Control of Hypoallergenicity by Animal Models

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The normal immune response of experimental animals to ingested antigens is usually an increased mucosal immunity associated with an active suppression of the systemic response (1-3). Orally induced tolerance through the persistent feeding of antigens should prevent the hypersensitivity phenomenon. However, the wide use of formulae most frequently based on modified cow's milk is linked to the frequent incidence of cow's milk allergy in infants (4). The pathogenesis of food allergies may be governed by various immunologic mechanisms: immediate manifestations mediated by reagins, inflammatory reactions caused by immune complexes, or delayed hypersensitivity associated with specific T-lymphocytes. One major component of the pathomechanism involved in food allergy is a deficient or immature immune regulation. Children with cow's milk allergy have a decreased immune suppressor T-cell activity, and the immaturity of this control system is suggested by the usually transient character of the disease. The global allergenicity of cow's milk is contributed, to some degree, by the different protein fractions, which are all potential allergens. Among the most likely candidates, we should mention caseins, beta-lactoglobulin (β-LG), alpha-lactalbumin, serum albumin, and immunoglobulins. The large number of milk proteins, in addition to the different immunologic mechanisms possibly involved, are all contributing factors of complexity in the pathogenesis of the disease. This is reflected by a multiplicity of symptoms in various intestinal, respiratory, dermatologic, and hematologic disorders (5).

GENERAL CONCEPTS FOR HYPOALLERGENIC FORMULAE

The ability of mother's milk to prevent the development of atopic diseases in childhood has been suggested by a number of well-documented clinical studies. Exclusive breast-feeding during the first few months of life appears to be the best measure to prevent, or to delay the onset and to attenuate, the symptoms of early food allergy (6,7). However, the excellent prognosis of breast-feeding is highly dependent on an appropriate maternal diet during late pregnancy and lactation.
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(e.g., complete avoidance of the most offending foods such as milk and eggs) (8,9). When high-risk newborns cannot be breast-fed, a formula with reduced allergenic properties is the major alternative that can be recommended. Modulating the immune system of the neonate by other dietetic manipulations remains premature and debatable. Protein chemists and immunologists of the food industry have considered several concepts in the development of hypoallergenic infant formulae. The most reasonable proposals until now involve technological procedures such as heat denaturation and enzymatic proteolysis. Protein sources other than cow's milk have also been frequently substituted in a number of modern formulae. Soya preparations, for instance, have been considered to be less sensitizing than bovine milk, but their wider application has, in turn, increased the occurrence of allergies to soya (10).

Reduction in allergenicity of infant formulae must remain compatible with impeccable nutritional qualities and industrial feasibility. Currently, application of enzymatic in vitro hydrolysis to the milk casein or the whey protein fraction appears to be the most successful technique employed to guarantee a hypoallergenic source of nitrogen (11). Our contribution in product development has been to provide in vivo procedures for evaluating the sensitizing capacity of different food products as well as the potential influence of industrial treatments on subsequent immunologic reactivity. Two independent animal models have been developed in order to follow up the effect of enzymatic hydrolysis on reducing whey protein allergenicity. The hybridoma technology was applied to a mouse model with the production and experimental use of monoclonal IgE antibodies in a test of passive intestinal anaphylaxis (12). Independently, an oral screening procedure in the guinea pig was adapted to evaluate the sensitizing capacity of whey protein hydrolysates (13,14). Both model systems essentially diagnose Type-I hypersensitivity with the implication of reaginic antibodies in immediate systemic or local manifestations.

INTESTINAL ANAPHYLAXIS EXPLORED WITH A MOUSE MONOCLONAL IgE ANTIBODY AGAINST BOVINE MILK BETA-LACTOglobulin

In most cases of cow's milk allergy, children exhibit gastrointestinal disorders such as vomiting and diarrhea. These functional abnormalities are usually associated with alterations of the intestinal mucosa, mostly in the proximal jejunum, which are characterized by villous atrophy, hyperplasia of the crypts, and inflammation. Several animal models of intestinal anaphylaxis have previously been proposed to reproduce human symptoms (15,16). The manifestations of gastrointestinal hypersensitivity observed in rats and guinea pigs were mainly of the immediate type. However, reaginic antibodies involved in these reactions have not been fully characterized and might have been superimposed on other factors. The synthesis and use of a monoclonal IgE antibody against bovine beta-lactoglobulin
enabled us to select for an authentic IgE mediation in testing milk allergy. The IgE-secreting hybridoma was produced in our laboratory by fusion of NS1 myeloma cells with spleen cells of Balb/c mice immunized specifically against the pure milk protein. The secreted antibody was fully characterized and identified as an IgE. On radioimmunoassay, this antibody was found to react with both native and aggregated β-LG. However, positive in vivo reactions such as passive cutaneous anaphylaxis (PCA) were obtained with aggregated β-LG only. Approximately 1 ng of purified antibody was capable of passively sensitizing mast cells of local skin sites in the PCA test.

