

Effect of Ingestion of Cow's Milk Protein Hydrolysate Formulas on α -Casein-Specific Immunoglobulin E and G1 Antibody Responses in Naïve and Sensitized Mice

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ABSTRACT

Objectives: Cow's milk protein hydrolysate formulas are widely used for genetically predisposed atopic infants. Whether hydrolysate formulas can induce oral tolerance to α -casein was studied for the first time in naïve and sensitized mice.

Methods: Using immunoblotting, residual antigenicity to α -casein was examined for in animals fed hydrolysate formulas. Naïve mice fed hydrolysate formulas for 1 to 4 weeks were later sensitized with α -casein. Another group of mice sensitized first with α -casein were then fed hydrolysate formulas continually for 12 weeks.

Results: Oral tolerance measured by immunoglobulin (Ig)E and IgG1 antibody responses to α -casein was induced in naïve mice fed NAN for 1 week or NAN-HA for 4 weeks. IgE responses to α -casein were suppressed in mice fed NAN-HA for

1 week or Neoangelac FL for 4 weeks. In contrast, mice fed Alfare, Pepti-Junior, or Pregestimil for 1 to 4 weeks did not develop tolerance to α -casein. Antibody responses to α -casein were not significantly suppressed in sensitized mice fed NAN or hydrolysate formulas for 12 weeks.

Conclusions: Primary IgE responses to α -casein are readily suppressed in naïve mice first fed cow's milk formula or partially hydrolyzed formula for 1 week. Conversely, ongoing IgE, IgG1, and IgG antibody responses to α -casein are poorly suppressed in previously sensitized mice even after prolonged feeding of cow's milk formula or hydrolysate formulas. *JPGN* 41:438–444, 2005. **Key Words:** α -casein—Cow's milk formula—Hydrolysate formula—IgE-oral tolerance—Cow's milk allergy. © 2005 Lippincott Williams & Wilkins

Oral tolerance is the state of systemic immune hyporesponsiveness induced after feeding protein antigens to naïve adult animals and human beings (1–3). It is a physiologic defense mechanism to maintain the homeostasis between the body and dietary antigens. Breakdown of oral tolerance may lead to hypersensitivity to food proteins. It is a physiologic defense mechanism to maintain the homeostasis between the body and dietary antigens. Breakdown of oral tolerance may lead to hypersensitivity to food proteins (4–6). The pathogenesis of food allergy is unclear, but it is probable that factors abrogating the induction of oral tolerance predispose to food allergy.

Cow's milk allergy affects approximately 2.5% of children less than 3 years of age (7). Cow's milk protein hydrolysate formulas are designed to eliminate or minimize allergenicity and to reduce the potential risk of

oral sensitization (3,7). They are widely used for infants genetically predisposed to atopy and children with cow's-milk protein allergy (8–17). For primary prevention of atopy in infants with positive family history, some studies have found that partially hydrolyzed infant formulas are useful (8–12) and some have not (13,14). It has been hypothesized by some investigators that extensively hydrolyzed formulas may be effective in preventing cow's milk allergy in high-risk infants (7,13–15). For secondary prevention of cow's milk protein symptoms in sensitive infants and children, only extensively hydrolyzed formulas and amino acid derived formulas are recommended (7,16,17). Residual cow's milk proteins and large antigenic fragments are often present in partially hydrolyzed formulas (18–22). Therefore, they are not recommended for infants with cow's milk allergy (23–26).

Mice are commonly used in the animal models of oral tolerance (27–29). It has been shown that feeding of whey protein, bovine serum albumin (BSA) or casein can induce oral tolerance in naïve mice (28,29). Similar to reports that ingestion of tryptic fragments of BSA or β -lactoglobulin can induce oral tolerance in mice (30,31), recent reports have shown that ingestion of partially hydrolyzed formulas can induce oral tolerance to whey

