

Effect of ingestion of cow's milk hydrolysed formulas on whey protein-specific Th2 immune responses in naïve and sensitized mice

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Summary

Background For genetically predisposed atopic infants, cow's milk protein hydrolysed formulas have been widely used.

Objective Whether hydrolysed formulas can induce oral tolerance to whey proteins will be extensively studied in naïve and sensitized mice.

Methods Antigenicity of hydrolysed formulas was first studied using immunoblotting. Naïve mice fed hydrolysed formulas for 1–4 weeks were sensitized with whey allergens. In contrast, mice sensitized with whey allergens were fed hydrolysed formulas continually for 12 weeks.

Results Whey allergens were found in Nan and Neoangelac FL. Large whey peptides with antigenicity were found in Nan-HA. Profound suppression of IgE, IgG1 and IgG responses to whey allergens were induced in those fed Nan for 1 week, or Nan-HA for 4 weeks. IgE responses to whey allergens were suppressed in those fed Neoangelac FL for 4 weeks, or Nan-HA for 1–2 weeks. In contrast, those fed extensively hydrolysed formulas for 1–4 weeks failed to show decreased responses. On the other hand, IgE responses to β -lactoglobulin, but not to bovine serum albumin or α -lactalbumin, were decreased in sensitized mice fed Nan for 12 weeks. There was no suppression in sensitized mice fed hydrolysed formulas.

Conclusion Suppression of IgE responses to whey proteins was readily induced in naïve mice fed Nan or Nan-HA for 1 week. In contrast, it was hardly induced in sensitized mice even after prolonged feeding of Nan for 12 weeks, let alone hydrolysed formulas.

Keywords α -lactalbumin, β -lactoglobulin, BSA, cow's milk allergy, hydrolysed formula, IgE, oral tolerance

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Introduction

Oral tolerance is the state of systemic immune hyporesponsiveness induced after feeding protein antigens to naïve animals and humans [1–3]. It is a physiological defense mechanism to maintain the homeostasis between the body and food antigens. Breakdown of oral tolerance may lead to hypersensitivity to dietary protein antigens [4–6]. Although the pathogenesis of food allergy is unclear, it is likely that factors abrogating the induction of oral tolerance may predispose to food allergy.

Cow's milk allergy affects about 2.5% of infants [7]. Cow's milk protein hydrolysed formulas are designed to eliminate or minimize allergenicity and to reduce the potential risk of oral sensitization [3, 7]. They are widely used for genetically predisposed atopic infants and cow's milk-allergic infants [8–17]. For primary prevention of atopy in infants with positive family history, partially hydrolysed infant formulas are found useful to avoid cow's milk allergy and atopic symptoms in

some studies [8–12], but not in others [13, 14]. For secondary prevention in cow's milk-sensitive infants, only extensively hydrolysed formulas are recommended [7, 16, 17]. Using immunoblotting, residual cow's milk proteins and large antigenic fragments are well documented in partially hydrolysed formulas [18–22]. It is strongly recommended not to use partially hydrolysed formulas in allergic infants [23–26].

Mice are widely used in the studies of oral tolerance [27–29]. It has been shown that feeding of bovine serum albumin (BSA) can induce oral tolerance in naïve mice [28, 29]. Similar to the early report that ingestion of tryptic fragments of BSA can induce oral tolerance in mice [29], a recent report has shown that ingestion of a partially hydrolysed whey formula can induce oral tolerance to β -lactoglobulin (BLG) in rats [30]. In the present study, we have further demonstrated here that oral tolerance of IgE, IgG1 and IgG responses to three whey allergens BLG, α -lactalbumin and BSA can be all induced after feeding a cow's milk formula for 1 week, or a partially hydrolysed formula for 4 weeks, but not after feeding extensively hydrolysed formulas. Whereas 1-week feeding with the partially hydrolysed formula suppressed specific IgE responses only, prolonged feeding of it increased further oral tolerance of IgE, IgG1 and IgG responses in

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unsensitized mice. On the other hand, it is still unknown whether ongoing IgE responses to whey allergens can be suppressed or not after feeding cow's milk formulas or hydrolysed formulas to previously sensitized mice. The present study has further disclosed that suppression of ongoing IgE responses to BLG, but not to BSA or α -lactalbumin, can be induced in the sensitized mice after extremely prolonged feeding of a cow's milk formula for 12 weeks. However, there was no suppression in the sensitized mice after prolonged feeding of partially or extensively hydrolysed formulas.

Materials and methods

Animals

Adult female C3H/HeN mice (5–6 weeks old) and Sprague–Dawley rats (150–250 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, USA) in the Animal House of Veterans General Hospital-Taipei (Taiwan). Groups of seven mice were first used at 6–8 weeks of age.

Milk formulas

Nan (Nestlé Danmark A/S, Copenhagen, Denmark) is one of conventional cow's milk formulas for infants. Nan-HA (Nestlé Deutschland AG, Biessenhofen, Germany) is a partially hydrolysed whey formula. Alfare (Nestlé Nederland BV, Amsterdam, The Netherlands) and Pepti-Junior (Nutricia, Zoetermeer, The Netherlands) are extensively hydrolysed whey formulas. Pregestimil (Mead Johnson BV, Nijmegen, The Netherlands) and Neoangelac FL (United Pharmaceuticals SA, Paris, France) are extensively hydrolysed casein formulas.

