

Allergenicity of Cows' Milk Formula Hydrolysates

1. In Vitro Evaluation by Fast Inhibition

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ABSTRACT

Cows' milk allergy is a significant clinical problem in infants. Treatment until recently has used soy, which is allergenic, and a casein hydrolysate (CH), which is costly and relatively nonpalatable. Since the CH and the newer whey hydrolysates (WH) are capable of causing anaphylaxis, we have evaluated the allergenicity, by IgE binding capacity, of several CH and WH using the in vitro fluoroallergosorbent test (FAST) inhibition assay comparing them to nonfat dried milk (NFDM) and Similac. Using sera from patients with cows' milk anaphylaxis and high IgE activity to whole milk (CM), casein (C), beta-lactoglobulin (BLG), and alpha-lactalbumin (ALA), we evaluated the CH Nutramigen and Alimentum and the WH Good Start (GS), Ultra, and Alfaré. Using a cumulative score of percentage fast inhibition (FI), the rank order of allergenicity was NFDM > Similac > GS > Ultra > Nutramigen > Alfaré > Alimentum. In general, all hydrolysates bound significantly ($p < 0.05$) less IgE than NFDM and Similac, whereas Good Start bound significantly more ($p < 0.05$) than the other hydrolysates, and Alimentum appeared to bind the least IgE antibody. However, all hydrolysates were capable of binding IgE specific to CM, C, BLG, and ALA. It is concluded that although the newer CH and WH hold promise with reduced allergenicity, they must be used with great caution, since they may be capable of causing anaphylaxis in the highly milk-allergic infant. It is recommended that tests for allergenicity, such as FI, become part of the food industry's protocol in developing new infant formulas with reduced allergenicity, which deserve further evaluation.

INTRODUCTION

COWS' MILK ALLERGY continues to be a significant clinical problem in infants, affecting approximately 1% of all infants.⁽¹⁾ Diagnosis has been based on a good clinical history, suggesting a cause and effect relationship between milk ingestion and allergic symptoms, skin tests or in vitro testing to determine the presence of specific IgE antibodies, and elimination followed by challenge when this can be done safely.

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Treatment of the cows' milk-allergic infant has used hypoallergenic formulas, including soy protein isolates and casein hydrolysates (CH), since soy is not truly hypoallergenic.⁽²⁾ Since CH are expensive and not very palatable, there has been a recent search for new hypoallergenic formulas that are safe, yet less expensive and more palatable. Several whey hydrolysates (WH) have been developed to fill this need. However, anaphylactic type reactions have been reported with these preparations in the highly milk-allergic infant.⁽³⁾ Since there might be some danger in studying potentially hypoallergenic formulas by challenging these infants, we have evaluated the allergenicity of several cows' milk hydrolysates by IgE binding capacity, using the fluoroallergosorbent test (FAST) inhibition technique.⁽⁴⁾

MATERIALS AND METHODS

Source of CM allergic sera

Sera were collected from patients with anaphylactic type symptoms from ingesting cows' milk, including urticaria, choking, asthma, rhinitis, and eczema (Table 1). All patients had IgE antibodies to whole milk and individual cows' milk proteins as measured by the FAST method.⁽⁵⁾

Preparation of milk products

The following products were used in the study: Carnation Dried Milk (NFD) (commercial source), Good Start (GS) (provided by Carnation), Ultra (provided by Carnation) (not commercially available), Similac (Ross, commercial source), Alimentum (Ross, commercial source) (freeze-dried for study), Nutramigen (Mead Johnson, commercial source), Alfaré (provided by Carnation, produced by Nestlé in Europe).

Stock solution of 10 g/dl (100 mg/ml) was prepared from each product by weighing out 5 g of powder and dissolving it by continuous stirring in phosphate buffered saline (PBS), pH 7.4, and making it up to 50 ml. This concentration approximates the formula for reconstitution of their powdered products. The dissolved products were centrifuged at 3,000 g for 1 hour at 6°C. The fatty layer was discarded. The homogeneous layer was carefully removed, aliquoted, and kept frozen until use. Care was exercised not to lose any component that might contribute to the allergenicity of the product. For the FAST inhibition procedure, the milk products were used either at 10 mg/ml concentration or in serial 10-fold ($\log_{10}$) dilutions prepared in PBS, pH 7.4, starting at 100 mg/ml.