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proteins in mice and rats (32,33). In the present study, we found protein fragments with antigenic similarity to α -casein. Moreover, we have demonstrated that oral tolerance of immunoglobulin (Ig)E and IgG1 responses to α -casein can be induced by feeding NAN for 1 week or NAN-HA for 4 weeks, but not after feeding extensively hydrolyzed formulas. Because a 1 week feeding of NAN-HA suppressed specific IgE responses only, the present study has revealed that prolonged feeding is needed for the partially hydrolyzed formula NAN-HA to inhibit primary IgG and IgG1 responses to α -casein in unsensitized mice. It is unclear whether ongoing IgE responses to cow's milk proteins can be suppressed by feeding cow's milk formulas or hydrolyzed formulas to previously sensitized mice. The present study has further shown that ongoing IgE, IgG1, and IgG responses to α -casein cannot be hyposensitized in previously sensitized mice by prolonged feeding of NAN or hydrolyzed formulas for 12 weeks.

METHODS

Animals

Female adult C3H/HeN and BALB/c mice (5–6 weeks old) and Sprague-Dawley rats (150–200 g) were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). They were maintained on a cow's milk free diet (Lab diet; PMI Feeds, St. Louis, MO) in the Animal House of Veterans General Hospital-Taipei (Taiwan). Groups of seven mice were first used at 6 to 8 weeks of age.

Milk Formulas and α -Casein

NAN (Nestle, Vevey, Switzerland) is a conventional cow's milk formulas for infants. NAN-HA (Nestle) is a partially hydrolyzed whey formula. Alfare (Nestle) and Pepti-Junior (Nutricia, Schiphol Airport, The Netherlands) are extensively hydrolyzed whey formulas. Pregestimil (Mead Johnson, Evansville IN) and Neoangelac FL (United Pharmaceuticals S.A., Paris, France) are extensively hydrolyzed casein formulas. α -casein was purchased from Sigma (St Louis, MO).

Protein Precipitation with Trichloroacetic Acid

Cow's milk proteins of NAN and hydrolysate formulas were precipitated with trichloroacetic acid (TCA; 20% final) as previously described (21,22).

Electrophoresis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 13.5% separating gel and a 3% stacking gel. Ten micrograms of TCA-precipitated proteins were applied to each well. Electrophoresis was conducted for approximately 2 hours at constant voltage (100 V). Gels were stained with Coomassie Brilliant Blue. Molecular weight standards (Bio-Rad, Richmond, CA) were phosphorylase b (97.4 kDa); BSA (66.2 kDa); ovalbumin (45 kDa);

carbonic anhydrase (31 kDa); soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

Immunoblotting

After electrophoresis, TCA-precipitated proteins (NAN-HA, Alfare, Pepti-Junior, and Pregestimil: 100 μ g each; Neoangelac FL: 8 μ g; NAN: 2 μ g) were transferred from the gel onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA). It was blocked overnight with 10% chicken egg white (EW) in saline-Tween 20 and then immersed in 15 mL of 10% EW-ST₂₀ containing 1/5,000 diluted hyperimmune mouse anti- α -casein sera (collected after monthly sensitization for 3 months) for 30 minutes at room temperature. After washes, it was immersed in 10% EW-ST₂₀ containing horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1/4000; SBA, Birmingham, AL) for 30 minutes at room temperature. After washes, the signal of the reaction in each membrane was then developed with enhanced chemiluminescent detection reagent (Amersham Biosciences, Buckinghamshire, UK) and detected by autoradiography.

Systemic Sensitization

Mice were intraperitoneally sensitized with 10 μ g α -casein adsorbed onto 4 mg alum and suspended with 100 ng pertussis toxin in 0.5 mL of phosphate-buffered saline.

Specific IgG1 and IgG Antibody Responses

Specific IgG and IgG1 antibody responses were assessed using an enzyme-linked immunoadsorbent assay as previously described (27,28). Ninety-six-well vinyl plates were coated with 10 μ g/mL α -casein in 0.05 mol/L carbonate buffer, pH 9.6 (100 μ L/well) overnight at 4 degrees C. Free sites on the plates were blocked with 10% EW-ST₂₀ for 1 hour. After six washes, serum samples (1/100 to 1,000 dilution) and serially diluted standards (pooled hyperimmune mouse sera collected after monthly sensitization for 3 months) were incubated in duplicate for 5 hours at room temperature. After washes, HRP-conjugated goat anti-mouse IgG or IgG1 (1/4000; SBA) was added and the plates were incubated overnight at 4° C. Color development was performed and read at 492 nm as previously described (27,28).