Purified whey proteins

Cow's milk whey proteins α -lactalbumin, BLG and BSA were purchased from Sigma (St Louis, MO, USA) at the highest available purification level.

Cow's milk protein precipitation with trichloroacetic acid (TCA)

Cow's milk proteins of conventional and hydrolysed formulas were precipitated, respectively, with TCA (20% final) as previously described [21, 22].

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 about 2 h at constant voltage (100 V). Gels were stained with Coomassie Brilliant Blue. Molecular weight standards (Bio-Rad, Richmond, CA, USA) 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 PVDF membrane (Millipore, Bedford, MA, USA). It was blocked overnight with 10% chicken egg white (EW) in saline-Tween 20 (ST₂₀), and then immersed in 15 mL of 10% EW-ST₂₀ containing affinity-purified rabbit anti-BLG IgG, rabbit anti- α -lactalbumin IgG or goat anti-BSA IgG (1/6000, respectively; Bethyl Lab., Montgomery, TX, USA) for 0.5 h at room temperature (RT). After washes, it was immersed in 10% EW-ST₂₀ containing horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG or rabbit anti-goat IgG (1/6000, respectively; Jackson ImmunoResearch Lab, West Grove, PA, USA) for 0.5 h at RT. After washes, the signal of the reaction in each membrane was developed with enhanced chemiluminescent detection reagent (ECL; Amersham Biosciences, Buckinghamshire, UK) and detected by autoradiography.

Systemic sensitization

Mice were intraperitoneally sensitized, respectively, with 10 μ g BLG, BSA or α -lactalbumin adsorbed onto 4 mg alum and then suspended with 100 ng pertussis toxin in 0.5 mL of PBS.

Assessment of specific IgG and IgG1 antibody responses

Serum-specific IgG and IgG1 antibody responses were assayed using an ELISA as previously described [27, 28]. Briefly, 96-well vinyl plates were coated with 20 μ g/mL BLG, α -lactalbumin or BSA in 0.05 M carbonate buffer, pH 9.6 (100 μ L/well) overnight at 4 °C. Free sites on the plates were blocked with 10% EW-ST₂₀ for 1 h. After six washes, serum samples (1/100 to 1000 dilution) and serially diluted standards (pooled hyperimmune mouse sera collected after monthly sensitization for 3 months) were incubated in duplicate for 5 h at RT. After washes, HRP-conjugated goat anti-mouse IgG or IgG1 (1/4000; SBA, Birmingham, AL, USA) was added and the plates were incubated overnight at 4 °C. After washes, orthophenylenediamine (OPD; 0.5 mg/mL) in citrate-carbonate buffer containing 0.015% H₂O₂ was added and the plates were incubated in the dark. Colour development was stopped by 4 N H₂SO₄. Absorbance at 492 nm was read using a reader (SPECTRAMax 250, Molecular Devices, Sunnyvale, CA, USA).

Passive cutaneous anaphylaxis (PCA)

Specific IgE antibody responses to whey proteins were assessed in triplicate by the PCA test in male Sprague–Dawley rats. First, 0.1 mL of twofold dilutions of pooled mouse serum samples (1/5–640) was intradermally injected in rats. All rats were challenged 48 h later by an intravenous injection of 1 mL of 0.9% saline solution containing 5 mg Evans Blue and 2 mg BLG, α -lactalbumin or BSA. The reaction was read 30 min after challenge. The PCA titre was the highest dilution giving a positive reaction of at least 5 mm in diameter and expressed as means $\pm$ SEM.

Statistical analysis

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

Results

Electrophoresis and immunoblotting of hydrolysed formulas

TCA-precipitated freeze-dried weights of six formulas (1 g each) were very different (Alfare: 14.2 mg; Nan: 219.8 mg; Pregestimil: 4.6 mg; Neoangelac FL: 99.6 mg; Nan-HA: 44.1 mg; Pepti-Junior: 5.7 mg). Ten micrograms of them were analysed by SDS-PAGE. Bands corresponding to intact cow's milk proteins are found in Nan and Neoangelac FL. Large

protein fragments were found in Nan-HA, but not in Alfare, Pepti-Junior or Pregestimil (data not shown). Using antibodies specific for BSA, BLG and α -lactalbumin, respectively, the antigenicity of six formulas was further analysed using immunoblotting (Fig. 1). Well-defined antigen-antibody complexes corresponding to BSA, BLG and α -lactalbumin are found in Neoangelac FL and Nan. Large fragments with antigenic reactivity to whey proteins were found in Nan-HA, but not in Alfare, Pepti-Junior or Pregestimil.

Effect of feeding of hydrolysed formulas on reagenic antibody responses to BLG, α -lactalbumin or BSA 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, respectively. One week after the last feed, they were sensitized with BLG, α -lactalbumin or BSA, respectively. Their specific antibody responses were assessed 2 weeks after sensitization and are shown in Figs 2, 3 or 4, respectively.

