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# Timing of *Bifidobacterium* administration influences the development of allergy to ovalbumin in mice

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**Abstract**

C3H/HeJ mice were sensitized with ovalbumin (OVA) and choleratoxin (CT) for 5 weeks, and then *Bifidobacterium bifidum* BGN4 was administered continuously for 7 weeks, starting 2 weeks before (pre-treatment group) and 2 weeks after (post-treatment group) the initial sensitization. After sensitization, the OVA-induced (sham group) mice showed growth inhibition and had scab-covered tails which was associated with serum levels of $9887 \pm 175$ ng OVA-specific IgE/ml and $758 \pm 525$ ng IgG1/ml. The sera of the pre-treatment group had $4805 \pm 245$ ng OVA-specific IgE/ml and $193 \pm 87$ ng IgG1/ml, as well as less severe tail symptoms. The sera of the post-treatment group had $5723 \pm 207$ ng OVA-specific IgE/ml but the IgG1 and IgG2a levels were the same as those of the sham group. In spleen cultures, both pre-treatment and post-treatment increased the levels of IFN-$\gamma$ but decreased the levels of IL-6 and IL-18. Taken together, the *in vivo* and *in vitro* results show that treatment with *Bifidobacterium* before OVA sensitization suppresses or modulates the allergic response more effectively than treatment with Bifidobacterium following OVA sensitization.

## Introduction

Food allergy is characterized by an abnormal immunologic reactivity to food components. It often begins in the first 1–2 years of life with the process of sensitization in which the immune system responds to specific food proteins, most often with the development of allergen-specific immunoglobulin E (IgE) (Matsuda & Nakamura 1993). The hygiene hypothesis (Gavett *et al.* 1995, Kirjavainen *et al.* 1999) proposes that the Th2-biased immune systems of new-born infants (Martinez & Holt 1999) might be changed to the Th1 type by exposure to gut bacteria after birth. A recent study showed that the administration of probiotics induces anti-inflammatory, tolerogenic immune responses (Schultz *et al.* 2003). Among the diverse probiotics, *Bifidobacterium* is one of the most promising, since it is the most predominant bacterium in infants (He *et al.* 2001). On the other hand, the timing of exposure to antigens or infections is widely accepted to be of primary importance in determining the phenotype of Th1 cell responses in neonates (Hosono *et al.* 1997, Holt & Sly 2002).

In the present study, to investigate the effect of timing of the administration of the probiotic bacteria, *Bifidobacterium* was administered to ovalbumin-induced mice at different time schedules. *Bifidobacterium bifidum* BGN4, which lowers the production of allergy-related cytokines from mouse cells (Kim et *al.* 2005), was used. The results show that the administration of *Bifidobacterium* before OVA sensitization was more

efficient in blocking the allergic response than administration after OVA sensitization.

## Materials and methods

### *Mice*

Three-week-old female C3H/HeJ mice, 11–13 g, were sensitized at 5 weeks of age and each group included six mice of similar weight. Mice were allowed free access to water, and maintained on a 12:12 h light:dark cycle in an environmentally controlled animal chamber at $23 \pm 1$ °C and $55 \pm 10\%$ humidity. The animal experimentation guidelines of Seoul National University were followed.

### *Microorganisms*

*Bifidobacterium bifidum* BGN4 (BGN4) was anaerobically cultured in MRS broth (Difco). After centrifugation ($4000 \times g$, 40 min, 4 °C), bacteria were dried by lyophilization and mixed with mouse food (0.2% w/w). The total cell number of BGN4 was $3 \times 10^9$ c.f.u./g. The daily intake of diet for each mouse was about 3.0 g and the amount of bacteria intake for each mouse was approx. 6 mg.

### *Intragastric antigen sensitization and treatment*

Mice were deprived of diet for 2 h preceding the oral sensitization. Sensitization was performed by intragastric administration of 50 $\mu$g ovalbumin (OVA, Sigma) with 10 $\mu$g cholera toxin (CT, Sigma) using a stainless blunt feeding needle. Four groups of mice were used in this study (Figure 1).

