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FACS-sorted naive CD4+ T cells (CD44low CD25− CD4+ T cells) were stimulated with anti-CD3 mAb/anti-CD28 mAb under Th1-, Th2-, and Th17-polarizing conditions, IL-21–producing cells were detected under Th17-polarizing conditions with a high frequency and under Th2-polarizing conditions with a moderate frequency (Fig. 2), which is consistent with the levels of IL-21 in the supernatants of activated CD4+ T cells under the corresponding culture conditions (Fig. 1 A). Under Th2-polarizing conditions, a significant number of IL-4–producing cells simultaneously produced IL-21 and >70% of IL-21–producing CD4+ T cells were positive for intracellular IL-4 (Fig. 2). Under Th17-polarizing conditions, a significant number of IL-17A- and IL-17F–producing cells also simultaneously produced IL-21, but >60% of IL-21–producing CD4+ T cells were negative for intracellular IL-17A and -17F (Fig. 2). IL-21–producing CD4+ T cells under Th17-polarizing conditions were also negative for intracellular IL-4 and IFN-γ (Fig. 2). These results suggest that although IL-21–producing CD4+ T cells develop preferentially under Th17-polarizing conditions, the majority of IL-21–producing CD4+ T cells do not produce IL-17A or -17F.

IL-6 induces, but TGF-β inhibits, the development of IL-21–producing CD4+ T cells

Because IL-21–producing CD4+ T cells developed preferentially under Th17-polarizing conditions as compared with Th1- or Th2-polarizing conditions (Fig. 1 A and Fig. 2), we next examined the role of IL-6 and TGF-β in the development of IL-21–producing CD4+ T cells. Naive CD4+ T cells were stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of IL-6, anti–IL-4 mAb, and anti–IFN-γ mAb with or without TGF-β. We found that IL-6 together with the blocking antibodies to IL-4 and IFN-γ strongly induced the development of IL-21–producing CD4+ T cells without the induction of IL-4, IFN-γ, IL-17A, or IL-17F production (Fig. 3 A). Furthermore, unexpectedly, TGF-β inhibited the development of IL-21–producing CD4+ T cells in a dose-dependent manner (Fig. 3 A). Measurement of IL-21 levels in the supernatants confirmed the TGF-β–mediated inhibition of IL-21 production (Fig. 3 B). TGF-β also inhibited the development of IL-21–producing CD4+ T cells under

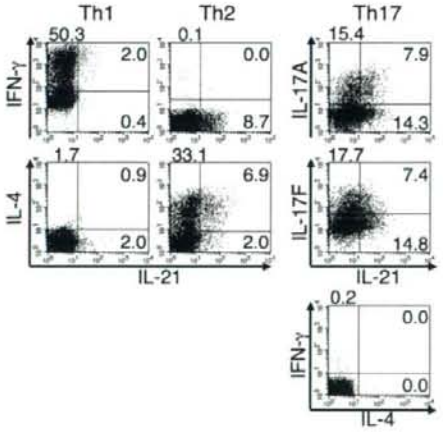

Figure 2. CD4+ T cells producing IL-21, but not IL-17A/IL-17F, are present under Th17-polarizing conditions. Naive CD4+ T cells from lymph nodes of C57BL/6 mice were stimulated with anti-CD3 mAb/anti-CD28 mAb under Th1-, Th2-, and Th17-polarizing conditions for 5 d. Cells were evaluated for the expression of the indicated cytokines by intracellular cytokine staining as described in the Materials and methods. Data are representative of three independent experiments.