Specific antibody responses to BLG (Fig. 2) Compared with control mice, those fed Nan for 1, 2 or 4 weeks showed suppression of IgG ($P = 0.001$ or 0.002 , respectively), IgG1 ($P = 0.001$, respectively) and IgE antibody responses. Whereas those fed Nan-HA for 4 weeks had suppression of IgG, IgG1 ($P = 0.001$, respectively) and IgE responses, those fed Nan-HA for 1 or 2 weeks had decreased IgE but not IgG or IgG1 responses. Those fed Neoangelac FL for 4 but not 1 or 2 weeks also had suppressed IgE responses. In contrast, those fed Alfare, Pepti-Junior or Pregestimil for 1–4 weeks did not show any suppression.

Specific antibody responses to BSA (Fig. 3) Compared with control mice, those fed Nan for 1–4 weeks or Nan-HA for 4 weeks had suppression of IgG ($P = 0.001$, respectively), IgG1 ($P = 0.001$, respectively) and IgE antibody responses. Those fed Neoangelac FL for 2 or 4 weeks also had suppressed IgE antibody responses. In contrast, those fed Alfare, Pepti-Junior, or Pregestimil for 1–4 weeks did not have suppressed IgG, IgG1 or IgE antibody responses to BSA.

Specific antibody responses to α -lactalbumin (Fig. 4) Compared with controls, those fed Nan for 1–4 weeks had suppression of IgG ($P = 0.001$, respectively), IgG1 ($P = 0.001$, 0.01 or 0.001 , respectively) and IgE responses, and those fed Nan-HA for 4 weeks had decreased IgG ($P < 0.05$), IgG1 ($P = 0.001$) and IgE antibody responses. Those fed Nan-HA for 2 weeks or Neoangelac FL for 4 weeks also had suppressed IgE responses. In contrast, those fed Alfare, Pepti-Junior, or Pregestimil for 1–4 weeks did not have suppression of specific antibody responses.

Effect of feeding of hydrolysed formulas on ongoing antibody responses to BLG, BSA or α -lactalbumin in previously sensitized mice

Groups of C3H/HeN mice ($n = 7$) were sensitized with BLG, BSA or α -lactalbumin, respectively. Two weeks later, they were fed different formulas (2%), respectively, or water only for 12 weeks. Their specific antibody responses were assessed biweekly after sensitization and are shown in Figs 5 and 6, respectively.

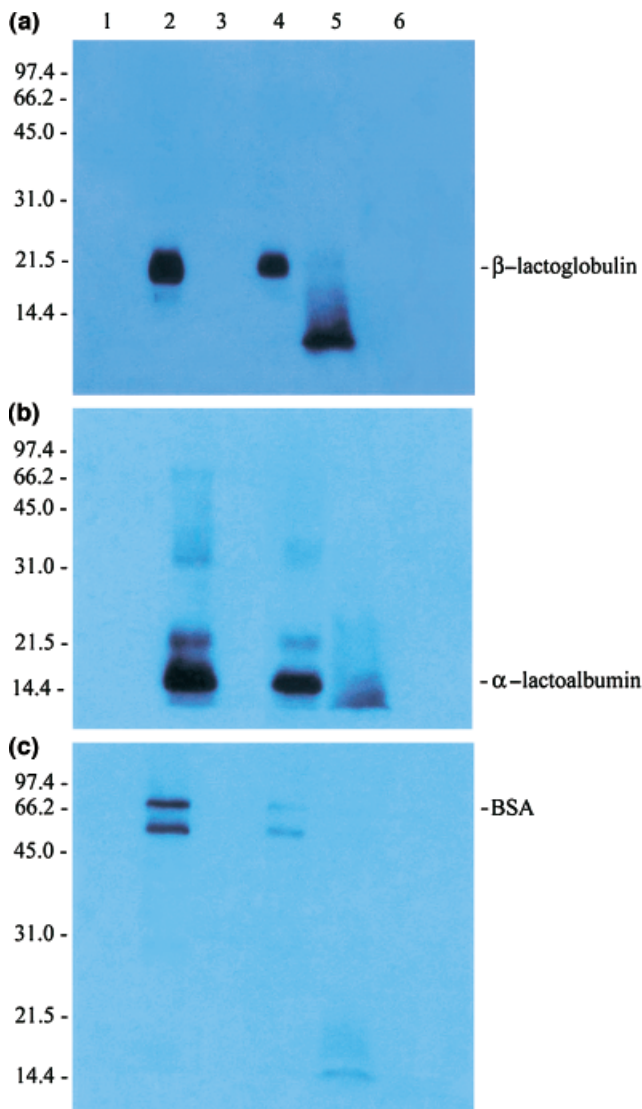


Fig. 1. Immunoblotting of Alfare (1), Nan (2), Pregestimil (3), Neoangelac FL (4), Nan-HA (5) and Pepti-Junior (6). Affinity-purified IgG antibodies against whey protein allergens β -lactoglobulin (BLG), bovine serum albumin (BSA) or α -lactalbumin were used, respectively.