### *Measurement of OVA-specific and total immunglobulins in serum and in fecal samples*

To determine serum antibody responses, tail vein blood was obtained biweekly following initial sensitization. Sera were collected and stored at −80 °C. Extracts of fecal pellets were prepared as described by Marinaro *et al.* (1997). Antibody levels were determined by ELISA according to the manufacture's protocol (Pharmingen).

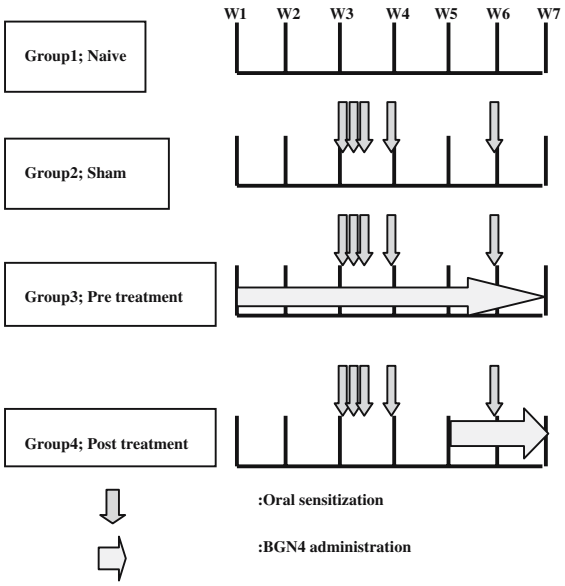


*Fig. 1.* Experimental protocol: Intragastric ovalbumin sensitization and BGN4 administration. Mice were sensitized at weeks 3, 4, and 6 with ovalbumin and choleratoxin. Mice were fed 0.2% of lyophilized *Bifidobacterium bifidum* BGN4 in diet pellets starting 2 weeks before (group 3) or after (group 4) the initial sensitization until sacrifice. The naive mice in group 1 received phosphate buffer saline (pH 7.2) instead of ovalbumin and choleratoxin as a negative control. Mice in group 2 were administered cornstarch instead of bacteria powder as a sham treatment. Each group included six mice.

### *Measurement of IL-6, IL-18, and IFN-$\gamma$* levels in spleen

Spleens were isolated from mice from each group ($n = 6$) at week 7. Spleen cells from mice were separated using glass slides and a 200 gauge stainless mesh. Cells were cultured in RPMI medium and stimulated with concanavalin A (10 $\mu$g/ml) for 48 h in 24-well flat bottom plates at a density of $5 \times 10^6$ cells/ml under 5% $CO_2$. ELISA kits (Pharmingen) were used for detecting the various cytokines.

### *Assessment of hypersensitivity reactions*

Allergic symptoms were evaluated after sacrifice utilizing a scoring system: 0, no symptoms; 1, puffiness of the tail; 2, 1–2 scabs on the tail; 3, 3–4 scabs on the tail; 4, 5–6 scabs on the tail; 5, more than 7 scabs on the tail. The scoring of the symptoms was evaluated by 10 individuals who were unaware of sample identity in a blind manner.