The monoclonal IgE was examined for its capacity as a mediator of intestinal anaphylaxis in a mouse model. Mice (immunologically virgin hosts) were systemically sensitized with the amount of IgE antibodies giving a positive reverse PCA reaction (about 500 μg of monoclonal IgE per mouse). After 24 hr of fasting, the animals were challenged with aggregated β-LG (8 mg in 0.4 ml) by gastric intubation. Gut manifestations were evaluated after 1 hr following intravenous injection of colloidal carbon black (0.5 ml of Pelikan biological ink diluted 1:10 in saline) in order to demonstrate increased vascular permeability (17). Thirty minutes later, the gut was removed and flushed with ice-cold Carnoy’s fixative for histological studies. Carbon leakage was most pronounced in the jejunum and was of decreasing intensity along the small intestine. Histology showed edema of the villi but showed no detectable modifications of the epithelial cells. The vessels of the submucosa were heavily labeled with carbon, mainly along the bottom of the crypts but to a lesser extent along the villous axis. Intestinal anaphylaxis following passive sensitization of mice occurred without any obvious morphologic alterations of the mucosae or cellular infiltration. Vascular leakage in the submucosae was a transient phenomenon observable within 2 to 3 hr post-challenge. It seems likely that more chronic anaphylactic reactions of this type within the gastrointestinal tract would also result in mucosal damage. This model system helps to demonstrate that certain gastrointestinal manifestations of food allergy could be mediated by reaginic antibodies alone. However, at this point of our investigation, it does not provide a routine procedure for the control of hypoallergenicity in modified milk formulae. One major problem would be to produce, for instance, a battery of monoclonal reagins against the most significant epitopes of the different milk proteins.

**ORAL SENSITIZATION TO FOOD PROTEINS IN A GUINEA PIG MODEL**

A convenient in vivo model was necessary to determine orally the global allergenicity contributed to all protein fractions of cow’s milk also present in infant formulae. The oral screening procedure worked out in our laboratory was a modification of the guinea pig model proposed by Coombs and co-workers in Cambridge (18,19). Guinea pigs and humans may both become anaphylactically
sensitized to dietary proteins. Oral sensitization can be assessed by hypersensitiv-
ity reactions of the immediate type upon systemic challenge with these proteins.
The complex symptoms of food allergy in humans, such as distinctive respiratory
and cutaneous manifestations, may be partially reproduced in this model.

Weaned Dunkin-Harley guinea pigs (Madörin AG, CH-4414 Füllingsdorf) were
used in the model (13). The animals originated from a breed that was maintained
on a cow’s-milk-free and soya-free diet (Kliba Sodi 3000, Klingenthalmühle AG,
CH-4303 Kaiseraugst) for a minimum of three generations. Test diets were given
in liquid form instead of water over 2 weeks. The occurrence of hypersensitivity
was then tested by intravenous challenge for systemic anaphylaxis (16–18 mg pro-
tein/0.5 ml) and passive cutaneous anaphylaxis (PCA) (1–2 mg/0.5 ml), after an
additional week on Sodi 3000 and water.

Optimal sensitization was achieved by oral presentation of food proteins in solu-
tion as mentioned by the Coombs protocol. In these conditions, protein intake was
at least twice as high as that from a pelleted diet. Also, natural bacterial contami-
nants could proliferate (up to $10^9$ total germs and $10^7$ enterobacteria per milliliter)
and possibly enhance the reaginic response. The immunostimulating properties of
their amphipathic products (LPS, peptidoglycans) have been established (20).
Food allergies have often been seen as a sequel to acute gastroenteritis (21). How-
ever, feeding did not by itself trigger any local or systemic reactions and caused
no apparent damage in the intestinal mucosa, although sensitization had been in-
duced by the same route. These observations indicate that other factors in addition
to infection might be leading to oral sensitization.

Feeding spray-dried milk initially failed to induce oral sensitization to milk pro-
teins. Guinea pigs are usually raised on commercial feeds containing 1% to 2%
milk whey complement (roughly 0.1% of the diet in terms of milk proteins). This
unforeseen complication was probably not encountered by the Cambridge scien-
tists, who never mentioned this factor in their own development of this model sys-
tem. Diet supplementation with small amounts of milk proteins appeared to be
sufficient in suppressing subsequent sensitization to these allergens. Pregnant fe-
males were transferred to a milk-free diet at midgestation, and further breeding
was resumed under these conditions. Their F1 offspring could only be partially
sensitized by feeding, and anaphylactic manifestations were transient and weak.
The F2 and later generations became fully responsive to oral exposure to cow’s
milk or moderately processed whey proteins.