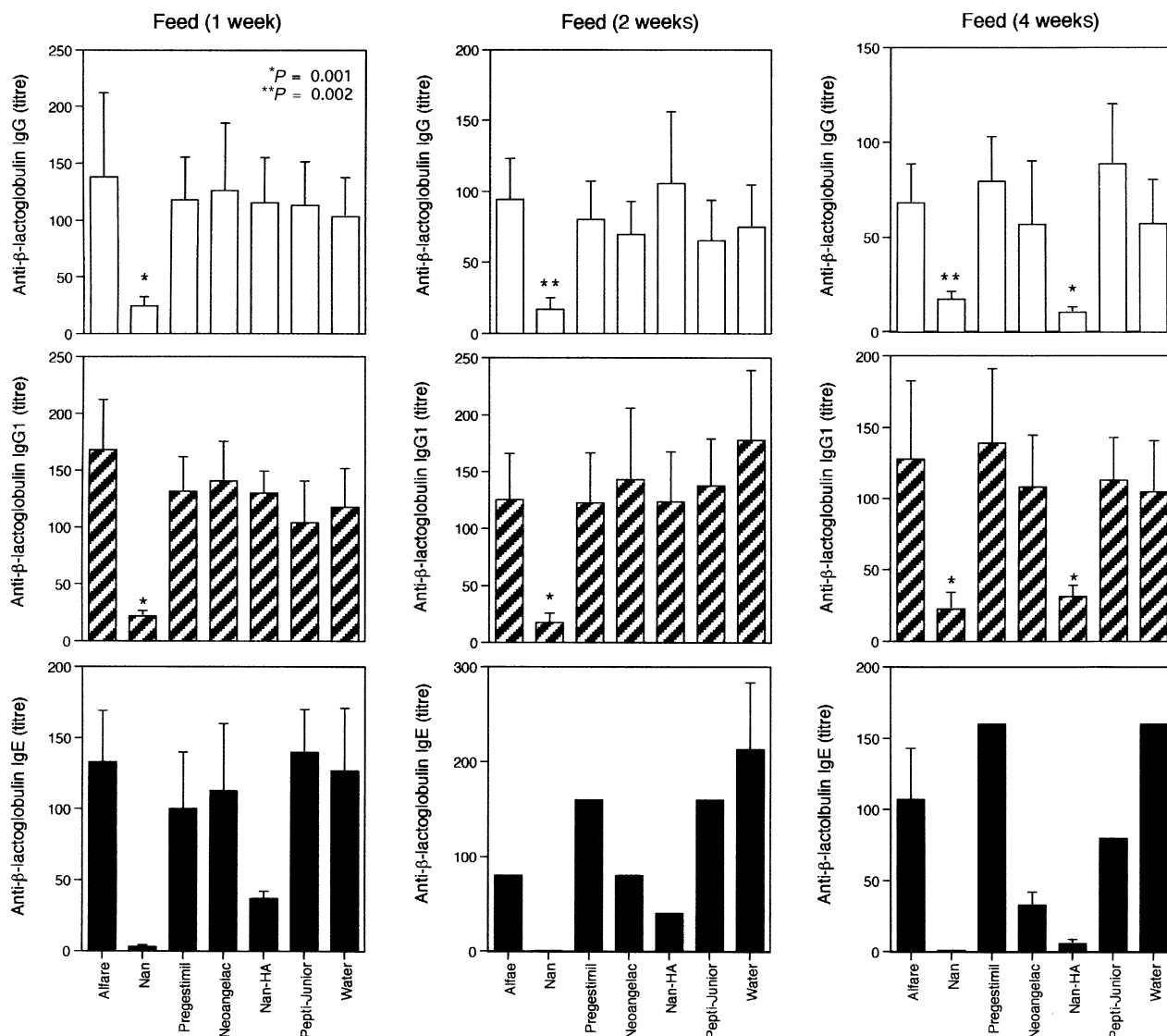


Fig. 2. Effect of ingestion of hydrolysed formulas on Th2 antibody responses to β -lactoglobulin (BLG) in unsensitized mice. Groups of C3H mice ($n = 7$) were fed different formulas for 1, 2 or 4 weeks, respectively. One week after the last feed, they were sensitized with BLG. Their antibody responses were assessed 2 weeks after sensitization.

Ongoing antibody responses to BLG (Fig. 5) Compared with control mice, those fed Nan for 2–12 weeks showed greatly enhanced ongoing IgG and IgG1 antibody responses (Fig. 5a). Whereas there were no differences in ongoing IgE antibody responses between the control mice and those fed Nan for 2–8 weeks (data not shown), the ongoing IgE antibody response of the mice fed Nan for 12 weeks was lower than that of the controls (Fig. 5b). Those fed Nan-HA for 2–12 weeks had no suppression of ongoing IgE antibody responses, let alone those fed extensively hydrolysed formulas.

Ongoing antibody responses to BSA (Fig. 6) Compared with control mice, those fed Nan for 2–8 weeks showed greatly enhanced ongoing IgG and IgG1 antibody responses (Fig. 6a). In contrast, there were no differences in ongoing IgE antibody responses between the control mice and those fed Nan for 2–12 weeks (data for the 12th week shown in Fig. 6b; data for 2–8 weeks not shown). On the other hand, those

fed Nan-HA for 12 weeks had no suppression of ongoing IgE antibody responses, let alone those fed extensively hydrolysed formulas.

Specific antibody responses to α -lactalbumin Similar to the result of BSA, there were no differences in ongoing IgE antibody responses to α -lactalbumin between the controls and those fed Nan or hydrolysed formulas for 2–12 weeks (data not shown).

