

Evaluation of the immunogenicity of protein hydrolysate formulas using laboratory animal hyperimmunization

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The rabbit hyperimmunization model has previously been used to evaluate candidate hypoallergenic protein ingredients. Use of the model has been expanded to include the evaluation of protein hydrolysate formulas. Each formula's immunological reactivity was determined by ELISA measurement of formula-specific rabbit antibody. Animals hyperimmunized with formulas containing extensively hydrolyzed proteins (Alimentum, Nutramigen, and Pregestimil) generated very weak immune responses (<100 fold antibody increase). Products containing intact or partially hydrolyzed proteins (Similac, Enfamil, Good Start, Beba HA, and Nidina HA) elicited high level (>10,000 fold increase) immune responses. Immunogenicity results were then compared to measurements of residual antigen content (by inhibition ELISA) and clinical performance. Correlation of formula immunogenicity, antigenicity and clinical performance indicates that the rabbit model is useful for screening "hypoallergenic" formulas to predict allergenic reactivity.

**C. T. Cordle¹, G. Duska-McEwen¹,
L. M. Janas¹, W. T. Malone¹,
M. A. Hirsch²**

¹Ross Laboratories, Division of Abbott Laboratories
Columbus, Ohio, ²Hazleton Washington Vienna,
Virginia, USA

C. T. Cordle
Ross Laboratories
625 Cleveland Avenue
Columbus
OH 43215-1724
USA

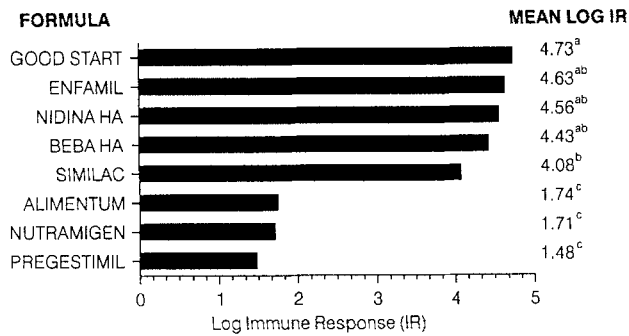
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Recommendations by the Subcommittee on Nutrition and Allergic Disease of the American Academy of Pediatrics for clinical testing of hypoallergenic infant formulas were recently summarized, in part, by Kleinman, et al. (1). The subcommittee has advised that hypoallergenic formulas must be shown not only to be nutritionally adequate, but to be safe for food allergic individuals as demonstrated in appropriate clinical trials using double-blind, placebo-controlled food challenges (2). Because of the risk of anaphylaxis, limited capacity to perform studies in food-allergic patients, and high cost, such clinical testing procedures are unsuitable for early product development or screening new formulas. For such purposes, preclinical testing is necessary to characterize the potential immunological reactivity of the experimental protein sources.

Numerous methods are available to assess the potential allergenic reactivity of protein ingredients. These can be classified as measuring antigenicity, immunogenicity or allergenicity. Understanding the definition of these terms and the differences between each response is necessary to

assess the suitability of a particular method. Antigenicity is the capability of molecules (antigens) to interact with immunologically-specific antibodies and is defined by the three-dimensional chemical structure of relatively small segments of the molecule which actually contacts the antibody during binding. Enzyme-linked immunosorbent assay (ELISA) is a method that measures antigenicity. Immunogenicity refers to the capability of molecules (immunogens) to elicit the production of specific antibodies and/or initiate cellular immune responses and is affected by molecular weight, chemical complexity, "foreignness", dose, and method of immunization. Laboratory animal hyperimmunization is an example of a method that measures immunogenicity. In this method a number of immunochemical procedures may be used to detect and quantitate the immune response (precipitation tests, radio immunoassays, ELISAs, etc.). Allergenicity is the capacity of molecules (allergens) to initiate an allergic response, which is controlled by the same elements that affect immunogenicity plus other factors, including route of exposure, and unknown genetic factors that are species-specific.