FAST inhibition assay

The IgE FAST inhibition studies were carried out according to manufacturers' suggested protocol for determining relative potencies of allergenic extracts with minor modifications.⁽⁴⁾

TABLE 1. CHARACTERISTICS OF TEST SERA

Subject	Age	Symptoms	Total IgE (IU/ml)	Allergen-specific IGE-IU/ml			
				CM	Casein	ALA	BLG
1	25 years	Eczema, asthma, rhinitis	10,705	46 (IV) ^a	174 (IV)	7 (III)	40 (IV)
2	8 years	Anaphylaxis	349	230 (IV)	216 (IV)	16 (III)	185 (IV)
3	14 years	Anaphylaxis	77	44 (IV)	60 (IV)	1 (I)	10 (III)
4	12 years	Anaphylaxis	94	36 (IV)	44 (IV)	1 (I)	10 (III)
5	13 months	Anaphylaxis	106	8 (III)	2 (II)	<1 (0)	2 (III)
6	5 years	Anaphylaxis	241	82 (IV)	70 (IV)	8 (III)	136 (IV)

^a(), traditional 0-IV class RAST scoring system.

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Black microtitration wells were used as a solid support for antigens (whole milk, casein, alpha-lactalbumin, and beta-lactoglobulin) in the FAST test. FAST reagents were supplied in kit form by 3M Diagnostic Systems, Santa Clara, CA.

From the stock solutions of 100 mg/ml milk or formula, at least three 10-fold dilutions were prepared. In some cases, up to seven serial dilutions were made in diluent buffer. In glass tubes, 100 μ l of each dilution of milk products was mixed with 100 μ l of positive sera. The sera, when the IgE antibody content was very high, were prediluted with diluent buffer. The positive control (0% inhibition) consisted of 100 μ l of buffer and 100 μ l of positive serum. All the tubes were incubated in a 37°C waterbath for 1 hour. Then the tubes were centrifuged at 6°C for 30 minutes.

Antigen-coated microtitration wells were presoaked for 3–5 minutes with wash buffer. After removal of all fluid, 50 μ l of the diluted milk product–antiserum mixtures were pipetted into the wells. One well received diluent buffer (negative control), two wells received the serum–diluent mixture (positive control). From each milk product–serum mixture 50 μ l was pipetted into appropriate wells (in duplicate for the pooled serum study). The wells were incubated at 37°C for 1 hour. The wells then were washed three times with wash buffer. Then 100 μ l anti-IgE–enzyme–conjugate was added and incubated again for 1 hour at 37°C. After another washing, 100 μ l of fluorogenic substrate (4-methylumbelliferyl phosphate) was added and incubated until an appropriate fluorescent signal was developed. At that time, the fluorescence was recorded.

Calculation. The percent FAST inhibition was calculated according to the following formula.

$$\frac{\text{Average FSU of positive control} - \text{FSU of sample}}{\text{Average FSU of positive control} - \text{FSU of negative control}} \times 100$$

where FSU equals fluorosorbent unit.

The percent FAST inhibitions (ordinate) was plotted on a semilog paper against the concentration of the milk products (abscissa) for dose–response experiments. The intraassay variability was determined using 12 replicates and found to be approximately 8%.

Statistical analysis

A one-way analysis of variance (ANOVA) repeated measures design was used to compare FAST inhibition of milk proteins among all milk preparations at the 10 mg/dl concentration (Fig. 1). For dose–response experiments, the dose–response curves were converted to a linear expression by using a best fit analysis (Fig. 2).

RESULTS

Characteristics of sera used for FAST inhibition

Most subjects had high total serum IgE levels for their respective ages (Table 1). Specific IgE was high (class III or IV) for most subjects for whole milk protein (CM), casein (C), and beta-lactoglobulin (BLG) but were elevated in only three sera for alpha-lactalbumin (ALA). IgE levels were low for bovine serum albumin (BSA) for all subjects (data not shown). This prevented us from using FAST inhibition for studying the BSA content of the milk preparations.