Discussion

We have demonstrated here that the unsensitized mice fed a cow's milk formula (Nan) for only 1 week or a partially hydrolysed whey formula (Nan-HA) for 4 weeks develop oral tolerance of IgE, IgG1 and IgG antibody responses to whey

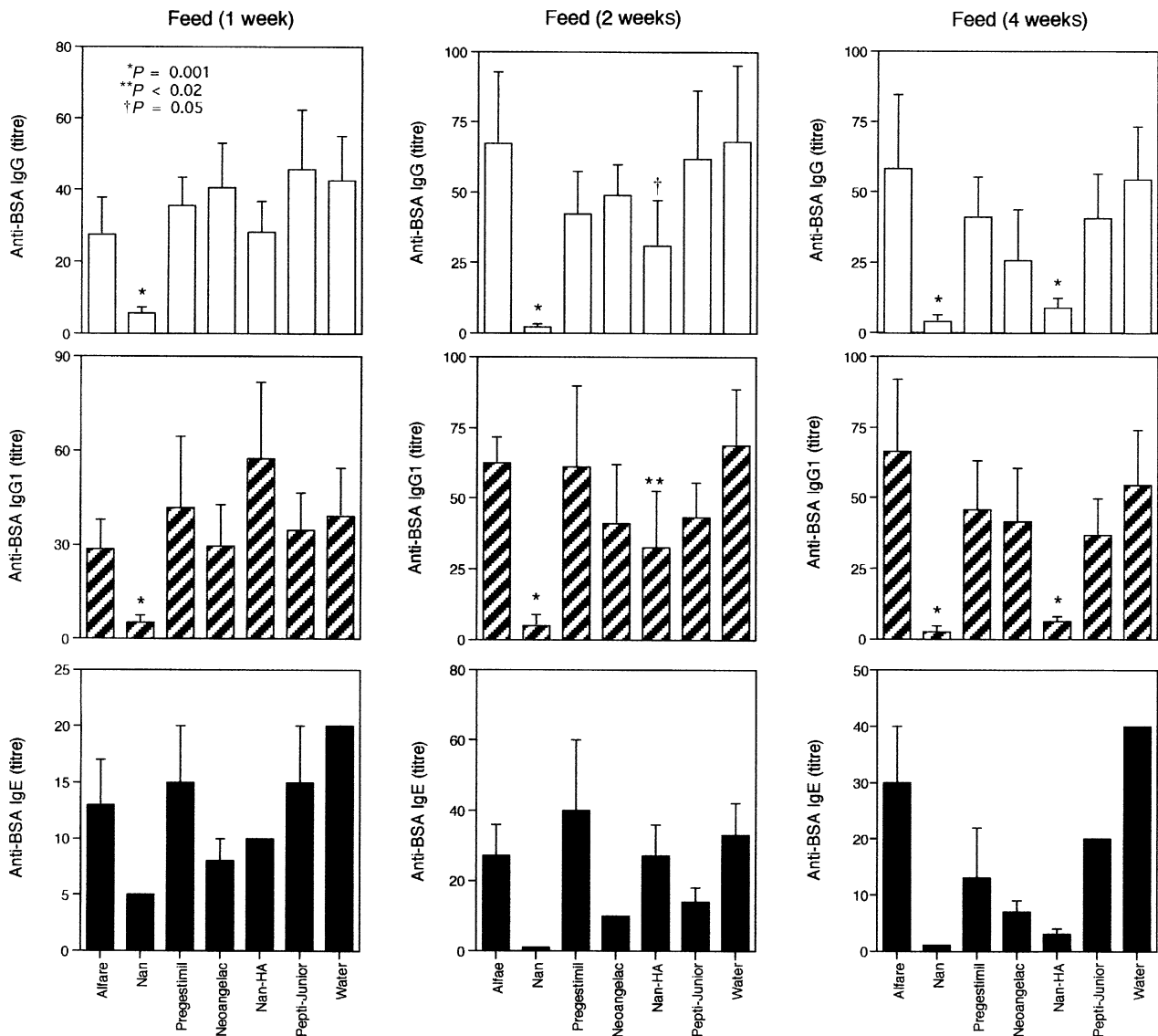


Fig. 3. Effect of ingestion of hydrolysed formulas on Th2 antibody responses to bovine serum albumin (BSA) in unsensitized mice. Groups of C3H mice ($n = 7$) were fed different formulas for 1, 2 or 4 weeks, respectively. One week after the last feed, they were sensitized with BSA. Their specific antibody responses were assessed 2 weeks after sensitization.

allergens BLG, BSA and α -lactalbumin. Nevertheless, 1-week feeding with Nan-HA can suppress specific IgE responses in unsensitized mice. On the other hand, those fed extensively hydrolysed whey formulas (Alfare and Pepti-Junior), or an extensively hydrolysed casein formula (Pregestimil) for 1–4 weeks failed to develop oral tolerance to whey allergens. These findings are consistent with the early report in a rat model that oral tolerance to BLG can be induced by partially hydrolysed whey formula BEBA-HA, but not by extensively hydrolysed whey formula Alfare [30]. Moreover, we have further shown in the sensitized mice that ongoing IgE antibody responses to BLG, but not to α -lactalbumin or BSA, can be suppressed after prolonged feeding of Nan for 12 weeks. Suppression of ongoing IgE responses was hardly induced in sensitized mice fed hydrolysed formulas.