* Titers with the same letter are not significantly different.

Fig. 1. Immunogenicity of various infant formulas.

Guinea pig oral sensitization/systemic anaphylaxis has been used to measure allergenicity (3). However, because of significant differences between humans and guinea pigs, both in terms of immunogen sensitivity and allergy mechanism (significant IgG1 contribution in guinea pigs vs IgE in human) this model has failed to identify some reactive formulations (4–9). Although the specific molecular and genetic mechanisms of allergenicity and the development of an IgE response in humans are not completely understood (10–12), by definition, allergens are required to be immunogenic. In evaluating candidate hypoallergenic protein sources, any protein source that is poorly or not immunogenic in a vigorous hyperimmunization study seems likely to be hypoallergenic. Such a protein source would be a good candidate for clinical studies in infants and children for proof of its hypoallergenic performance.

To overcome the limitations in the guinea pig system, a rabbit model was developed to assess immunogenicity following vigorous systemic hyperimmunization. This model has been used previously to evaluate candidate hypoallergenic protein ingredients (13). Low immunogenicity of hydrolyzed casein and soy protein measured in these studies was used to predict that they would be good candidates for hypoallergenic infant formulas (13). The purpose of the present study was to extend the observations from protein hydrolysate ingredients studied previously to whole infant formulas and to compare these results with measurements of antigenicity and clinical performance. It was also important to determine the utility of the method for evaluating complete formulations and to confirm that processing the hydrolysate in the presence of numerous other ingredients did not change the relative immune potential of the formula.

Material and methods

Infant formulas

Similac with Iron®, lot SW8631, and Alimentum®, lot HF8711; Ross Laboratories, Columbus Ohio, USA. Enfamil with Iron®, lot MKN85, Nutramigen®, lot BCN15, and Pregestimil®, lot BGH10; Mead Johnson, Evansville, Indiana, USA. Good Start®, lot LK81716547N; Carnation, Los Angeles, California, USA. Beba HA®, lot 01.89WKRAA, and Nidina HA®, lot 05.89; Nestle, Vevey, Switzerland.

Alimentum, Nutramigen, and Pregestimil are all manufactured using highly hydrolyzed bovine casein. Good Start, Beba HA, and Nidina HA are based on partially trypsin-hydrolyzed and heat-treated bovine whey protein. Similac with Iron and Enfamil with Iron contain intact cow's milk proteins.

Immunogenicity

Immunogenic reactivities of formulas were determined using the rabbit hyperimmunization model described previously (13). Briefly, groups of five or more rabbits were hyperimmunized with sufficient product to deliver 5 mg protein (or protein equivalent) per immunizing dose. Primary and booster immunizations were delivered on Days 0 and 21, both using Complete Freund's Adjuvant. Serum samples were obtained on Days 0 and 35. Serum antibody levels were quantitated using ELISA methods as previously described. ELISA plates were coated with the immunizing formula. In all cases block titrations were done to determine optimal (saturating) antigen plate-coating concentrations. The antibody titers measured reflect a composite immune response to all active components of the immunogen.

Statistical analysis of immunogenicity data

Some variability was observed in nonspecific (background) interaction between different products coated on the ELISA plates, the pre-immune antisera, and the ELISA immunochemical detection system. While the magnitude of this non-specific interaction was small, its product-to-product variability should be accounted for in the data analysis. Therefore, a valid comparison of rabbit antibody responses to the infant formulas was best obtained after adjusting 0-time titers to a common starting point. To make the adjustment, the mean of 0-time log titers for each formula was subtracted from each rabbit's day 35 log titer. This is equivalent to analyzing the log of each rabbit's fold increase in antibody level at day 35 over its group's