Specific IgE Antibody Responses

Specific IgE antibody responses to α -casein were assessed in triplicate by passive cutaneous anaphylaxis tests in male Sprague-Dawley rats. First, 0.1 mL of twofold dilutions of pooled mouse serum samples (1/5–1,280) was injected intradermally in rats. Rats were challenged 48 hours later by intravenous injection of 1 mL of 0.9% saline solution containing 5 mg Evans Blue and 2 mg α -casein. The reaction was read 30 minutes after challenge. The passive cutaneous anaphylaxis titer was the highest dilution giving a positive reaction of at least 5 mm in diameter and expressed as means $\pm$ 1 SEM.

Statistical Analysis

Specific IgG and IgG1 antibody titers were expressed as means + 1 SD. All group comparisons for specific IgG and IgG1 antibody responses were made using two-tailed Wilcoxon tests.

RESULTS

Electrophoresis and Immunoblotting of Hydrolysate Formulas

Ten micrograms of TCA-precipitated freeze-dried proteins of NAN or hydrolysate formulas were analyzed by SDS-PAGE and are shown in Figure 1A. Bands corresponding to intact cow's milk proteins are found in NAN and Neoangelac FL. Large protein fragments are found in NAN-HA but not in Alfare, Pepti-Junior, or Pregestimil. Using hyperimmune mouse sera specific for α -casein, antigenicity of NAN and hydrolysate formulas was further analyzed using immunoblotting (Fig. 1B). Well-defined antigen-antibody complexes corresponding to α -casein were found in Neoangelac FL and NAN. Large fragments with antigenic reactivity to α -casein

were found in NAN-HA but not in Alfare, Pepti-Junior, or Pregestimil.

Effect of Feeding Hydrolysate Formulas on Primary Reaginic Antibody Responses to α -Casein in Unsensitized Mice

Groups of C3H/HeN mice ($n = 7$) were fed different cow's milk formulas (2%) or water only for 1, 2, or 4 weeks. One week after the last feed, they were sensitized with α -casein. Specific antibody responses were assessed 2 weeks after sensitization and are shown in Figures 2, 3, and 4, respectively.

Feeding for 1 Week

Compared with control mice, those fed NAN had suppressed IgE and IgG1 ($P < 0.05$) antibody responses. Those fed NAN-HA had decreased IgE antibody responses only. Those fed Neoangelac FL, Alfare, Pepti-Junior, or Pregestimil did not show any suppression (Fig. 2).

Feeding for 2 Weeks

Compared with control mice, those fed NAN showed suppression of IgG ($P = 0.001$), IgG1 ($P = 0.01$) and IgE antibody responses; those fed NAN-HA also had suppressed IgE antibody responses only. Those fed Neoangelac FL, Alfare, Pepti-Junior, or Pregestimil did not have suppressed IgG, IgG1, or IgE antibody responses (Fig. 3).

Feeding for 4 Weeks

Compared with controls, those fed NAN or NAN-HA had suppression of IgG, IgG1 ($P = 0.001$ or < 0.05 , respectively), and IgE antibody responses. Those fed Neoangelac FL also had decreased IgG ($P < 0.05$, respectively) and IgE antibody responses. Those fed Alfare, Pepti-Junior, or Pregestimil did not show suppression at all (Fig. 4).

Effect of Feeding Hydrolysate Formulas on Ongoing Reaginic Antibody Responses to α -Casein in Sensitized Mice

Groups of C3H/HeN mice ($n = 7$) were first sensitized with α -casein. Two weeks later, they were fed different formulas (2%) or water only for 12 weeks. Their specific antibody responses were assessed biweekly after sensitization. The data after feeding different formulas for 12 weeks are shown in Figure 5. Similar to control mice, those fed NAN or hydrolysate formulas showed no suppression of ongoing IgE, IgG1, or IgG antibody responses.