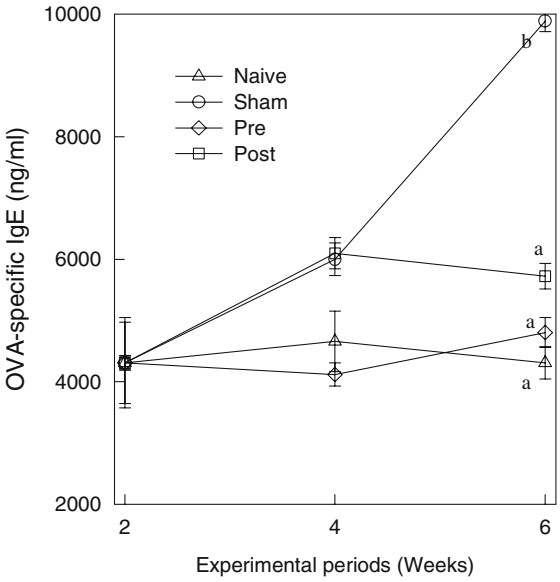


*Fig. 2.* Effect of *Bifidobacterium* BGN4 administration on production of ovalbumin specific IgE in serum from ovalbumin-sensitized mice. Sera from all groups of mice were obtained biweekly following initial ovalbumin sensitization. IgE levels were determined by ELISA.

*Statistical analysis*

All data are presented as the mean ± standard error of mean (SEM), indicated by bars in the figures. Data were analyzed using SAS (Release 8.01, O, USA). Differences between immunoglobulin and cytokine levels in the groups were analyzed by ANOVA followed by Duncan's multiple range test for multiple comparisons. $p$ values $< 0.05$ were considered significant.

## Results and discussion

*Effect of BGN4 on IgE production*

The OVA-specific IgE levels in sera of each group are presented in Figure 2. Pre-treatment with BGN4 markedly inhibited the production of OVA-specific IgE at week 6 (Naive, $4310 \pm 263$ ng/ml; Sham, $9887 \pm 175$ ng/ml; Pre, $4805 \pm 245$ ng/ml; Post, $5723 \pm 207$ ng/ml). Since IgE levels in the pre-treatment group did not increase compared with the sham group during the experimental period, pre-treatment with BGN4 would appear to block the induction of

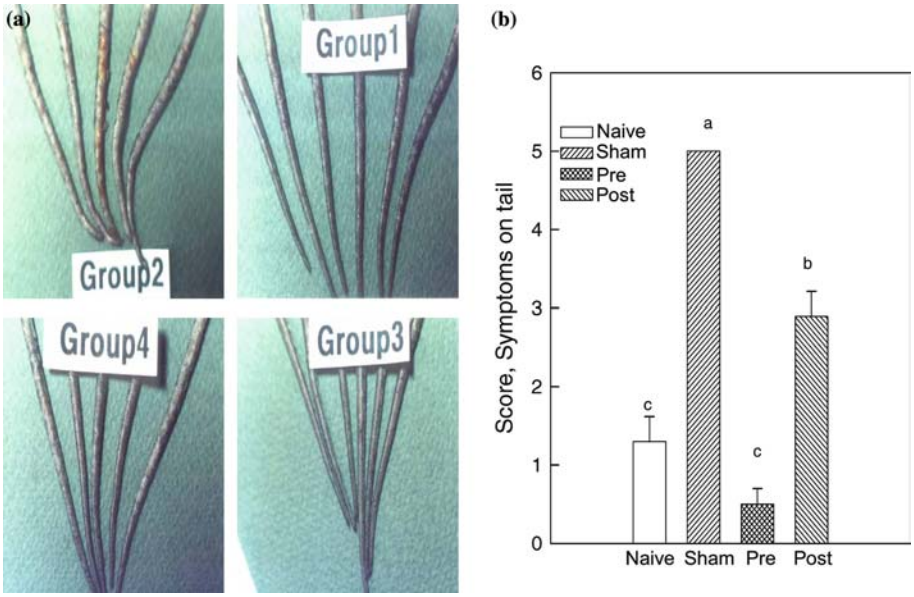


*Fig. 3.* Severity of tail allergy symptoms in ovalbumin-sensitized mice treated with *Bifidobacterium bifidum* BGN4 (BGN4) at week 7. Immunized mice showed marked tail bruising and scabs. A: Pictures of the tails from ovalbumin-sensitized mice treated with BGN4. Group 1: naive group; Group 2, sham group; Group 3, BGN4 pre-treatment group; Group 4, BGN4 post-treatment group. B: Severity of tail allergy symptoms in ovalbumin-sensitized mice was evaluated utilizing a scoring system in a blind manner.

