

Dietary Nucleic Acid and Intestinal Microbiota Synergistically Promote a Shift in the Th1/Th2 Balance toward Th1-Skewed Immunity

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Key Words

CpG motif · Food allergy · Germ free mice · Microbiota · Nucleic acids

Abstract

Background: Intestinal microbiota are known to play an important role in the establishment of oral tolerance, thereby protecting the organism from food allergies. Dietary intake of nucleic acid (NA) is also reported to have such an anti-allergic effect; however, one unsolved question is whether or not dietary NA would act through a process of toll-like receptor 9 signaling activated by DNA containing a CpG motif, a well-known sequence leading to immunostimulatory activity. In this study, we focused on the question of whether the addition of dietary NA lacking CpG motifs would allow continued modulation of the Th1/Th2 balance. **Methods:** Germ free (GF) and *Bifidobacterium-infantis*-monoassociated BALB/c mice were maintained on either an NA-free casein diet or on an NA-supplemented casein diet for 4 weeks. Thereafter, both the in vivo anti-casein antibody levels and in vitro splenocyte cytokine secretion pattern were evaluated. **Results:** Feeding with a casein diet elicited a substantial increase in the serum anti-casein-spe-

cific IgG1, IgG2a, and IgE levels of GF mice fed the NA free-diet. The in vitro cytokine production profile showed that enhanced IL-4 production in the GF mice fed the NA free-diet was markedly reduced by the supplementation with dietary NA in both the GF and *B.-infantis*-monoassociated mice. In addition, IFN- γ secretion increased in the *B.-infantis*-reconstituted mice fed the diet containing NA. **Conclusions:** These results suggest that dietary intake of NA devoid of CpG motifs may prevent the development of allergies via acceleration of Th1-dominant immunity.

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Introduction

Food allergy is the manifestation of an abnormal immune response to a food antigen delivered by the oral route. In order to avoid mounting such a response against harmless or potentially beneficial materials, gut mucosa is generally guarded by the following strong physical barriers: the presence of luminal enzymes that alter the nature of the antigen itself; the phenomenon of systemic unresponsiveness following oral presentation of the antigen, termed 'oral tolerance'; and the production of an

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antibody, secretory immunoglobulin A, that is highly suited to the hostile environment of the gut [1]. Together they protect against the development of allergic sensitization.

A number of factors are known to participate in the maturation of the mucosal immune system. As demonstrated by our previous work with germ free (GF) animals [2], intestinal microbiota, which colonize rapidly after birth, play a principal role in the postnatal maturation of the mammalian immune system. In addition, an optimal mucosal barrier function in the neonatal period is accomplished by an adequate supply of breast milk. In fact, breast milk contains a wide variety of immunomodulating and anti-inflammatory components, such as cytokines and prostaglandins [3]. One such breast-milk-derived factor, nucleic acid (NA), has also been shown to have the ability to promote a shift in the Th1/Th2 balance toward Th1-dominant immunity [4, 5]. In this study, considering recent topics regarding immunostimulatory DNA containing CpG motifs [6], we examined whether or not the addition of dietary NA lacking CpG motifs would still modulate the flora-induced maturation of the mucosal immune system.

Materials and Methods

Mice

GF BALB/c mice were originally obtained from Japan Clea (Tokyo, Japan) and were then bred in our facility. These GF mice were maintained in Trexler-type flexible-film plastic isolators with sterile food and water until just before death, as described previously [2]. To obtain *Bifidobacterium-infantis*-associated mice at the neonate stage, the parent GF mice were orally administered *B. infantis*, and then their offspring, which thus became infected with this bacterium at the neonate stage, were used in this study. Bacteriological examination of fecal samples revealed that *Bifidobacterium* colonization was completely accomplished by 3 weeks after birth. All experiments were approved by the Ethics Committee for Animal Experiments of the Kyushu University.