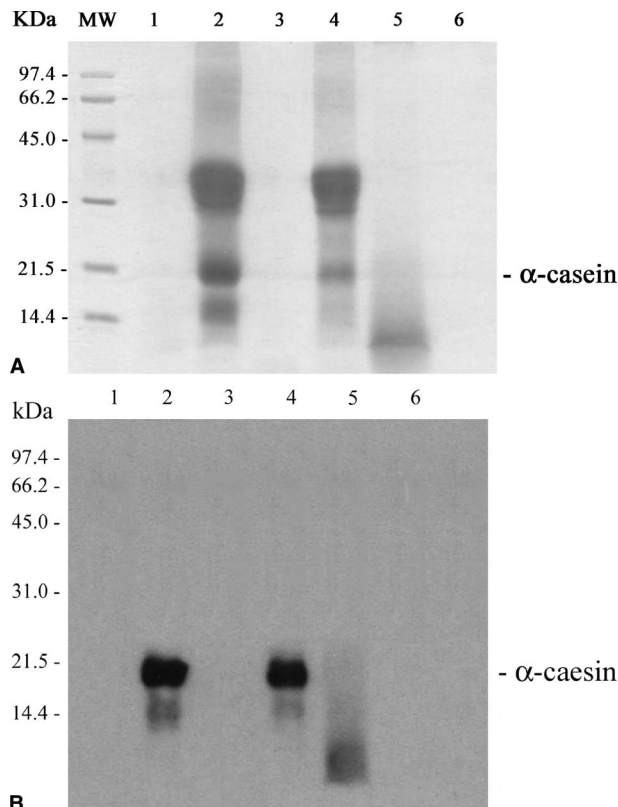


FIG. 1. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (A) and immunoblotting (B) of trichloroacetic acid (TCA)-precipitated proteins in cow's milk protein hydrolyzed formulas: 1. Alfare; 2. NAN; 3. Pregestimil; 4. Neoangelac FL; 5. NAN-HA; 6. Pepti-Junior.