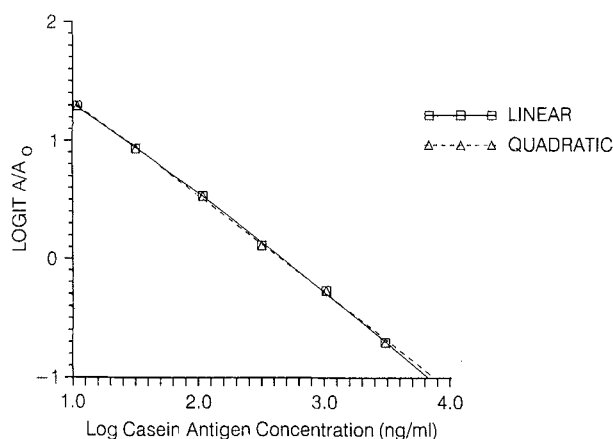


Fig. 2. Sample standard curve for Casein ELISA assay.

0-time geometric mean and is expressed as log immune response (IR).

A one-way analysis of variance was performed to test the hypothesis that mean 35 day log IRs for each immunization group were equal. This hypothesis was rejected. The Tukey multiple-comparison procedure was then used to separate group mean IRs by identifying those that were significantly different (confidence level set at 95%). For this data set, the Tukey minimum significant difference criterion for group mean IR comparisons was 0.6332. Statistical groupings shown in Fig. 1 are derived from this analysis.

Formulas antigen content by ELISA

An indirect (inhibition) ELISA was used to quantitate immunologically active bovine casein (IAC) or immunologically active bovine whey protein (IAW) in the formulas. These assays measure the level at which test products block binding of specific antibody to plate-bound intact antigen. This inhibition is then related to the sample's active antigen concentration using known antigen standards. The assay measures "immunologically active" antigen, and does not require that antigen be completely intact. The only antigenic structural requirement is that individual epitopes present on intact casein or whey retain their ability to be bound tightly by specific antibody. This is important because monovalent antigen fragments that typically will not stimulate immune responses nor mediate allergic reactions will be measured by these methods.

Assay specifics are as follows: Reagents: High-titered polyclonal anti-casein and anti-whey protein antisera were made in rabbits as previously described (13). Intact bovine casein standard; Del-town, Fraser, NY, USA. Intact bovine whey proteins standard; Milei, Stuttgart, Germany. Immulon 2 96-well ELISA plates; Dynatech, Chantilly,

VA, USA. Detector antibody, peroxidase-labelled goat-anti rabbit IgG; Sigma, St. Louis, MO, USA. Substrate, 3,3' 5,5' tetramethylbenzidine; Kirkegaard & Perry, Gaithersburg, MD, USA. Protocol: ELISA plates were coated with 100 μ l/well of a 100 ng/ml solution of casein or whey protein dissolved in 0.05M sodium bicarbonate buffer, pH 9.6 by incubating overnight at 4°C. Coated plates were washed 3 $\times$ using Wash Solution (Kirkegaard & Perry). Plates were then blocked to minimize non-specific binding using phosphate-buffered saline (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.0, PBS) which contained 0.07% crystalline ovalbumin (200 μ l/well, 1 h, room temperature (RT)). Test solutions (formulas) and standards (intact casein or whey) were made by incubating 400 μ l of an appropriate dilution of the standard or test product with 400 μ l of a 1:150,000 dilution of the anti-casein or anti-whey protein rabbit antisera at 37°C for 2 h (all dilutions done using PBS containing 0.1% Tween 20 plus 0.05% ovalbumin). The blocked plates were washed 3 $\times$, test solutions were then loaded (100 μ l/well, in triplicate) and incubated (1 h, RT). Plates were washed 4 $\times$ and 100 μ l of a 1:2000 dilution of detector antibody was added. After a 1 h, RT incubation, the plates were washed 3 $\times$ and 100 μ l of the enzyme substrate was added. The enzyme reaction was stopped after 10 min, by adding 100 μ l of 1 M phosphoric acid and the well absorbance at 450 nm was determined. Average absorbencies were calculated for the test samples, standards, assay control samples, and a "no antigen" sample (Ao). A plot was then made of Logit A/Ao for the standards versus log of the standard concentration. Sample concentrations were determined from data points within this standard curve and then adjusted for sample dilution (Fig. 2).