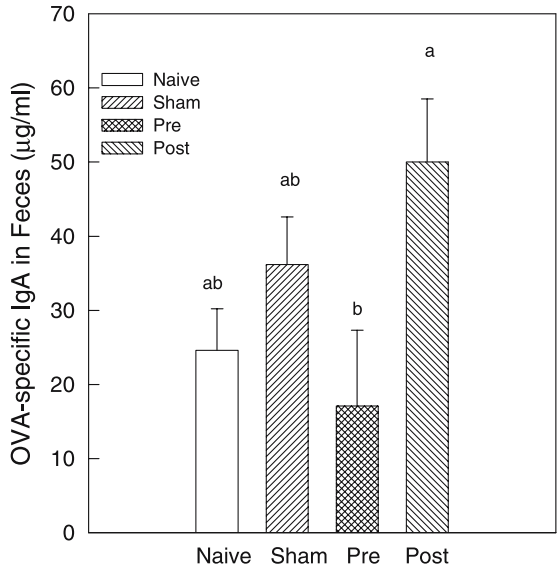


*Fig. 4.* Effect of *Bifidobacterium* on the production of ovalbumin specific IgA in fecal samples from ovalbumin-sensitized mice treated with BGN4. Fecal extracts were prepared from fresh fecal pellets of each group and ovalbumin-specific IgA levels were detected by ELISAs .

OVA-specific IgE entirely. On the other hand, treatment with BGN4 after initial sensitization reduced OVA-specific IgE levels less extensively.

*Tail symptoms*

After sensitization, sham mice started to scratch their tails, resulting in injury, including bleeding (Figure 3). The tail symptoms of the pre-treatment group were markedly reduced compared to the severe symptoms of the sham group at week 7, and those of the post-treatment group were decreased compared to those of the sham group, although the former still showed slight tail injuries. Therefore, the pre-treatment group showed the most effective attenuation of the tail injuries.

*Ovalbumin specific mucosal IgA*

The OVA-specific IgA level in the post-treatment group was not significantly different from that in the sham and naive groups at week 7 (Figure 4). However, the OVA-specific IgA level in the pre-treatment group was decreased compared to that of the sham group (Naive, $24.6 \pm 5.6$ $\mu$g/ml; Sham, $36.2 \pm 6.4$ $\mu$g/ml; Pre, $17.1 \pm 10.2$ $\mu$g/ml; Post, $54.6 \pm 8.5$ $\mu$g/ml, respectively).

*Levels of IgG1 and IgG2a in sera*

The levels of OVA-specific IgG1 in the post-treatment group were different from those of the naive group and pre-treatment group at week 7 (Figure 5a). On the other hand, only the levels of OVA-specific IgG2a in the pre-treatment group were different from those of the other groups. (Figure 5b). However, there were no differences in the levels of total IgG1 and IgG2a between the groups (Figure 5c, d).

The decreased levels of OVA-specific IgA, IgG1 and IgG2a in the pre-treatment groups are consistent with those of Mandic *et al.* (2004) who reported that the levels of IgG and IgA are high in allergic asthma patients compared to healthy children.

*Table 1.* Body weights (g) of ovalbumin-sensitized mice fed a diet containing (w/w) 0.2% of BGN4 lyophilized powder before or after initial sensitization for 7 weeks.[1]