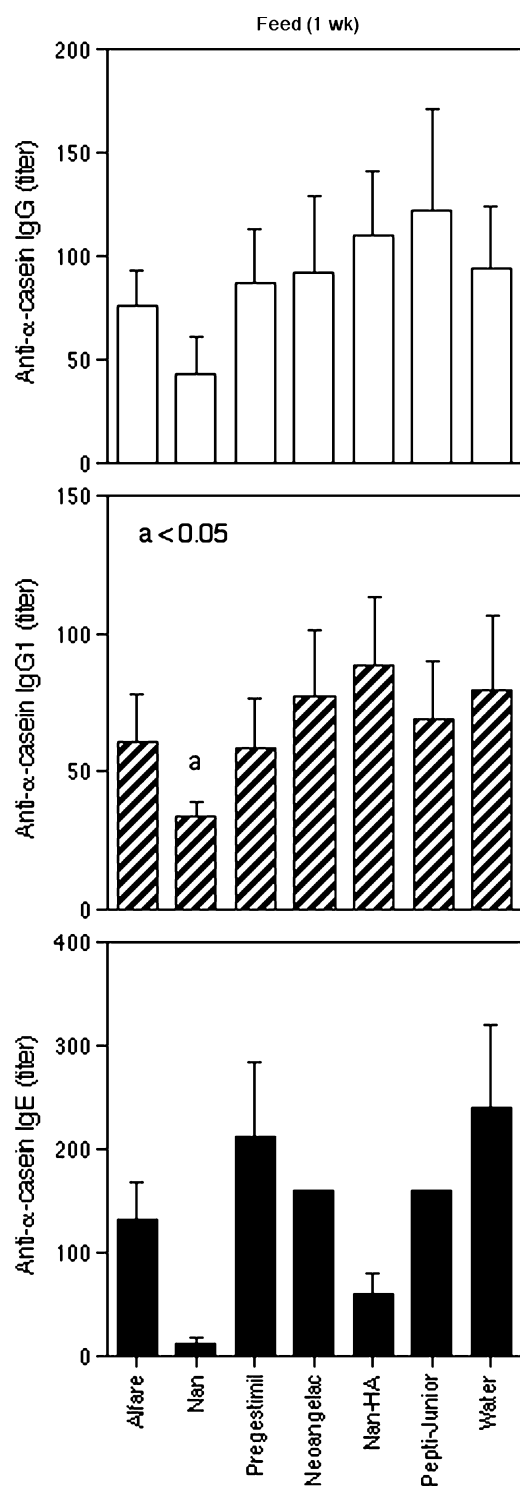


FIG. 2. Effect of 1 week feeding of hydrolysate formulas on Th2 antibody responses to α -casein in previously unsensitized mice. Groups of C3H mice ($n = 7$) were fed different formulas for 1 week. One week after the last feed, they were sensitized with α -casein. Antibody responses were assessed 2 weeks after sensitization.

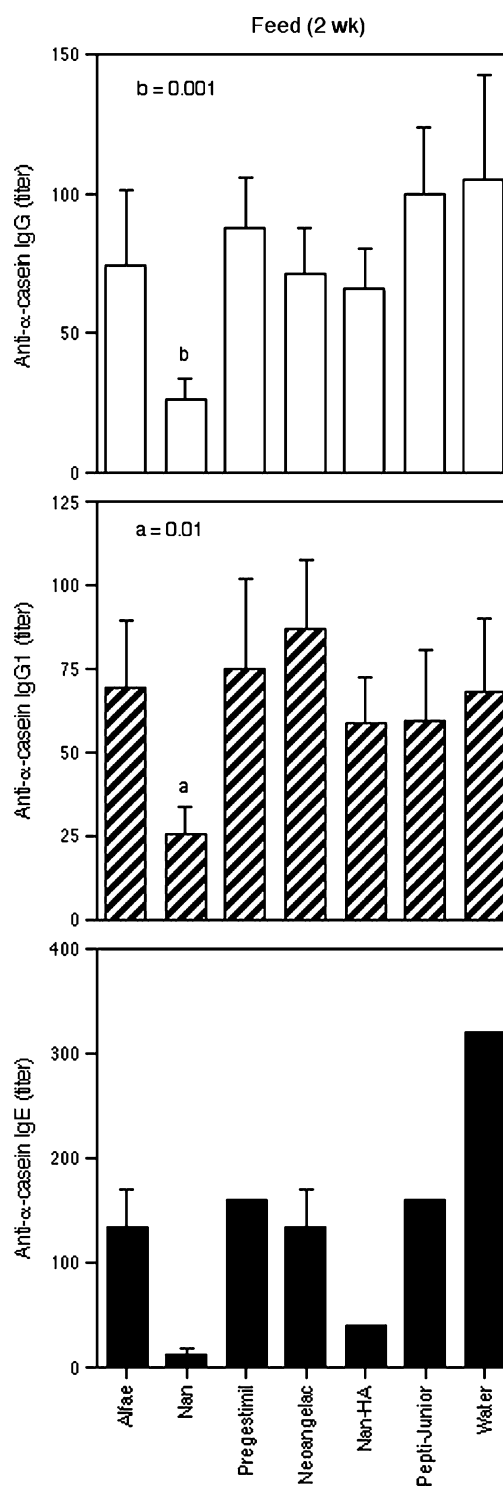


FIG. 3. Effect of 2 week feeding of hydrolysate formulas on Th2 antibody responses to α -casein in previously unsensitized mice. Groups of C3H mice ($n = 7$) were fed different formulas for 2 weeks. One week after the last feed, they were sensitized with α -casein. Antibody responses were assessed 2 weeks after sensitization.