Specificity of FAST inhibition assay

The specificity of this assay system was shown in two ways. First, the binding of perennial rye grass-specific IgE was not inhibited in perennial rye grass coated wells by prior incubation with cows' milk protein. Second, commercially available cows' milk protein, including BLG and C and CM, inhibited the

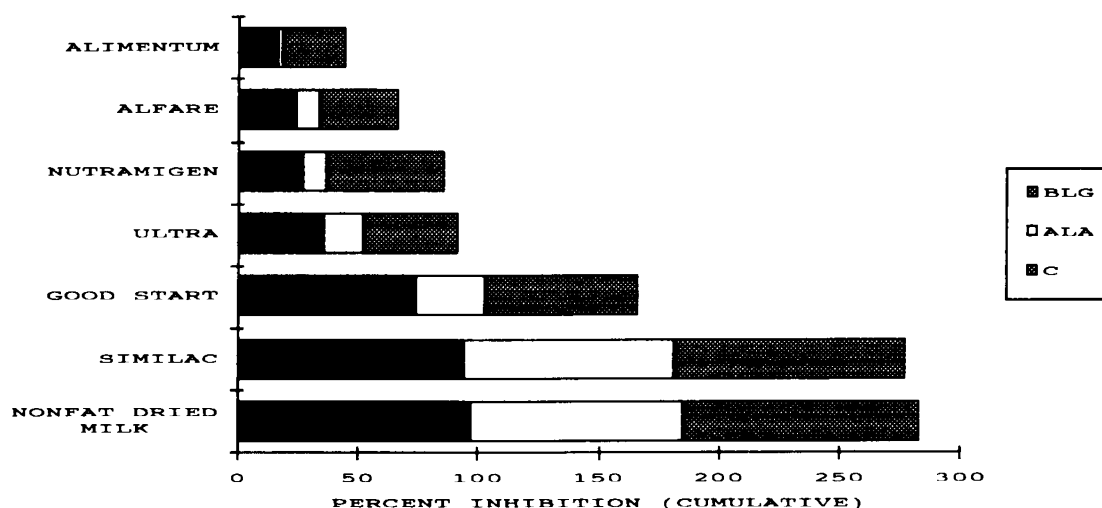


FIG. 1. Cumulative FAST inhibition. Shown are the total and individual milk protein FAST inhibition categories for all milk preparations, including beta-lactoglobulin (BLG), alpha-lactalbumin (ALA), and casein (C), using 10 mg/ml milk product concentration.

binding of milk-specific IgE to the milk protein coated wells in a dose-response fashion. For example, shown in Figure 2 is the dose-response FAST inhibition by BLG compared to the other milk products.

FAST inhibition (FI) profile

The FAST inhibition (FI) profiles for the milk formulas are shown in Figure 1. All preparations are compared for each of the major milk proteins—C, BLG, and ALA. Shown is the mean value of at least three subjects for each category. An overall score was developed by adding the percentage FI for each category for each milk preparation. The greater the score, the more allergenic the preparation. NFDM (283) and Similac (279) were very similar, and both were considerably greater than the hydrolysates. For the WH, GS (167) was greater than Ultra (90) and Alfaré (67), whereas for the CH, Nutramigen (86) was more allergenic than Alimentum (46). For whole milk protein (CM), all hydrolysates were significantly less allergenic than NFDM and Similac ($p < 0.05$). GS was significantly more allergenic ($p < 0.05$) than the other hydrolysates, whereas Alfaré and Alimentum appeared to be the least allergenic (data not shown). For individual milk proteins, the pattern was similar in regard to C. For BLG, all hydrolysates were less allergenic ($p < 0.05$) than Similac and NFDM. There were no significant differences, however ($p > 0.05$), between GS and Ultra or Nutramigen, but there was between GS and Alfaré or Alimentum ($p < 0.05$). For ALA, the pattern was similar to BLG, but only Alimentum was significantly less allergenic than GS ($p < 0.05$).

Relative allergenicity of formulas

In order to study more precisely the relative allergenicity of each formula, the six sera, characterized in Table 1, were pooled, and a dose-response curve was performed to determine the concentration of each formula that would result in 50% FI (FI_{50}) (Table 2, Fig. 2). From these curves, the relative FI_{50} was calculated by arbitrarily assigning NFDM a value of 1 μ g/ml for simplicity.

For any of the milk protein categories (CM, C, BLG, and ALA), Similac appears to be approximately one tenth as allergenic as NFDM. The hydrolysates were several orders of magnitude (10^3 – 10^5) less allergenic than either Similac or NFDM. Several formulas did not produce 50% inhibition at the highest concentration used (100,000 μ g/ml). GS appears to be at least 10-fold more allergenic than the other hydrolysates. However, in general, there were no specific differences between CH and WH as a group in regard to casein or whey protein categories studied.