Allergy is characterized by sensitization of allergen-specific Th2 cells. Th2 cell-regulated antibody responses are mainly

IgE production in humans, as well as IgE and IgG1 production in mice [1]. It has been suggested that oral tolerance to BLG can be induced in naïve rats by large whey fragments in BEBA-HA [30]. Using electrophoresis, large protein fragments were found in BEBA-HA and other hydrolysed formulas [20–22, 30]. In the present study, they were also found in Nan-HA, but not in extensively hydrolysed formulas. Similar to the previous study of BLG [30], oral tolerance to BSA and α -lactalbumin was also found in the naïve mice after 4-week feeding of Nan-HA. Whereas it has been suggested that the same doses of the cow's milk formula and the partially hydrolysed formula induce the same levels of oral tolerance [30], the present result has conversely revealed a time-dependent discrepancy between them. Oral tolerance of IgE, IgG1 and IgG responses was readily induced after 1-week feeding of Nan or 4-week feeding of Nan-HA, but only suppression of IgE responses was found after 1- or

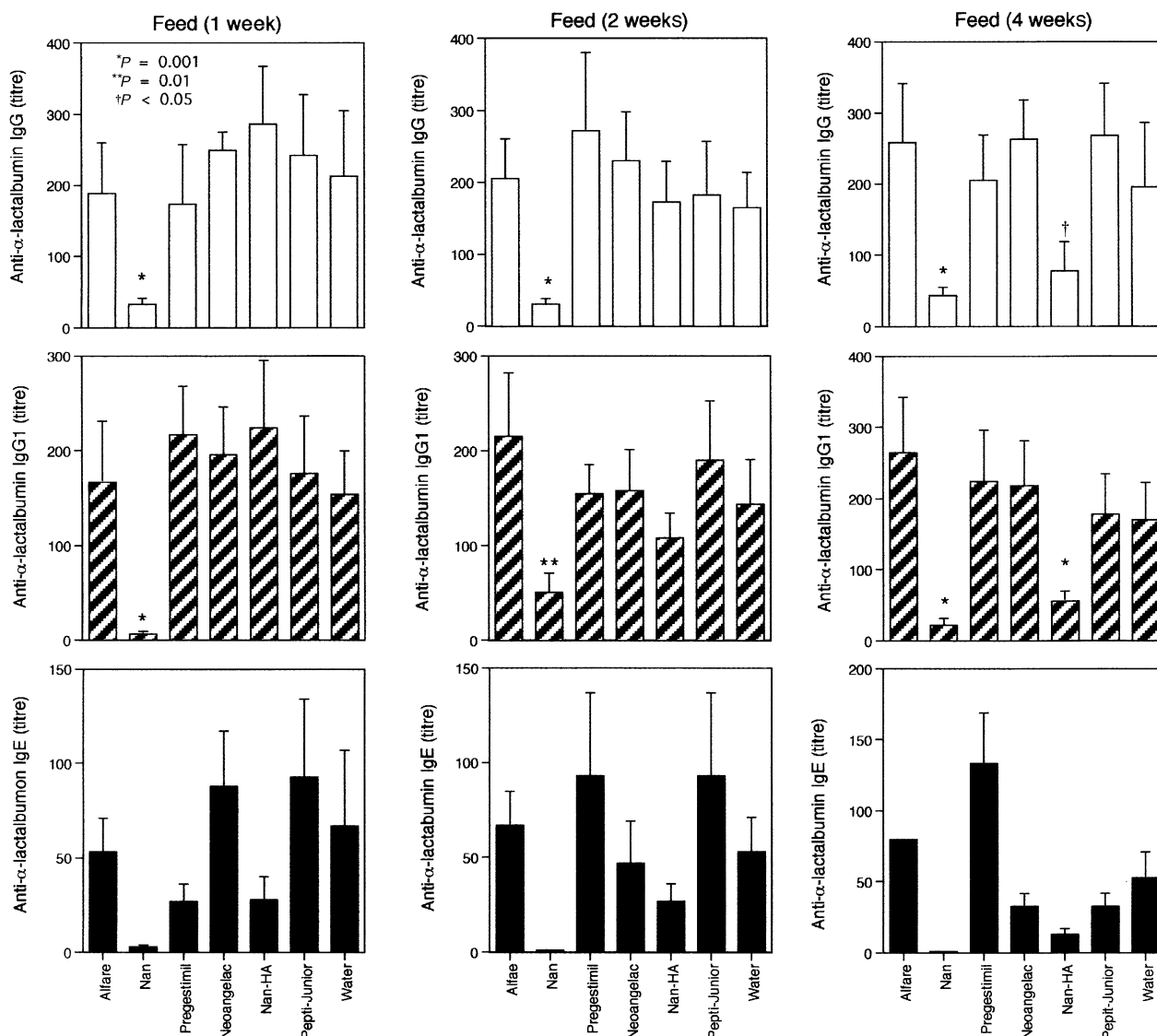


Fig. 4. Effect of ingestion of hydrolysed formulas on Th2 antibody responses to α -lactalbumin in unsensitized mice. Groups of C3H mice ($n = 7$) were fed different formulas for 1, 2 or 4 weeks, respectively. One week after the last feed, they were sensitized with α -lactalbumin. Their specific antibody responses were assessed 2 weeks after sensitization.