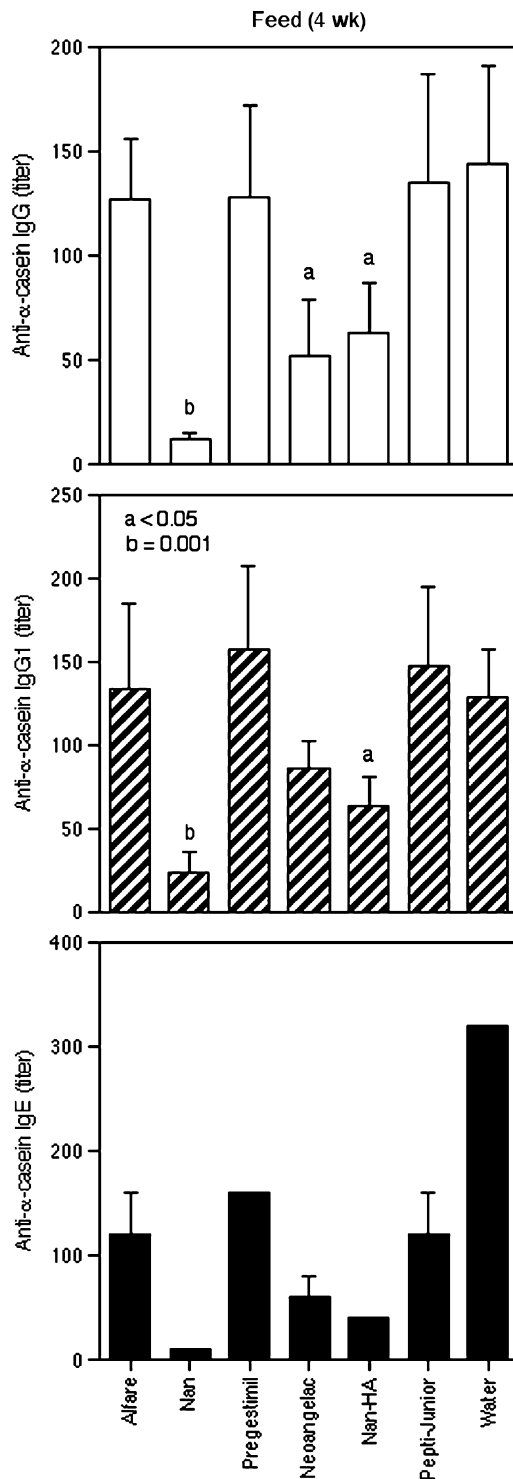


FIG. 4. Effect of 4 week feeding of hydrolysate formulas on Th2 antibody responses to α -casein in previously unsensitized mice. Groups of C3H mice ($n = 7$) were fed different formulas for 4 weeks. One week after the last feed, they were sensitized with α -casein. Antibody responses were assessed 2 weeks after sensitization.