Results

Immunogenicity

Similac and Enfamil (intact milk protein-based formulas) were used as positive controls in this study. Rabbits hyperimmunized with Similac with Iron and Enfamil with Iron developed high level antigen-specific IgG immune responses (Fig. 1). Rabbits immunized with Beba HA and Nidina HA also developed hyperimmune IgG immune responses that were not significantly different from Similac with Iron or Enfamil with Iron. Good Start elicited the highest immune response, but one that was not significantly different from that of Enfamil, Beba HA or Nidina HA. In the present study the immune response to Good Start was higher than the immune response to Similac with Iron. This difference, while statistically significant, was of rela-

Table 1. Average μg IAC and IAW per gram of protein for infant formulas.

Formula	μg IAC/g protein	μg IAW/g protein
Enfamil [†]	400,000	600,000
Similac [†]	820,000	180,000
Good Start	1060	214,000
Beba HA	NT	NT
Nidina HA	1210	140,000
Pregestimil	8.39	19.1
Nutramigen	12.2	25.6
Alimentum	7.68	20.7

[†] Values for unhydrolyzed protein-based products calculated using whey: casein ratios of the protein sources.

NT = Not Tested

tively small absolute value. Rabbits immunized with Alimentum, Nutramigen, and Pregestimil demonstrated low-level IgG immune responses that were not significantly different at the 95% confidence level. The immune response to these highly hydrolyzed formulas was significantly lower than the immune response to the partially hydrolyzed or intact formulas.

Antigen concentration

The concentration of immunologically active casein (IAC) and of immunologically active whey (IAW) was determined for selected infant formulas using ELISA methods. Results are summarized in Table 1. Pregestimil, Nutramigen and Alimentum were found to have low concentrations of IAC and IAW. Good Start and Nidina HA had levels of IAC that were approximately 100 fold greater, and levels of IAW that were as much as 11,200 fold greater than Pregestimil, Nutramigen and Alimentum.

Discussion

Results of this study demonstrated that infant formulas made with extensively hydrolyzed casein, (Alimentum, Nutramigen, and Pregestimil) generated very weak immune responses (< 100 fold antibody increase) in animals hyperimmunized with relatively large doses of these materials. In contrast, the products containing partially hydrolyzed whey protein or intact cow's milk proteins elicited high-level (> 10,000 fold) antigen-specific IgG immune responses typical of hyperimmunization with strong immunogens. The relative response of the products containing intact and extensively hydrolyzed casein protein is similar to results reported previously for intact compared with extensively hydrolyzed casein and soy proteins used alone (13). Therefore, processing and other ingredients combined with extensively hydrolyzed casein during product manufacture did not en-

hance the immunogenic potential of the final formulation.

Antigen content data presented in this study are similar to those reported by Sampson and associates (14). Comparisons of the immunogenicity data with the antigen content results show a lack of correlation. Antigen quantitation results clearly reflected the destruction of active antigen by the partial hydrolysis used to manufacture Good Start and Nidina HA (80–85% reduced). This substantial decrease in antigenicity was not reflected in the immunogenicity of these products which is still very high, and not lower than the intact protein-based products. In the present study the immune response to Good Start was statistically greater than the response to Similac with Iron. We do not believe that this statistical difference is of clinical significance. Both infant formulas generated vigorous immune responses and both are judged to be highly immunoreactive. Available information suggests that partial whey hydrolysate formulas may be highly allergenic as well (8, 9, 14, 15). The data presented in this study suggest that there is a threshold in the relationship between antigen content and immunogenicity. At the standard antigen immunizing dose used here, destruction of 80–85% of the antigen's reactivity left sufficient antigen to fully stimulate an antigen-specific immune response. In the highly hydrolyzed products where the antigen had been reduced to 0.001–0.002%, immunogenicity was dramatically reduced. Further study will be required to clarify the relationship between increasing antigen content and immunogenicity. An antigen content cut-off point for significantly reduced immunogenicity is not known at this time.