Consequently, oral sensitization proved to be dependent on the absence of an
anamnestic response caused by previous maternal exposure to the food antigens.
Maternal factors transferred either in utero or in milk appeared to be responsible
for a suppression of reaginic responsiveness in the offspring. In the young rat, sup-
pression of the IgE response was linked to specific IgG antibodies (22,23) and
maybe also to secretory IgA and other factors transferred in milk from immunized
mothers. Though responsiveness in guinea pigs or rats may be suppressed by ma-
ternal immunity, clinicians usually doubt that human beings may acquire reaginic
tolerance in the same manner (6–9,24,25). However, the amounts of bovine milk
proteins (0.1%) contained in standard guinea pig feed are minute in comparison with Western human diets, where these proteins are usually present in many hidden forms as well as in dairy products. Maternal control over the reaginic responsiveness of the infant might be overtaken by an excessive load of food antigens. Transmucosal transfer of intact macromolecules or antigenic fragments from the intestinal lumen to the mammary secretions is well documented (26,27). These conditions would then favor sensitization to these antigens and would probably lead to a decreased transfer of maternal suppressive factors, either transplacental or from breast milk.

The systemic and local allergic reactions observed in this system (bronchospasm, cutaneous anaphylaxis) can be associated with antibodies differing from the IgE reagin. Previous work by Coombs et al. on a similar model suggested that the sensitizing antibodies belonged to the IgG1a isotypes but not to the IgE class (28). Our own findings support this statement, although no formal identification of the isotype by serology has been performed. The reaginic activity can be fully recovered from guinea pig serum by two types of affinity gels, with the immobilized ligand being either Protein A or specific antigens (ovalbumin, β-LG). The affinity-purified antibodies against ovalbumin were characterized by heavy chains with a molecular weight of approximately 55,000 to 60,000 on SDS polyacrylamide. However, cytophilic activity cannot be attributed to the whole population of isolated IgG antibodies on this sole basis. Trace amounts of specific IgE undetectable by electrophoresis cannot be ruled out. When testing for circulating reagins, skin reactions are optimal after a sensitization phase of 4 to 18 hr prior to intravenous challenge in the PCA test. The skin reactions remain faint or undetectable after 72 hr. The conformational stability and the biological activity of the reagins seemed unaffected by thermal treatment at 56°C for 4 hr or by acid elution in affinity chromatography.

The animal model was expected to provide a sensitive in vivo evaluation of dietary proteins and technologies aiming at the prevention of food allergies. Oral screening was initially applied to milk products. The specificity of the systemic immune response induced orally was determined by a screening of the circulating reaginic antibodies. Thus, serum from animals orally sensitized against whey proteins was tested with pure fractions in the PCA test. The reaginic antibodies produced by orally exposed guinea pigs demonstrated a specific activity associated with β-LG, α-lactalbumin, and IgG1 but demonstrated no detectable allergenicity that could be attributed to bovine serum albumin. However, the brunt of the in vivo reactivity was carried by β-LG in this animal model (Table 1).

The sensitivity of the animal model was evaluated by oral sensitization and systemic challenge. The lowest protein concentration range for a detectable oral induction of the reaginic response was of the order of 200 to 500 μg protein per milliliter for whole milk but only 10 to 20 μg/ml for whey proteins. The minimal allergen concentration in the provocation phase was roughly 20 to 50 μg/ml for whey proteins and 5 to 10 μg/ml for pure β-LG. By comparison, radioimmunoassays could detect 0.01 to 0.1 μg β-LG per milliliter in processed whey, which is
TABLE 1. Anaphylactic sensitization to milk whey proteins and to the hypoallergenic formula based on its tryptic hydrolysate

<table>
<thead>
<tr>
<th>Challenge with specific milk proteins</th>
<th>PCA titers in recipient animals following:</th>
<th>Oral sensitization to untreated whey proteins</th>
<th>Parenteral sensitization to hypoallergenic formula</th>
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<tr>
<td></td>
<td></td>
<td>500–1,250</td>
<td>500–1,000</td>
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<tr>
<td>Beta-lactoglobulin</td>
<td></td>
<td>500–1,250</td>
<td>500–1,000</td>
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<tr>
<td>Alpha-lactalbumin</td>
<td></td>
<td>1–15</td>
<td>0</td>
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<tr>
<td>Colostral IgG₁</td>
<td></td>
<td>1–10</td>
<td>0–10</td>
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<tr>
<td>Serum albumin (BSA)</td>
<td></td>
<td>0</td>
<td>0–10</td>
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<tr>
<td>Sodium caseinate</td>
<td></td>
<td>0</td>
<td>0–5</td>
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*Intravenous injection of 1 mg of specific protein in 0.5 ml of physiological saline including 2% Evans Blue.

