of ordinary infant formulas, whole and skimmed milk were diluted 10^4 -fold, and the hypoallergenic infant formulas were diluted from 10^1 to 10^3 -fold. The primary antibody

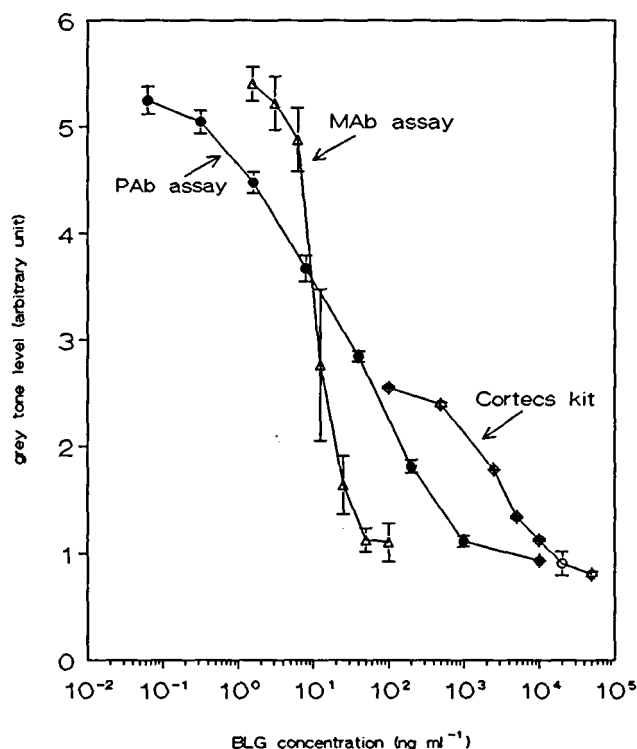


FIG. 1. Comparison of the standard curves of three indirect competitive ELISAs for β -lactoglobulin. The first assay used a rabbit PAb, the second used a mouse MAb and the third was a commercial assay using a sheep antibody (Cortecs kit).

was diluted 3×10^4 -fold for the PAb assay or 5×10^4 -fold for the MAb assay. All dilutions were carried out in TBS/Tw.

The coated plate was washed twice with TBS/Tw before use. Standards, samples and control ($100 \mu\text{l well}^{-1}$) were pipetted in triplicate by an automated laboratory station (Biomek 1000, Beckman Inc., Palo Alto, CA, USA) followed by the addition of the diluted primary antibody ($100 \mu\text{l well}^{-1}$). The sealed plate was incubated for 20 h at 4°C and washed five times. The sealed plate was then incubated for 1.5 h at room temperature with alkaline phosphatase-conjugated rabbit anti-mouse Ig (D-314, DAKO, A/S, Glostrup, Denmark) diluted 2×10^3 -fold in the MAb assay or alkaline phosphatase-conjugated swine anti-rabbit Ig (D-306, DAKO, Denmark) diluted 2×10^4 -fold in the PAb assay ($200 \mu\text{l well}^{-1}$). Polyethylene glycol (6000 Dalton, 4%, w/v) was added to the diluent to enhance the interaction between the primary and the secondary antibodies (Salonen & Vaheri, 1981). The plate was washed seven times before the substrate reaction was started.

A substrate amplification system was used according to the manufacturer's instructions (code 9589SA, Gibco BRLLife, Technologies, Gaithersburg, MD, USA). It consisted of NADPH as phosphatase substrate. The NADH formed is linked to a redox cycle driven by diaphorase and alcohol dehydrogenase, resulting in formation of the purple-coloured formazan dye. The substrate amplifier system runs $20 + 20$ min in the PAb assay and $10 + 10$ min in the MAb assay. This substrate amplification system can lower the detection limit 10 to 30 times compared with *p*-nitrophenyl phosphate (Self, 1985), and gives an purple colour which is excellent for our video digital image analyzer, when equipped with a complementary green filter (Cokin A004, Chromofilter SA, Paris, France).