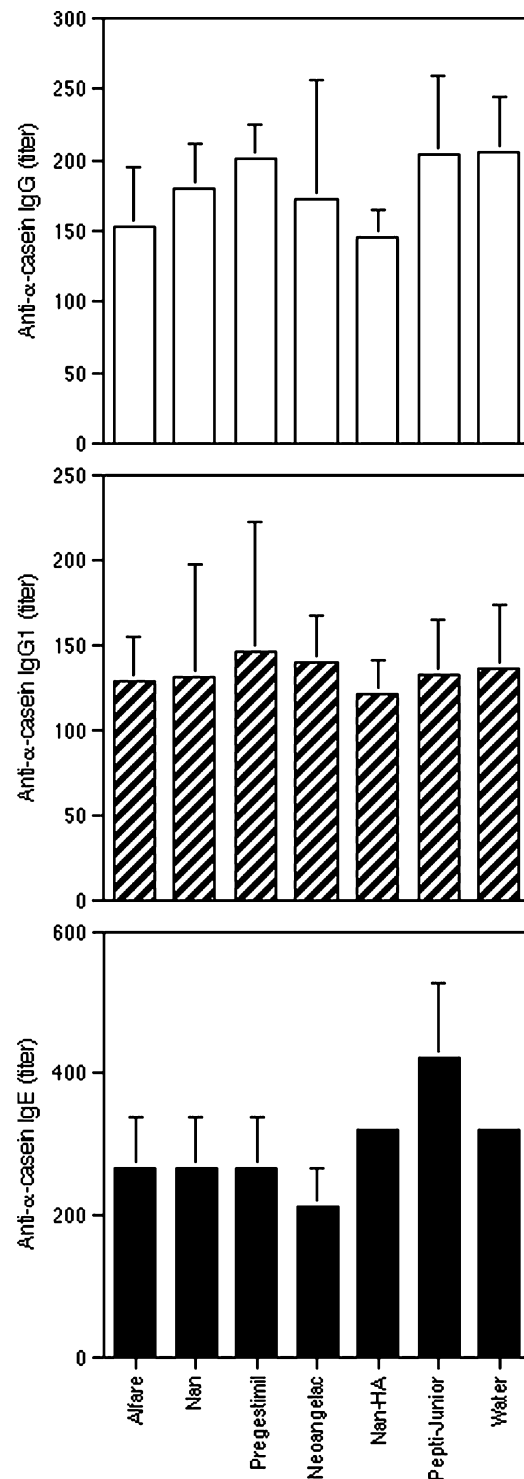


FIG. 5. Effect of feeding hydrolysate formulas on Th2 antibody responses to α -casein in previously sensitized mice. Groups of C3H mice ($n = 7$) were first sensitized with α -casein. Two weeks later, they were fed different formulas for 12 weeks and their specific immunoglobulin (Ig)E, IgG1, and IgG antibody responses were assessed after the last feed.

DISCUSSION

We have shown that naive mice fed a cow's milk formula (NAN) for 1 week or partially hydrolyzed formula (NAN-HA) for 4 weeks have oral tolerance of the primary IgE and IgG1 antibody responses to α -casein. Naïve mice fed NAN-HA for 1 to 2 weeks also had suppressed specific primary IgE responses but not IgG1 or IgG responses. On the other hand, those fed extensively hydrolyzed formulas Alfare, Pepti-Junior, or Pregestimil for 1 to 4 weeks failed to suppress primary IgE, IgG1, or IgG responses to α -casein. These findings are consistent with recent studies in mice and rats showing that oral tolerance to whey allergens can be induced by partially hydrolyzed formulas but not by extensively hydrolyzed formulas (32,33). We have further shown that in previously sensitized mice, ongoing IgE, IgG1, and IgG responses to α -casein cannot be suppressed by prolonged feeding cow's mild formula or hydrolysate formulas for 12 weeks.

Oral tolerance is readily induced after feeding proteins to naive adult animals and humans (1–3,27,28). It is a physiologic mechanism to maintain the homeostasis between the body and many food allergens. It is possible that absence of oral tolerance induction may predispose to food allergy. Because cow's milk proteins may trigger Th2-cell-regulated IgE antibody responses in genetically predisposed atopic infants (3,23–26), it is suggested that breast-feeding is the best means by which to avoid cow's milk allergy (3,7). When maternal milk is not available, cow's milk protein hydrolysate formulas are widely used alternatives for infants at risk of cow's milk allergy (3,7). Hydrolysate formulas are widely felt to be hypoallergenic and to prevent IgE mediated atopic disorders in infants with positive family history. Theoretically, oral administration of such formulas would be a good choice for infants either suffering from or at risk of cow's milk allergy. However, there is so far no formula proven to hyposensitize the infants with cow's milk allergy.

We have previously shown that the kinetics of oral hyposensitization of Th1-cell-regulated immune responses in primed mice are determined by immune status and the timing, dose, and frequency of antigen administration (27). Feeding of antigen to the mice immunized 2 weeks earlier with ovalbumin/complete Freund's adjuvant not only failed to suppress Th1-cell-regulated delayed-type hypersensitivity but also boosted IgG antibody responses (27). We have further shown here that ongoing Th2-cell-regulated IgE responses to α -casein were not suppressed during 12 week feeding of NAN. Similarly, specific IgG and IgG1 responses were boosted after 2 to 4 weeks of feeding of NAN to previously sensitized mice (data not shown). However, they were no longer enhanced after long-term feeding of NAN for 12 weeks (Fig. 5). Neither did those fed hydrolysate formulas have increased IgG and IgG1 antibody responses (Fig. 5). Ongoing IgE responses were never suppressed during 12 week feeding of hydrolysate formulas (Fig. 5). Similar data were found