| Groups | Week 2 | Week 3 | Week 4 | Week 5 | Week 6 | Week 7 |
|---|---|---|---|---|---|---|
| Naïve | $17.2 \pm 0.6^{b}$ | $18.8 \pm 0.5^{a}$ | $19.1 \pm 0.8^{a}$ | $22.4 \pm 0.6^{a}$ | $22.9 \pm 0.7^{a}$ | $23.3 \pm 1.1^{a}$ |
| Sham | $18.8 \pm 0.4^{b}$ | $18.5 \pm 0.3^{ab}$ | $19.9 \pm 0.3^{a}$ | $20.5 \pm 0.5^{b}$ | $21.2 \pm 0.4^{b}$ | $21.0 \pm 0.6^{b}$ |
| Pre | $19.5 \pm 0.3^{a}$ | $19.7 \pm 0.4^{a}$ | $20.9 \pm 0.3^{a}$ | $21.2 \pm 0.2^{ab}$ | $21.8 \pm 0.4^{ab}$ | $22.9 \pm 0.4^{a}$ |
| Post | $16.9 \pm 0.3^{b}$ | $19.0 \pm 0.2^{a}$ | $20.6 \pm 0.4^{a}$ | $21.5 \pm 0.3^{ab}$ | $21.2 \pm 0.5^{b}$ | $21.9 \pm 0.1^{ab}$ |

[1]Experimental diets:
Naive: 0.2% of corn starch.
Sham: 0.2% of corn starch.
Pre: 0.2% of *Bifidobacterium bifidum* BGN4 ($3 \times 10^9$ c.f.u./g) starting 2 weeks before initial sensitization.
Post: 0.2% of *Bifidobacterium bifidum* BGN4 ($3 \times 10^9$ c.f.u./g) starting 2 weeks after initial sensitization.
Values are given as mean ± SEM of six mice per group.
Different superscripts indicate significant differences determined by Duncan's multiple range test ($p < 0.05$).