TABLE 1. Parameters of the three indirect competitive ELISAs for BLG-calculated by the Immunofit statistic software using a four-parameter logistic curve fit. The measuring range is estimated from the precision profile curve corresponding to a CV% of less than 33%

	Polyclonal rabbit Ig antibody against heat-denaturated BLG PAb	Monoclonal mouse Ig antibody against BLG MAb	Polyclonal sheep Ig antibody against BLG Cortecs kit
Detection limit (ng ml ⁻¹)	0.08	3.2	144
Measuring range (ng ml ⁻¹)	0.1–1000	4–50	260–50 000
Correlation coefficient	0.9954	0.9935	0.9997

Whey Protein Test Kit

The whey protein test kit (Cortecs Diagnostics, Deeside, UK) was used according to the manufacturer's instructions except that the standard BLG dilution was extended in order to obtain the low concentration plateau of the assay. The standard range was then 100–50 000 ng ml⁻¹ BLG. The assay is an indirect competitive ELISA using a sheep antibody against BLG as detector and an anti-sheep Ig conjugated with peroxidase as secondary antibody. The ABTS substrate gave a green colour, which was measured with a complementary red filter (Cokin A003) by the video image analyzer. Some performance characteristics of this assay are given in the kit manual. Figure 1 shows that the standard curve of this assay is flat below 500 ng ml⁻¹ BLG.

Microtiter Plate Reading by Video Digital Image Processing

The end-point colour development was read by digital image processing using a CCD video-camera. The CREAM software, EIA module (Kem-En-Tec, A/S, Copenhagen, Denmark), was used to obtain the microplate scanning data in grey scale values (Brogren *et al.*, 1992; Sølve *et al.*, 1994). For further statistical analysis the scanning data were transferred to statistical software (Immunofit, Beckman, USA), which contained several curve-fitting features, carried out the calculation of detection limit, printed standard curves with outlier functions and plotted the precision profile of the assays.

RESULTS

The standard curves, one for the PAb assay, one for the MAb assay and one for the Cortecs kit, are shown in Figure 1. Comparison of performance of the three ELISAs shows that the detection limit is superior for the PAb assay (Table 1). Figure 2 illustrates the results of a parallelism study using the PAb assay and proves that matrix effects were not present in the diluted samples. The differences in residual BLG in the hypoallergenic infant formulas are shown in Table 2. Comparison of the absolute BLG content in the samples measured by the three assays show significant differences (Tables 2 and 3), but the relative contents with Nidina 1 or cows' milk as a reference were in better agreement.

DISCUSSION

The absolute BLG content quantified was strongly dependent on the test antibody used. This should be remembered when standardizing such assay procedures. In general, the ordinary infant formulas showed a slight variation in the BLG content and was at the same

TABLE 2. Comparison of BLG content in hypoallergenic infant formulas calculated by competitive ELISA using the PAb assay, the MAb assay and a Cortecs kit

Type	Source	Name	PAb assay		MAb assay		Cortecs kit	
			BLG ($\mu\text{g ml}^{-1}$) ^a	Nidina 1 (%)	BLG ($\mu\text{g ml}^{-1}$) ^b	Nidina 1 (%)	BLG ($\mu\text{g ml}^{-1}$) ^b	Whole milk (%)
Hypoallergenic infant formula	Hc	Nutramigen	0.2	0.1	0.1	0.05	4	0.1
	Hw	Alfaré	52	31	3	1	341	9
		Nan HA	67	40	3	1	658	17
		Profylac	3	1.8	0.3	0.1	22	1
Ordinary infant formula	Wm, Dw	Nidina 1	167	100	213	100	—	—
Bovine milk	Wm	Whole milk	157	94	—	—	3730	100

Hc, hydrolyzed casein; Hw, hydrolyzed whey; Wm, whole milk; Dw, de-salted whey.

^aAverage value of seven assays.

^bValue of one assay.