*No detectable oral sensitization to trypsin-hydrolyzed whey protein and to the hypoallergenic formula.

*Three weekly injections (~10 mg of hydrolyzed protein or 10–30 μg of immunoreactive peptides) in 0.15 ml of physiological saline ± 0.15 ml 2% AL(OH)₃ intraperitoneally (injections 2 and 3). Parenteral sensitization with the tryptic hydrolysate alone gives similar titers.

at least 100 times more sensitive than the in vivo model. The relatively low response to whole milk suggests a rather minor contribution of caseins to the global allergenicity of milk in the guinea pig.

It was assumed that lactic bacteria might reduce the allergenicity of milk proteins by acidification, partial proteolysis, improved protein digestibility, and a barrier effect against pathogenic microorganisms (29). Microbial acidification by different lactic strains (effect due to S. lactis) had a negligible effect on cow’s milk allergenicity. The reaginic antibodies produced in these animals had a specially high affinity for bronchiolar mast cells. This characteristic was demonstrated indirectly during PCA testing (titers 1:25–1:50), because intradermal injection of the test sera produced anaphylactic shock in the recipient animals, associated mainly with bronchospasm. Reagin diffusion out of the skin site and into the blood stream was sufficient to cause passive systemic anaphylaxis. Heat treatment of milk proteins usually leads to a reduction of their antigenicity (30). It was suggested that a well-adapted heat process could produce a nonsensitizing cow’s milk formula (31). Knowing the heat stability of casein, a similar treatment applied to whey proteins looked more promising (32). A significant reduction of in vivo reactivity affecting both the sensitization and the provocation steps could be achieved by heat treatment of whey proteins. However, this effect did not reach a level acceptable for clinical applications.

In modern infant nutrition, hypoallergenicity refers to a strict avoidance of intact protein determinants from the diet. Hypoallergenic formulae based on this concept usually include hydrolyzed proteins (casein, whey) or crystallized amino acids as a nitrogen source. Pancreatic and tryptic hydrolysis of whey proteins completely abolished their capacity to sensitize orally and to trigger systemic or cutaneous
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anaphylaxis (PCA). A 1,000-fold reduction of immunoreactivity in radioimmunoassays seemed sufficient to avoid oral sensitization and allergic manifestations in the guinea pig (13,14).

Without any detectable oral sensitization to trypsin-hydrolyzed whey, the potential allergenicity of the peptide pool was then evaluated by parenteral sensitization. In these conditions, the tryptic peptides of β-LG are responsible for most of the residual immunoreactivity in the hydrolysate. Minor or no observable reactivity was linked to peptides from other milk proteins (Table 1).

The experimental conditions established for oral sensitization to cow’s milk proteins were also applied for the evaluation of protein allergenicity from soya, carob germ, and egg white. According to reagin titration by PCA, egg white was comparatively the most sensitizing protein source, followed by milk, soya, then carob germ (Table 2). By administration of egg albumin, lethal systemic anaphylaxis was shown to affect all test animals, with a very quick onset of shock manifestations. The sensitizing capacity of soya was dependent upon the different alternatives involved in technological processing: milling, solvent extraction, and heat treatment. The highest level of sensitization concerned 60% to 80% of the animals, including 30% to 40% lethal shock. Carob germ flour was the least sensitizing product by the oral route, with two weakly positive responses out of 12 animals tested.

Animal models provide comparative indications on the allergenicity of proteins from various sources and on the impact of different technologies affecting their immunoreactivity in vivo. The validity of these model systems is restricted by the limited information concerning the prevailing epitopes in humans and animals (33). The introduction of new conformational determinants by enzymic proteolysis (34) appears to be considerably less significant than the elimination of native epitopes in food proteins. The role of other epitopes and control mechanisms in delayed-type hypersensitivity equally deserves to be closely examined in the future.

<table>
<thead>
<tr>
<th>TABLE 2. Oral sensitization to food proteins</th>
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<tr>
<td>Proteins*</td>
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<td>-----------</td>
</tr>
<tr>
<td>Egg albumin</td>
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<tr>
<td>Whey proteins</td>
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<tr>
<td>Soya cold extract</td>
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<td>Carob germ</td>
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*Oral sensitization by ad libitum feeding of protein solutions (~20 mg/ml). Challenge: intravenous injection of 5 mg of protein in 0.5 ml of saline + 2% Evans Blue.

*PCA titrations in immunologically virgin hosts with crude unfractionated food proteins.
REFERENCES