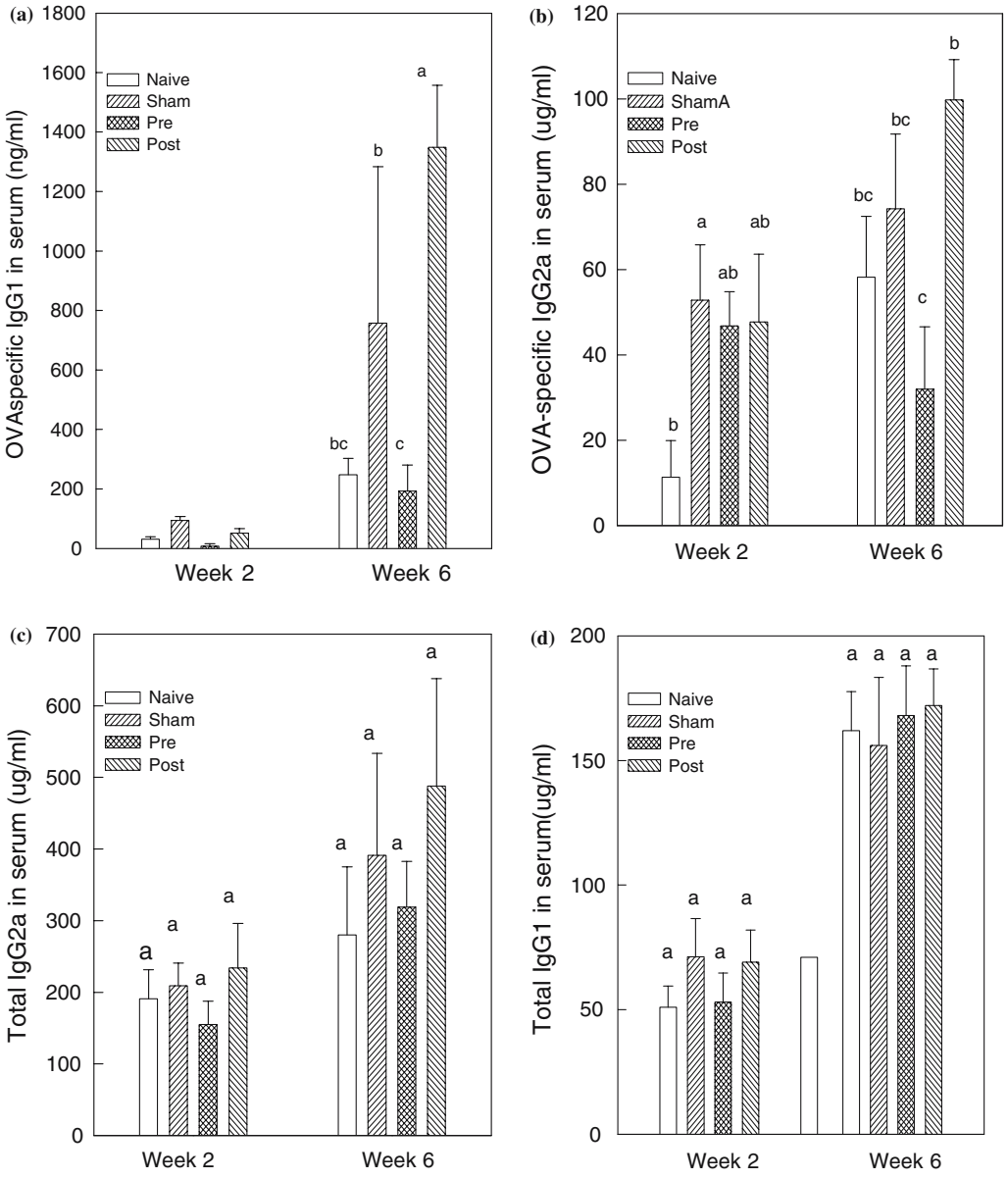


*Fig. 5.* Effect of *Bifidobacterium* on the production of ovalbumin-specific IgG1 and total IgG1 and ovalbumin-specific IgG2a and total IgG2a in sera from ovalbumin-sensitized mice treated with BGN4. Levels of antibodies were detected by ELISAs.

*Levels of cytokines in spleen cultures*

The levels of IFN-$\gamma$, IL-6, and IL-18 are given in Figure 6. In the *in vitro* spleen culture assay both pre-treatment and post-treatment with BGN4 markedly increased Th1 type cytokine IFN-$\gamma$ and decreased Th2 type cytokines IL-6 and IL-18 compared with the sham group. Recent reports indicate that IL-18 can directly stimulate IL-4 production and histamine release from basophils (Yoshimoto *et al.* 1999), enhance IL-4 and IL-13 production from both NK and T cells in synergy with IL-2 (Leite-De-Moraes *et al.* 2001), and induce IgE expression by B cells (Yoshimoto *et al.* 2000).