Until recently, there have been no appropriately conducted studies to support the use of either casein or whey hydrolysates for either treatment or prophylaxis in infants with cow's milk sensitivity (16), even though casein hydrolysate formulas have been used for this purpose. To demonstrate that an infant formula is effective in the treatment of cow's milk sensitivity, the Subcommittee on Nutrition and Allergic Disease of the American Academy of Pediatrics has recommended that double-blind, placebo-controlled food challenge (DBPCFC) studies be conducted according to standardized procedures (1, 17). In the case of protein hydrolysate formulas made from cow's milk, infants and children with documented sensitivity to cow's milk should be studied in DBPCFC followed by open challenges. For alternate protein sources, subjects with documented sensitivity to the intact protein source should be studied similarly. A sufficient number of subjects should be studied such that it can be predicted with 95%

confidence that 90% of cow's milk allergic infants and children will not react to the product.

In line with the recommendations (1), Sampson and associates have conducted a study in 23 subjects showing the safety of an extensively hydrolyzed casein formula (Alimentum) using Nutramigen as the placebo control (14). Another series of DBPCFC studies was conducted by Oldaeus and associates using the patient's own formula as the placebo control (18). Cow's milk allergy was confirmed in 11 subjects in this study and one of them reacted with urticaria to Alimentum administered in soy formula after 106 mL was consumed. The patient who reacted had a positive RAST to Alimentum, which supports that the reaction was to the formula. Although rare, allergic reactions can occur to extensively hydrolyzed casein-based formulas in highly sensitized individuals (19–23) and the formulations should not be considered non-allergenic. Oldaeus and colleagues (18) have suggested that hydrolysates be first fed to allergic patients using an open challenge procedure under medical supervision.

Data presented here show the utility of the animal hyperimmunization model for screening candidate "hypoallergenic" infant formulas to predict allergenic reactivity. The model shows strongly positive results with partially hydrolyzed products that demonstrate substantial *in vivo* and *in vitro* immunological activity (24, 25) and that are known clinically not to be "hypoallergenic" according to accepted standards (2, 8). Poor immunogenicity is measured for extensively hydrolyzed products that have generally not reacted in DBPCFC (14, 18) and have performed clinically as hypoallergenic. These results do not agree with some data reported earlier using the guinea pig sensitization model that showed the partially hydrolyzed whey protein product to have low reactivity (4–6). Recently the same whey hydrolysate product was tested in a more rigorous feeding study (37 days feeding vs 15 days) and found to be more reactive (26). By comparison, the guinea pig anaphylaxis test seems to be less sensitive than the animal hyperimmunization model as a screening tool for hypoallergenic clinical performance, particularly if the challenge is given only by the oral route and for a relatively short duration (27, 28). Even when the more rigorous feeding schedule is used, the extensively hydrolyzed products did not cause anaphylaxis in guinea pigs (28). These products did create measurable immune responses in the rabbits. It is therefore possible that the rabbit model could be used to differentiate the current hypoallergenic products from improved products not yet available which might be non-allergenic.

Using this model, a negative result would not prove hypoallergenic performance. This can only be done with the double-blind, placebo-controlled food challenge as per the recommendations of the Subcommittee on Nutrition and Allergic Disease of the American Academy of Pediatrics (2). However, the model described here seems valuable in minimizing the risk, time, and cost associated with large scale DBPCFC studies. Very low immunogenicity results (< 100 fold increase in serum antibody content) should provide useful guidance in assessing the clinical risk of testing new products in food-allergic patients. A strongly positive result (> 10,000 fold antibody increase) in the model seems to be a good predictor of allergenic reactivity. Therefore, great caution should be used when clinically testing strongly immunogenic products in highly allergic patients.

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