in sensitized mice fed whey allergens α -lactalbumin and BSA (unpublished data). Only ongoing IgE antibody responses to β -lactoglobulin were suppressed by 12 week feeding of NAN (unpublished data). It is possible that more prolonged feeding is required for the induction of oral hyposensitization to α -casein, α -lactalbumin, and BSA. These data may imply that it is not feasible to give conventional or hydrolysate formulas for oral immunotherapy in infants with active cow's milk allergy. In fact, only three of nine double-blind, placebo-controlled trials of oral immunotherapy for children with allergic rhinitis had a significant improvement of symptoms (34). Because high doses of allergens are required, oral immunotherapy is not cost effective and thus rarely performed (34).

Large protein fragments have been repeatedly found in partially hydrolyzed formulas by protein electrophoresis (20–22,31). It has been suggested that oral tolerance to whey allergens can be induced in naïve rats by the peptide fragments in partially hydrolyzed formulas (32,33). We have found large antigenic fragments of α -casein in NAN-HA but not in extensively hydrolyzed formulas (Fig. 1). This may explain why primary IgE responses to α -casein can be suppressed in naïve mice after 1 week feeding of NAN-HA (Fig. 4). The same doses of the cow's milk formula and the partially hydrolyzed formula might lead to different levels of oral tolerogenicity (Figs. 2–4). The present data have further revealed a time-dependence to the immune response to feeding. After 1 week feeding, primary IgE and IgG1 responses are both suppressed by NAN, but only primary IgE responses are suppressed by NAN-HA. It is possible that the tolerogenicity of intact proteins in NAN is better than that of tryptic peptides in NAN-HA. Most allergens are proteins with molecular weights between 10 and 100 kDa, but peptides smaller than 5 kDa are possibly not immunogenic. By electrophoresis, peptides between 5 and 10 kDa are present in NAN-HA (Fig. 1) and other partially hydrolyzed formulas (20–22,30). By immunoblotting in our study, peptides with much reduced but still positive antigenicity were found in NAN-HA (Fig. 1) and in other partially hydrolyzed formulas (20–22). Because antigenic peptides in partially hydrolyzed formulas may trigger allergic reactions in cow's milk sensitive subjects (7,13–15), only extensively hydrolyzed formulas and amino acid derived formulas are the correct choices for infants with high risk of atopy or with cow's milk allergy.

All the mice fed extensively hydrolyzed formulas failed to develop oral tolerance. It is likely that small peptides in those extensively hydrolyzed formulas are so highly fragmented that they are readily degraded by intestinal enzymes into tiny peptides and amino acids and thus sustain neither tolerogenicity nor immunogenicity. Thus, they are the correct choices for infants with active cow's milk allergy (7,13–15). It is likely that they are also candidates for infants with serious atopic family history to avoid possible of oral sensitization (7,16,17). Because a small amount of residual cow's milk proteins was found

in Neoangelac FL (Fig. 1), suppression of IgE and IgG antibody responses to α -casein was first found after 4 week feeding of Neoangelac FL. Nevertheless, oral tolerance of primary IgE responses was induced after 1 to 2 weeks of feeding of NAN but not Neoangelac FL. This discrepancy may be because only small amounts of α -casein are present in Neoangelac FL.

There are so far no reports on the induction of oral tolerance in neonatal infants and mice. It is likely that neonates may be not intestinally mature enough to develop oral tolerance. Bottle-fed infants develop systemic immune responses to cow's milk proteins, but they are gradually decreased by continual ingestion after the age of 9 months (35). Most children with cow's milk allergy also outgrow their sensitivity by their third birthday (7,35). It is suggested that hyposensitization may be induced in cow's milk allergic children when their intestinal immune systems are mature enough (35). Therefore, avoidance of ingestion of cow's milk allergens is more crucial for primary prevention of atopy in infants with positive family history. In other words, induction of oral tolerance or hyposensitization to cow's milk allergens may play a role only in early childhood but not in early infancy. Partially hydrolyzed formulas are designed to minimize allergenicity. Because they are inexpensive, they are good choices for the primary prevention of cow's milk allergy.

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