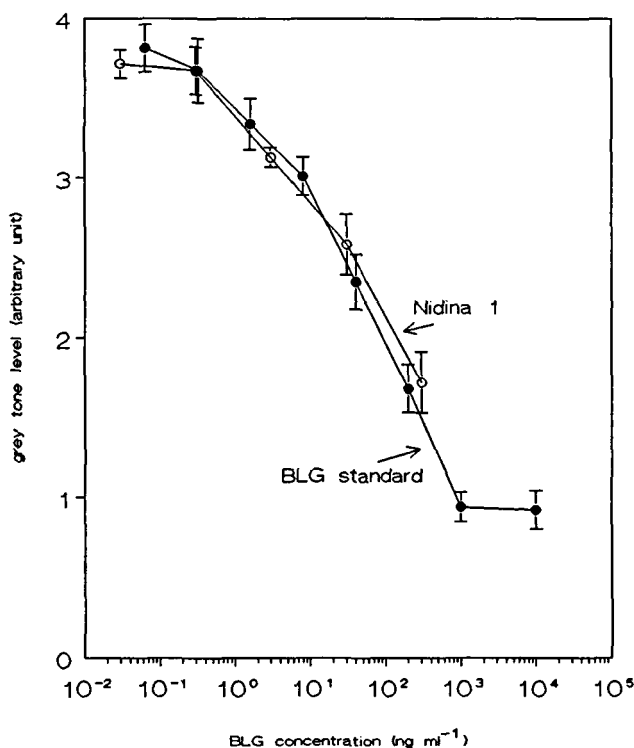


FIG. 2. Parallelism study performed with serial dilution of the β -lactoglobulin standard and the infant formula Nidina 1 measured by indirect competitive ELISA.

(level as in ordinary cows' milk. In contrast, the hypoallergenic infant formulas varied in BLG content from 0.1% (Nutramigen) to 40% (Nan HA) compared to Nidina 1 in the PAB assay. Almost the same relative BLG content was found with the Cortecs kit, showing 0.1% Nutramigen) and 17% (Nan HA) compared with whole milk. However, the MAb assay showed the BLG content to be about 10 times lower: from 0.05% (Nutramigen) up to 1% (Nan HA) compared with Nidina 1. These data were all in agreement with the expected lower level of BLG in the formulas based upon hydrolyzed whey or, in particular, if based upon hydrolyzed casein. The residual BLG was quantified easily in hypoallergenic infant formulas by our PAB-amplified ELISA, having a detection limit as low as 0.08 ng ml⁻¹. This was about 2000 times lower than the BLG content of Nutramigen.

A complete understanding of how the immune system recognizes BLG epitopes after heat treatment and gastrointestinal digestion was not the aim of this study. The epitope specificity or our rabbit antiserum is under evaluation to be sure that the specificity is related to the specificities of the IgE-mediated reactions in allergic patients. In preliminary studies, Hede and Helnov (1993) performed epitope mapping of BLG with our rabbit antibody on peptides obtained after trypsin digestion and separated by high-pressure liquid chromatography (HPLC). Several antibody-reactive peptides were found. The aim of our antibody production has been to obtain antibodies reactive to heat-stable BLG epitopes on hydrolyzed BLG to obtain a correct measurement of residual BLG, even in hypoallergenic infant formulas, after heat-denaturation which might occur during production or preparation of the infant formulas and after the intestinal digestion.

In addition, we also tested the mouse MAb 61B4 known to react with the epitope Thr¹²⁵-Lys¹³⁵ (Kaminogawa *et al.*, 1989) and found that the detection limit was not

TABLE 3. Comparison of BLG content in ordinary infant formulas calculated by competitive ELISA using the PAb assay and the MAb assay

Type	Source	Name	PAb assay		MAb assay	
			BLG ($\mu\text{g ml}^{-1}$) ^a	Nidina (%)	BLG ($\mu\text{g ml}^{-1}$) ^b	Nidina (%)
Ordinary infant formulas	Sm, C, Dwp	Allomin	189	113	—	—
		Allomin with lactulose	176	105	—	—
		Allomin with rice flour	159	95	318	149
	Dwp, Mp	Babymin A	177	106	233	109
	Dwp, Mp, Wpc	Babymin B	727	435	681	319
	Mc	Babymælk ^b	32	19	—	—
	Wm, Dw	Nidina 1	167	100	213	100
		Nan 1	194	116	246	115
	Wm	Nan 2	231	138	572	268
Bovine milk		Whole milk	157	94	—	—

Sm, skimmed milk; C, cream; Dwp, de-salted whey powder; Mp, milk powder; Wpc, whey protein concentrate; Mc, milk components; Wm, whole milk; Dw, de-salted whey.