2-week feeding of Nan-HA. Using immunoblotting, large polypeptides with positive antigenicity were found in Nan-HA (Fig. 1). It is likely that the tolerogenicity of intact proteins in Nan is better than that of tryptic polypeptides in Nan-HA. Most allergens are proteins with a molecular weight between 10 and 100 kDa, but polypeptides smaller than 5 kDa are possibly not immunogenic.

The mice fed extensively hydrolysed whey formulas failed to develop oral tolerance. It is likely that small peptides in those extensively hydrolysed whey formulas are so much fragmented that they are simply degraded by proteolytic enzymes into tiny peptides and amino acids and thus sustain neither tolerogenicity nor immunogenicity. On the other hand, small amount of whey proteins was found in Neoangelac FL (Fig. 1). Oral tolerance was induced after 1-week feeding of Nan, but not of Neoangelac FL. Suppression of IgE (but not IgG or IgG1) was found after 2- or 4-week feeding of Neoangelac FL. This discrepancy may be because

of the fact that only a small amount of whey proteins are present in Neoangelac FL. Therefore, this finding supports the reports that the induction of oral tolerance is dose-dependent [4, 27, 30].

Oral tolerance is readily induced after feeding protein antigens to naïve adult animals and humans [1–3, 27–30]. It is a physiological mechanism to maintain the homeostasis between the body and a lot of food allergens. It is possible that abrogation of oral tolerance induction may adversely predispose to food allergy. Cow's milk proteins may trigger Th2 cell-regulated IgE responses in genetically predisposed atopic infants [3, 23–26]. It is well accepted that breastfeeding is the best policy to avoid cow's milk allergy [3, 7]. When maternal milk is not available, cow's milk protein hydrolysed formulas are the most widely used alternatives for cow's milk-allergic infants [3, 7]. Thus, hydrolysed formulas are used as hypoallergenic foods to avoid IgE-mediated allergic reactions in atopic infants. Theoretically, oral administration of a

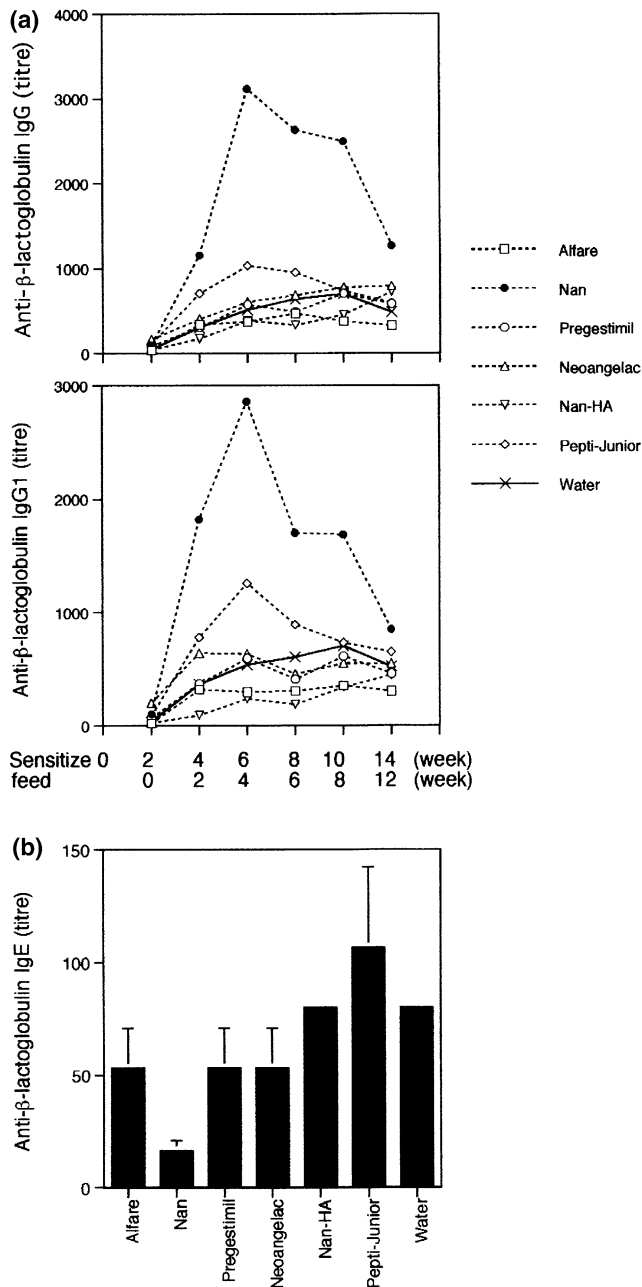


Fig. 5. Effect of ingestion of hydrolysed formulas on Th2 antibody responses to β -lactoglobulin (BLG) in sensitized mice. Groups of C3H mice ($n = 7$) were first sensitized with BLG. Two weeks later, they were fed different formulas for 12 weeks, respectively. Their specific IgG1 and IgG antibody responses were assessed biweekly (a). Specific IgE antibody responses after 12-week feeding of different formulas are shown in (b).

tolerogenic formula would be a preferred choice for infants 'at risk' of cow's milk allergy. However, there is so far no magic formula that can rapidly hyposensitize the infants with cow's milk allergy.