Study Protocol

GF and *B. infantis*-monoassociated mice at 5 weeks of age were maintained on either an NA-free diet (casein 24.5%, cornstarch 45.5%, granulated sugar 10.0%, corn oil 6.0%, crystal cellulose 3.0%, cellulose powder 2.0%, α -starch 1.0%, vitamin mixture 1.0% and mineral mixture 7.0%) or an NA-supplemented diet (casein 24.5%, cornstarch 44.3%, granulated sugar 10.0%, corn oil 6.0%, crystal cellulose 3.0%, cellulose powder 2.0%, α -starch 1.0%, vitamin mixture 1.0%, mineral mixture 7.0% and purified salmon milt DNA 1.2%). On the 4th week of the diet, these mice were killed by cervical dislocation. Blood samples were obtained by cardiac puncture, and the sera were kept at -80°C until the measurement of serum antibody levels. Spleen was also removed for analysis of in vitro cytokine production.

Determination of Serum Antibody Level and Cytokine Measurement

The serum levels of anti-casein IgG1, IgG2a, and IgE were determined by an ELISA technique as described elsewhere [7]. Briefly, serum samples with the serial dilutions were placed on ELISA plates coated previously with casein. This was followed by the addition of goat anti-mouse IgG1, IgG2a, or IgE conjugated with peroxidase (Caltag Laboratories, Burlingame, Calif., USA). Thereafter, the plates were developed using a substrate solution containing 0.04% *o*-phenylenediamine dissolved in phosphate citrate buffer (pH 5.0).

Cytokine production by spleen cells in vitro was also quantified using a sandwich ELISA technique as previously described [4, 8].

Statistical Analysis

Statistical differences between the groups were determined by the post hoc Dunnett's test after factorial analysis of variance.

Results and Discussion

As summarized in table 1, the serum anti-casein-specific IgG1 level of the GF mice fed the NA-free diet was substantially higher than that of those fed the NA-supplemented diet. Similarly, the casein diet also induced a small but significant elevation in serum anti-casein-specific IgG2a and IgE levels in the GF mice fed the NA-free diet, indicating that oral tolerance to casein was not fully induced in the absence of dietary NA intake and gut microbes. No such apparent elevation in the serum antibody level was found in the *B. infantis*-associated mice, regardless of whether or not the mice were supplemented with NA. However, the IgG1/IgG2a ratio, which was used as a marker of the Th1/Th2 balance in this study, was decreased by the addition of NA in both the GF and *B. infantis*-monoassociated animals. These results were further supported by the in vitro cytokine production profile of the spleen cells in which the addition of casein to the cultures led to the detection of IL-4 at an appreciable level in the GF mice fed the NA-free diet. IFN- γ was not detected in this group (table 2). Supplementation with dietary NA markedly reduced in vitro IL-4 production in both groups of mice, while elevating IFN- γ production only in the mice monoassociated with *B. infantis*.

Recently, accumulating evidence has shown bacterial DNA acting as a strong inducer of Th1-skewing immunity. Immunostimulatory DNA sequences are unmethylated CpG dinucleotides within consensus sequences, such as CpG motifs. CpG motifs are common in certain bacterial and viral genomes and are underrepresented in mammalian genomes [6]. The possible involvement of CpG motifs in the present data, in which dietary NA accelerated Th1-skewing immunity in concert with intestinal

Table 1. The effect of dietary NA on anti-casein-specific antibody titers

Study groups		Casein-specific antibody titer, EU			IgG1/IgG2a ratio
		IgG1	IgG2a	IgE	
GF	NA (-)	106 ± 12.8	44.8 ± 6.1	20.5 ± 3.8	2.5 ± 0.2
	NA (+)	29.9 ± 8.5***	20.3 ± 3.9**	3.5 ± 0.9***	1.7 ± 0.3*
BI	NA (-)	13.2 ± 1.8	15.5 ± 3.4	3.2 ± 1.2	1.0 ± 0.1
	NA (+)	9.1 ± 1.3	19.1 ± 3.7	2.9 ± 0.8	0.6 ± 0.1*

GF and *B. infantia* (BI)-monoassociated mice were fed the casein diet with or without dietary NA. The casein-specific antibody titer of the sample was calculated by comparing it with the internal standard. Standard serum was obtained by intraperitoneal sensitization of mice with casein plus aluminum hydroxide. The concentration of anti-casein IgG1, IgG2a, and IgE in the standard serum was arbitrarily assigned at 100 ELISA units (EU). All data are expressed as means ± SE (n=10/group). * p < 0.05, ** p < 0.01, and *** p < 0.001, vs. the corresponding value of the NA (-) group.