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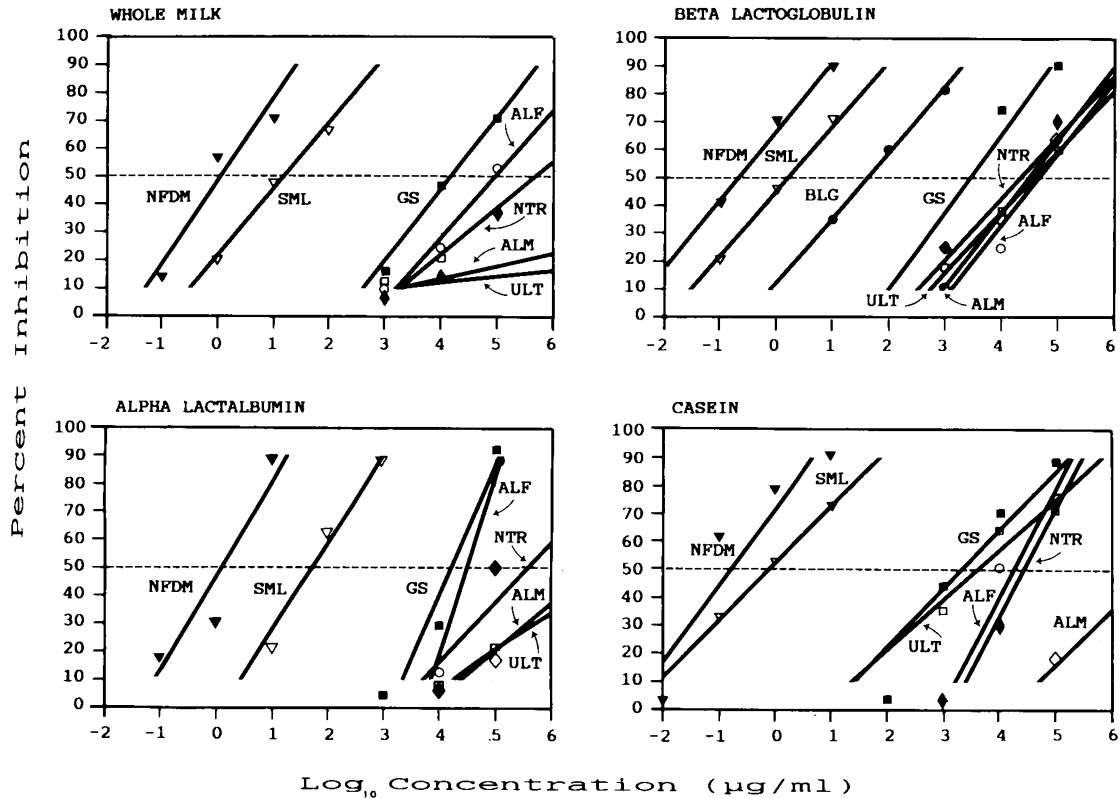


FIG. 2. FAST inhibition dose-response experiments to determine 50% inhibition (FI_{50}). Shown are dose-response curves converted to a linear expression by best fit analysis for whole milk protein, beta-lactoglobulin, alpha-lactalbumin, and casein, respectively, for each milk preparation. Abbreviations include nonfat dried milk NFDM, nonfat dried milk; SML, Similac; GS, Good Start; ALF, Alfaré; ULT, Ultra; NTR, Nutramigen; ALM, Alimentum.

TABLE 2. RELATIVE ALLERGENICITY OF MILK PREPARATIONS (FI_{50})

Milk preparation ^a	Milk proteins			
	CM	Casein	BLG	ALA
Nonfat dried milk	1.0	1.0	1.0	1.0
Similac	13.5	4.8	7.4	42.0
Good Start	13,000	12,000	14,000	12,000
Ultra	>100,000	25,000	>100,000	>100,000
Nutramigen	>100,000	>100,000	100,000	>100,000
Alfare	87,000	100,000	>100,000	22,000
Alimentum	>100,000	>100,000	>100,000	>100,000

^aConcentration of milk preparations (μg/ml) giving 50% FAST inhibition (FI_{50}), assuming the FI_{50} for nonfat dried milk is 1 μg/ml.