The kinetics of oral hyposensitization in primed animals are determined by immune status and the timing, dose and frequency of antigen administration [27]. Feeding antigen to the mice immunized 2 weeks ago not only failed to induce tolerance, but triggered secondary IgG responses [27]. Similar data are found in Figs 5a and 6a. Ongoing IgG and IgG1 responses to whey allergens were greatly boosted after 2–8-

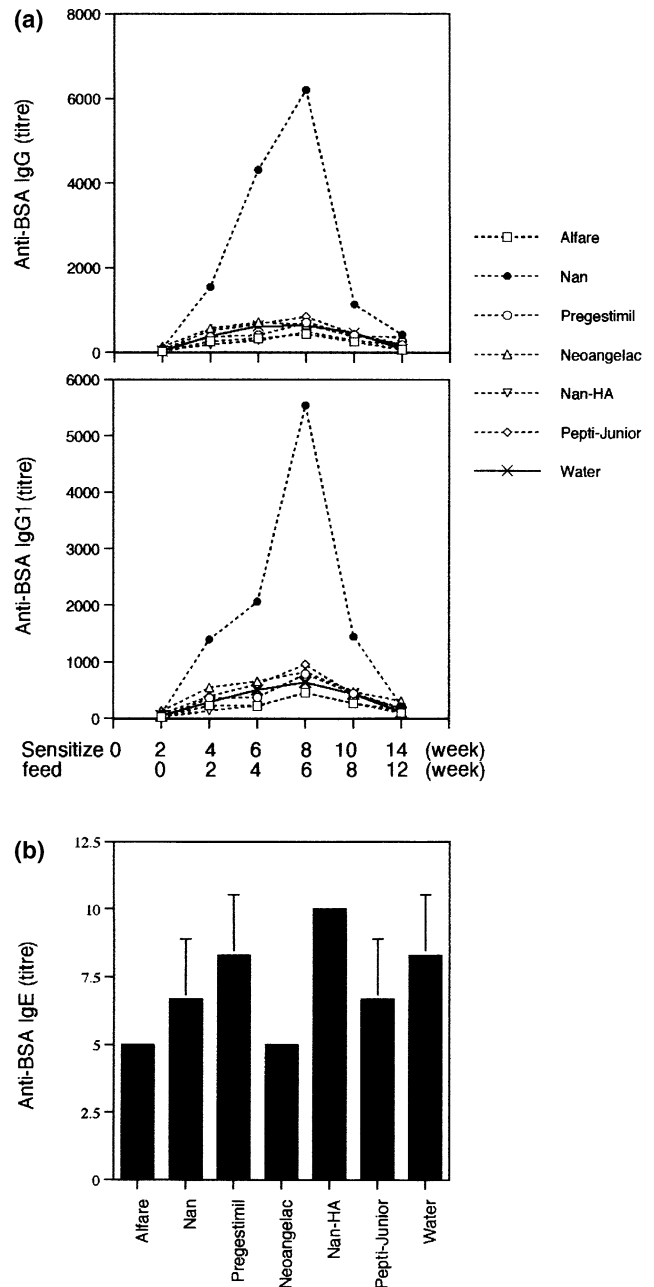


Fig. 6. Effect of ingestion of hydrolysed formulas on Th2 antibody responses to bovine serum albumin (BSA) in sensitized mice. Groups of C3H mice ($n = 7$) were first sensitized with BSA. Two weeks later, they were fed different formulas for 12 weeks, respectively. Their specific IgG1 and IgG antibody responses were assessed biweekly (a). Specific IgE antibody responses after 12-week feeding of different formulas are shown in (b).

week feeding of Nan, but not of hydrolysed formulas, to previously sensitized mice. Interestingly, their ongoing IgE responses were never increased after feeding Nan or hydrolysed formulas for 2–12 weeks.

In contrast to adult mice, oral tolerance cannot be induced in neonatal mice [6, 28]. Similarly, there is so far no report for the induction of oral tolerance in neonatal infants. It is probable that newborn infants may be not intestinally mature enough to develop oral tolerance. Indeed, systemic immune responses to cow's milk proteins are found in bottle-fed infants, but they are then depressed by continual exposure

after the age of 9 months [31]. Most atopic infants can overcome cow's milk allergy at the age of 2–3 years [7, 31]. This phenomenon is supported by our finding that the ongoing IgE response to BLG can be suppressed after prolonged feeding of Nan for 12 weeks. It is feasible that large antigenic polypeptides in partially hydrolysed whey formulas may be intestinally absorbed and thus leads to anaphylactic reactions [23–26]. Therefore, partially hydrolysed whey formulas are not recommended for infants with cow's milk allergy [7, 13–15]. Extensively hydrolysed formulas and amino acid-derived formulas are a better choice for infants with high risks or with cow's milk allergy [7, 16, 17]. As the price of extensively hydrolysed formulas is rather high, partially hydrolysed formulas are still a good choice for infants at a low risk to prevent atopy.

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