^aAverage value of seven assays.

^bValue of one assay.

satisfactory when compared with our rabbit PAb assay. It was probably due to lower antibody affinity of the MAb and that only one epitope on the BLG antigen is reactive.

The sample preparation from infant formulas and other food sources might be an important parameter in the assay reproducibility. The colloid structure of milk, micelle formation and the chelating properties of many constituents need special attention (Skerritt, 1990; B. A. Morris, 1993, personal communication). We have observed that addition of ethylene diamine tetra-acetic acid and/or filtration removed confounding matrix effects (data not shown). The use of centrifugal ultrafiltration created good results in the parallelism study.

One research team has recently published an ELISA to detect BLG in infant formulas (Mäkinen-Kiljunen & Palosuo, 1992). Inter-comparison studies and quality assurance of these assays are strongly recommended. Differences in antibody specificity are obviously due to the various immunogen preparations, the animals used and immunization procedures executed. Furthermore, quantitative protein identification methods for individual food proteins are strongly dependent on the epitope specificity, cross-reacting properties of the antibodies and the standard material used.

We hope these immunological assays and their ability to quantify specific potent allergens in infant formulas might lead to a more precise evaluation and declaration. Consequently, better recommendations for nutritional behaviour in early childhood might be given. Food allergen specific assays might result in improved prevention of food allergies in general through a lower challenge of the most potent food allergens especially in newborns and in early childhood. Furthermore, these methods might be useful in surveying and controlling infant formulas claimed to be hypoallergenic. A more objective classification according to allergenicity should be possible. Quantitative screening methods for potent milk allergens other than BLG should be developed and used on the same products. Casein and other milk proteins are also reported to be allergenic (Brodard *et al.*, 1993; Wal *et al.*, 1993). Therefore, even infant formulas based on hydrolyzed casein might have residual allergenicity (Sampson *et al.*, 1991).

Prevention of CMA by nutritional means is more complicated than simply avoiding potent milk allergens. Immune hypersensitivity disorders, such as coeliac disease (Brogren *et al.*, 1993a) and autoimmune diseases (e.g. type 1 [insulin-dependent] diabetes) (Brogren *et al.*, 1993b) might be polyclonally activated by potent immunogenic dietary proteins. An increased level of antibodies to BLG has also been linked to earlier onset of type 1 diabetes without correlation with an early introduction of cows' milk (Dahlquist *et al.*, 1992). Several studies indicate a linkage between diet and the incidence of diabetes (Scott *et al.*, 1989; Scott, 1990; Hoorfar *et al.*, 1992). The potential total immunogenicity from a complex food intake might initiate the immune-associated disease process in genetically allergy-susceptible individuals. Therefore, the type, number, immunogenicity and quantity of all food allergens digested might together be potent allergy initiators. However, a high challenge of specific allergens seems related to an increased incidence of the related allergies, such as milk allergy in Scandinavia, rice allergy in Asia and peanut allergy in the US.

Controlled feeding studies with more or less hydrolysed formulas have shown an effect on the incidence of atopic disease in high-risk infants (Chandra *et al.*, 1989) and in removing symptoms of allergy (Brasseur *et al.*, 1993). In contrast, IgE antibodies to hydrolyzates of cow milk proteins can still be detected in approximately half the children with CMA (Plebani *et al.*, 1990). Since prevention is better than treatment, the goal must be to lower the content of residual allergens in infant formulas.

In conclusion, our present analytical studies on hypoallergenic infant formulas have clearly shown that a residual allergen content exists to various extents depending on the protein source and of the production protocol. Finally, it should be remembered that exclusive or extended breast feeding is recommended as the absolute best prevention for atopic disease.

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