DISCUSSION

Using the FI technique, we have evaluated the allergenicity of several cows' milk hydrolysates in terms of their binding capacity to cows' milk IgE antibodies. Although these hydrolyzed formulas were several orders

of magnitude less allergenic than NFDM and Similac, they still retain epitopes that are capable of combining with IgE antibodies and potentially causing anaphylactic type reactions. Thus, the WH, GS, Ultra, and Alfaré, showed incomplete hydrolysis of the whey proteins BLG and ALA and surprisingly even had considerable C activity. GS appears to be the most allergenic of the group studied both in terms of the FI profile (Fig. 1) and the FI₅₀ (Fig. 2, Table 2). It is hydrolyzed by trypsin and heat. Ultra is prepared by a similar process but is also ultrafiltrated, resulting in reduced allergenicity compared to GS (Table 2). Alfaré hydrolyzed with pancreatic enzymes is similar in allergenicity to Ultra. It, like GS,⁽⁶⁾ however, has been reported to cause anaphylaxis in the highly milk-allergic infant.⁽³⁾ The CH include Nutramigen and Alimentum. Nutramigen, hydrolyzed by pancreatic enzymes and charcoal filtered, has been available since 1947. Although it is thought of as being very hypoallergenic, it has been reported to generate antibodies to milk proteins in laboratory animals⁽⁷⁾ and rarely has been reported to cause anaphylaxis in infants.⁽⁸⁻¹⁰⁾ Alimentum is new and has been reported to be reasonably safe in a study involving children with significant cows' milk allergy.⁽¹¹⁾ Both of these preparations appeared to be among the least allergenic of the hydrolysates studied, with Alimentum being especially hypoallergenic. It should be noted, however, that these preparations, including Nutramigen and Alimentum, show incomplete protein hydrolysis, as demonstrated by the ability to bind at least some IgE-specific cows' milk antibodies to casein and even the whey proteins BLG and ALA (Figs. 1 and 2) and to produce positive skin test results.⁽¹²⁾

Previous studies regarding cows' milk hydrolysate allergenicity are few indeed. The techniques used by the food industry involve chromatographic methods evaluating peptide size, relatively insensitive immunologic methods, such as gel diffusion precipitation, and animal models of anaphylaxis.^(7,13) Recent studies have used RAST inhibition with several enzyme-treated WH, which showed that trypsin digestion may not alter the protein significantly.⁽¹⁴⁾ In addition, basophil histamine release (BHR) techniques have demonstrated that these hydrolysates are not devoid of allergenic epitopes.⁽¹⁵⁾ Studies involving IgE allergenicity, however, have not been a routine part of the food industry's protocol in assessing new milk formulas. We believe this should be the most important of the assays to be used.

FI appears to be an appropriate technique to screen for the allergenicity of various milk preparations, since it correlates well with RAST inhibition,⁽⁴⁾ which has been established as a standard method for assessing relative allergenic potency.⁽¹⁶⁾

However, several issues in our study require further explanation. In Table 1, IgE antibody binding to individual milk protein antigens (C, BLG, ALA) in several cases was higher than to whole milk (CM). This suggests that the FAST assay may not be entirely quantitative, although it correlates well with RAST.⁽⁵⁾ In addition, purified BLG did not inhibit as well as NFDM or Similac, particularly in lower concentrations (Fig. 2). The reasons for this are not known but could include some degradation of BLG in the purification process, or perhaps milk contains a small quantity of additional epitopes that resemble BLG and can partially bind BLG-specific IgE antibodies. Nevertheless, at 10 mg/ml, BLG inhibited binding by 93%, which compares well with NFDM and Similac and was far more than was seen with the hydrolysates, suggesting that the specificity of the assay was reasonably good. In addition, best fit analysis of all dose-response data did not always result in parallel lines among the formulas. This might be expected, since the degradation process could easily cause altered as well as reduced allergenic epitopes.

Finally, using FI, one would predict that GS would be clinically more allergenic than several of the other hydrolysates, including Nutramigen, and this appears to be verified by recent observations. However, in spite of a low FI profile by Alfaré, approximately the same as Nutramigen, several cases of anaphylaxis have been reported with the former⁽³⁾ although rarely reported with the latter since it became commercially available in 1947.^(7,8) Plausible explanations include a recent increased awareness and reportability of formula-induced anaphylaxis and the fact that assessment of IgE binding capacity may not always reflect clinical allergenicity accurately.