Table 2. In vitro IL-4 and IFN-γ production by spleen cells

Study groups		IL-4, pg/ml	IFN-γ, pg/ml
GF	NA (-)	587 ± 118	ND
	NA (+)	152 ± 30.7**	ND
BI	NA (-)	115 ± 23.8	ND
	NA (+)	45 ± 10.4*	99.3 ± 14.2

For a cytokine production assay, single cell suspensions from the spleen were suspended at a final concentration of 2.5×10^6 /ml and cultured in 1-ml aliquots in 24-well plates in a medium with or without 100 µg/ml casein. Supernatants were collected either after 48-hour culture for IFN-γ or 72 h for IL-4, since at these times each cytokine production exhibited its peak level. Data shown are calculated by subtracting the value without casein from the value with casein, and are expressed as means ± SE (n = 5 per group). * p < 0.05, ** p < 0.01, and *** p < 0.001, vs. the corresponding value of the NA (-) group. ND = Not detected.

microbes, seems unlikely because the NA used in this experiment was derived from purified salmon milt DNA, which is reportedly unable to mount a CpG-motif-related biological event [9] owing to the absence of the CpG sequence. On the other hand, as demonstrated in a more recent paper by Rachmilewitz et al. [10], toll-like receptor 9 signaling was essential for exerting the anti-inflammatory effects of probiotics in murine experimental colitis; hence, the Th1-promoting effect of *B. infantis*, with a genome that actually contains CpG motifs [11], may be mediated by its own DNA rather than by its ability to colonize the gut.

How can such dietary NA lacking CpG motifs exert its effect on the gut? In general, dietary NA is generally degraded by various types of enzymes such as ribonucleases and deoxyribonucleases, and is subsequently absorbed mainly in the form of nucleosides, which thus suggests a possible mechanism by which the effect of dietary NA may be exerted at the mucosal barrier, specifically through a sensing pathway that involves purinergic signaling. Indeed, purinergic receptors have recently been found in the membrane of virtually all gastrointestinal epithelia, and moreover, extracellular adenosine-5'-triphosphate and adenosine have established roles as potent stimulators of fluid and electrolyte secretion in colon, gallbladder, pancreatic duct, and bile duct models [12]. A new project in our laboratory is now in progress that applies a DNA microarray technique to the GF animal model to further clarify the precise molecular mechanism whereby adenosine modulates the epithelial barrier function of the gut.

Although almost all the common allergic diseases have a hereditary component, quantitative differences in environmental factors may also influence the development of IgE antibodies and the resultant allergic diseases [13]. Many studies have focused on the influence of dietary manipulation on the occurrence of allergies. Some of these studies have suggested that prolonged exclusive breast-feeding of high-risk infants may provide both short- and long-term benefits in reducing the incidence of atopic disorders in infancy and later in childhood [14], although such an allergy-preventing effect of breast-feeding remains controversial. Because cow's milk contains lower concentrations of some NAs than human milk and

certainly has a different profile of NA [15], the NA level and profile in breast milk may be involved in the anti-allergic effect of breast milk.

Most human diets are rich in NA; however, the few exceptions to this would include patients receiving NA-free total parenteral or enteral nutrition and infants receiving solely NA-free formulas. Together with the present data in which antibody response to casein was not fully tolerated in the absence of both dietary NA intake and gut microbes, these findings thus indicate that unfavorable intestinal flora, which are often found after antibiotic treatment, could enhance an aberrant sensitization to food antigens in infants fed formula devoid of NA.

In summary, dietary NA, in concert with intestinal microbiota, may act as an environmental factor against the development of allergic sensitization and Th2-skewed

cytokine responses. Nonetheless, whether or not the effect of dietary NA is via a mucosal barrier function remains to be clarified. Clearly, further studies focusing on the cellular pathway are still needed to elucidate how dietary NA, particularly that lacking CpG motifs, interacts with the gut immune system. Such studies may extend existing options to effectively prevent high-risk infants from developing allergic diseases.

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