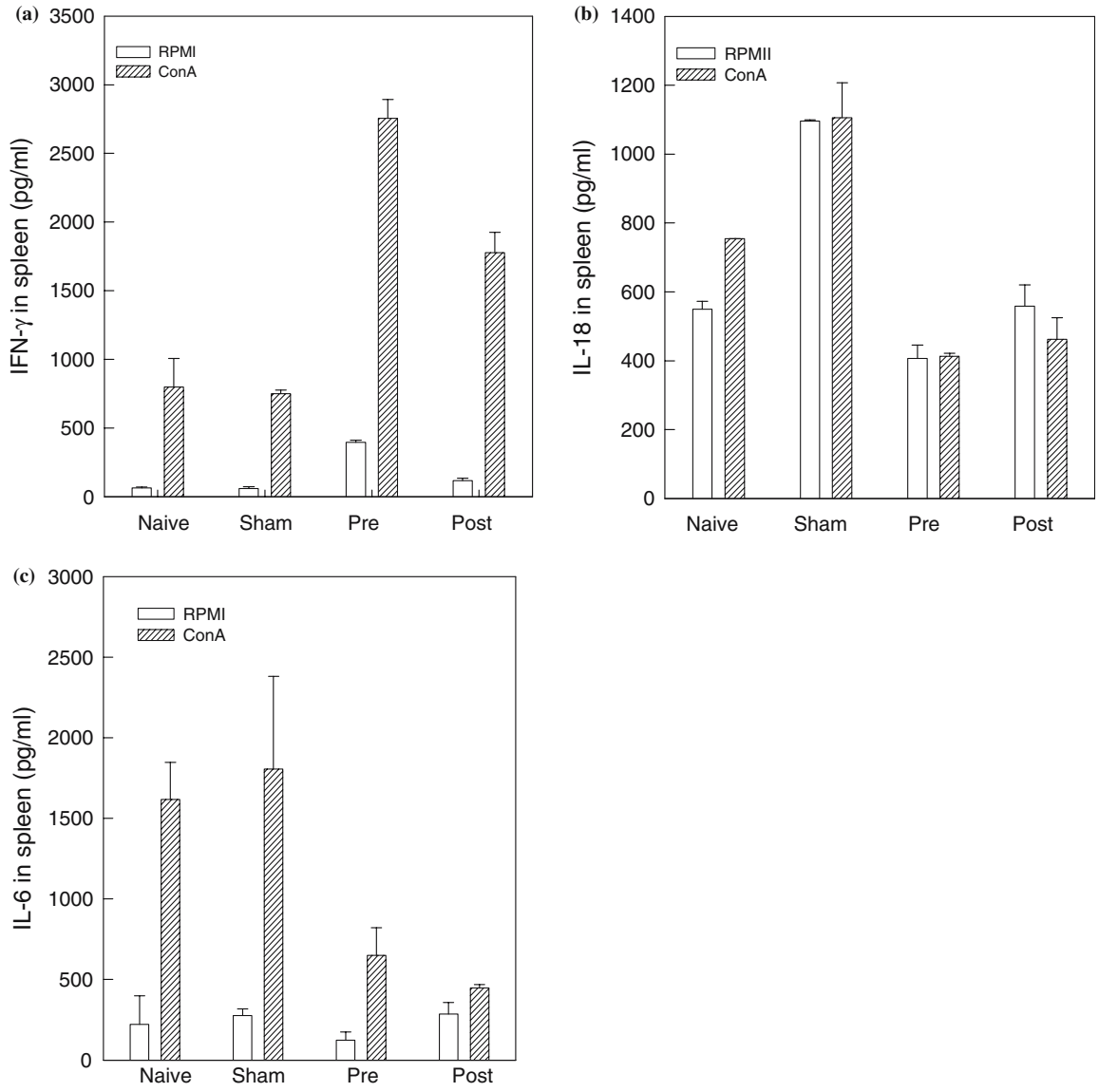


*Fig. 6.* Effect of *Bifidobacterium* on the production of IFN-γ, IL-6, IL-18, IgG1, and IgG2a from spleen cells of each group with BGN4 at week 7. Isolated spleen cells from different mouse groups were re-stimulated with concanavalin A (10 mg/ml).

*Effect of BGN4 administration on body weight*

The mean initial body weights did not differ significantly among the groups. Unlike the sham group, the pre-treatment group maintained similar body weights to the naive group during the experimental period (Table 1). BGN4 administration might decrease growth inhibition in the sham group.

Taken together, the *in vivo* and *in vitro* results show that pre-treatment with *Bifidobacterium* suppresses both the systemic and cellular allergic response, whereas post-treatment changes the cellular response in the spleen but only partially alters the systemic response.

**In conclusion**, these results demonstrate that oral administration of *Bifidobacterium* before

sensitization (or occurrence of allergy) profoundly prevents allergic response. However, the anti-allergenic effect of *Bifidobacterium* administered after outbreak of allergic disease is weaker than the effect of *Bifidobacterium* administered before administration of sensitization stimulus. Thus, intake of *Bifidobacterium* continuously from before the occurrence of allergic disease might be recommended to minimize allergic symptoms.

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