Our data show that all hydrolysates retained the ability to bind cows' milk IgE antibodies to some degree and, therefore, should be used with great caution in the highly cows' milk-sensitive child. They could be safe and effective, however, in allergy prophylaxis, although the data are preliminary and short term.⁽¹⁷⁾ We are planning to expand our protocol to include BHR and skin testing to assess IgE-mediated histamine release phenomena with these hydrolysates. In addition, since previous studies indicate allergenicity of BSA and bovine gamma globulin (BGG) in allergic infants,^(15,18) they should be included in evaluating

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formula allergenicity in spite of their heat lability. It is hoped that these procedures in conjunction with food challenge studies will provide the food industry with a method of selecting safer formulas for those infants at risk.

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REFERENCES

1. Buckley RA, Metcalfe D. Food allergy. *JAMA* 1982;248:2627–2631.
2. Committee on Nutrition. Soy-protein formulas. Recommendations for use in infant feeding. *Pediatrics* 1983;72:359–363.
3. Businco L, Contani A, Langhi A, Grampietro PG. Anaphylactic reactions to a cows' milk whey protein hydrolysate (Alfaré, Nestlé) in infants with cows' milk allergy. *Ann Allergy* 1989;62:333–335.
4. Jones LA, James RM, Sun JV, Tsay TG, Rock HS, Nicholson P. IgE-FAST inhibition: a rapid technique for the standardization of pollen allergen extract potency. *Ann Allergy* 1986;56:222–225.
5. Tsay YG, Halpern GM. IgE fluoroallergo sorbent (IgE-FAST[™]) Test: concept and clinical applications. *Immunol Allergy Pract* 1984;27:169–174.
6. Ellis MH, Short JA, Heiner DC. Anaphylaxis after ingestion of a recently introduced hydrolyzed whey protein formula. *J Pediatr* 1991;118:74–79.
7. Seban A, Konijn AM, Freier S. Chemical and immunological properties of a protein hydrolysate formula. *Am J Clin Nutr* 1977;30:840–846.
8. Lifschitz CH, Hawkins HK, Guerra C, Byrd N. Anaphylactic shock due to cows' milk protein hypersensitivity in a breast fed infant. *J Pediatr Gastroenterol Nutr* 1988;7:141–144.
9. Bock SA. Probably allergic reaction to casein hydrolysate formula. *J Allergy Clin Immunol* 1989;84:272.
10. Saylor JD, Bahna SL. Anaphylaxis to casein hydrolysate formula. *J Pediatr* 1991;118:71–74.
11. Sampson HA, Bernhisel-Broadbent J, Yang E, Scanlon SM. Safety of casein hydrolysate formula in children with cow milk allergy. *J Pediatr* 1991;118:520–525.
12. Schwartz RH, Keefe MW, Haris N, Witherly S. The spectrum of IgE-mediated acute allergic reactions to cow's milk in children as determined by skin testing with cow's milk protein hydrolyte formula. *Pediatr Asthma Allergy Immunol* 1989;3:207–215.
13. Pahud JJ, Monti JC, Jost R. Allergenicity of whey protein: its modification by tryptic in vitro hydrolysis of the protein. *J Pediatr Gastroenterol Nutr* 1985;4:408–413.
14. Asselin J, Hebert J, Amist J. Effects of in vitro proteolysis on the allergenicity of major whey proteins. *J Food Sci* 1989;54:1037–1039.
15. Wahn U. *In vitro* testing of cows' milk allergens and hypoallergenic formulas. In: RN Hamburger, ed. *Food Intolerance in Infancy*. Carnation Nutrition Ed. Series, Vol. 1. New York: Raven Press, 1989, pp 273–277.
16. Nordlee JA, Taylor SL, Jones RT, Yunginger JW. Allergenicity of various peanut products as determined by RAST inhibition. *J Allergy Clin Immunol* 1981;68:376–382.
17. Chandra RK, Singh G, Shridhara B. Effect of feeding whey hydrolysate, soy and conventional cow milk formulas on incidence of atopic disease in high risk infants. *Ann Allergy* 1989;63:102–106.

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18. Goldman AS, Sellars WA, Halpern SR, et al. Milk allergy II. Skin testing of allergic and normal children with purified milk proteins. *Pediatrics* 1963;32:572-579.

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