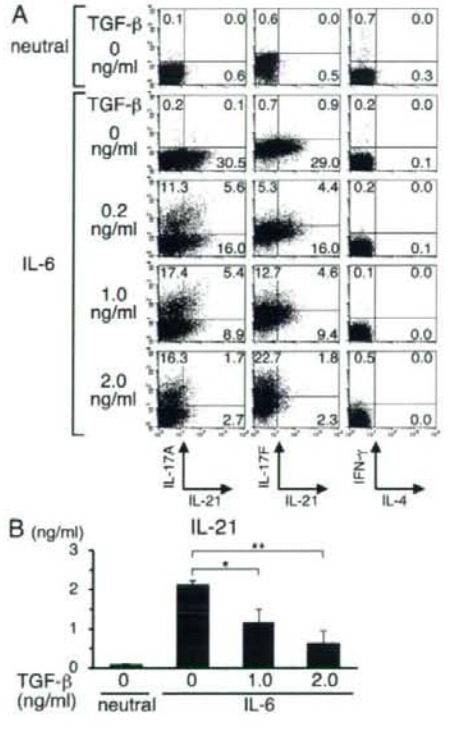

Figure 3. IL-6 induces, but TGF-β inhibits, the development of IL-21–producing CD4+ T cells. (A and B) Naive CD4+ T cells from C57BL/6 mice were stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of 10 μg/ml anti–IL-4 mAb and 10 μg/ml anti–IFN-γ mAb (neutral condition) with or without 100 ng/ml IL-6. Where indicated, 0.2–2 ng/ml TGF-β was added. (A) 4 d later, cells were stimulated with PMA/ionomycin, and intracellular staining for the indicated cytokines was performed. Shown are representative FACS profiles from three independent experiments. (B) 4 d later, cells were washed and stimulated with PMA/ionomycin for 8 h at 2 × 10^6 cells/ml. The levels of IL-21 in the culture supernatants were measured by ELISA. Data are the mean ± the SD ($n = 3$). *, $P < 0.05$; **, $P < 0.01$.

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Th2-polarizing conditions (unpublished data). In contrast, consistent with previous reports (20–22), TGF-β together with IL-6 induced the development of IL-17A– and IL-17F–producing Th17 cells (Fig. 3 A). These results indicate that IL-6 induces the development of IL-21–producing CD4+ T cells, but on the contrary, TGF-β inhibits IL-6–induced development of IL-21–producing CD4+ T cells.

We next examined the kinetics of IL-21, -17A, and -17F production from naive CD4+ T cells upon stimulation with anti-CD3 mAb/anti-CD28 mAb. In the presence of IL-6, anti–IL-4 mAb, and anti–IFN-γ mAb, IL-21–producing CD4+ T cells were detected at a low frequency at day 1 after stimulation, and then the frequency of IL-21–producing CD4+ T cells was increased until day 4 in a time-dependent manner without the production of IL-17A and -17F (Fig. 4). Under Th17-polarizing conditions, IL-21–producing CD4+ T cells were also detected at day 1, but the frequency of IL-21–producing CD4+ T cells was not increased thereafter (Fig. 4). On the other hand, IL-17A–producing CD4+ T cells were detected at day 2 under Th17-polarizing conditions (Fig. 4). IL-17F–producing CD4+ T cells were also detected at day 2 under Th17-polarizing conditions and were increased at day 4 (Fig. 4).

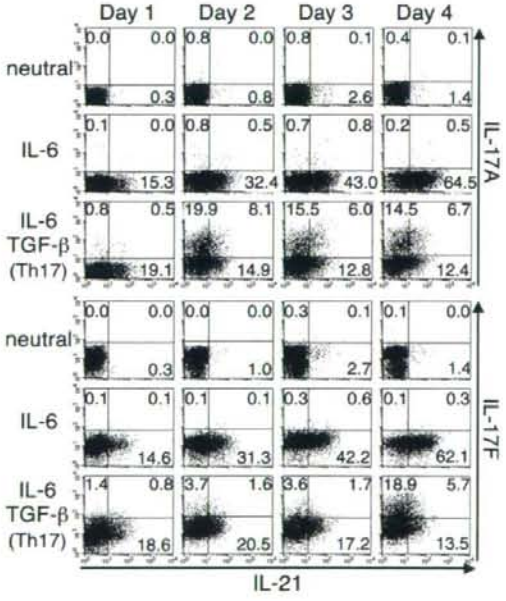

Figure 4. Kinetics of IL-21 production from CD4+ T cells. Naive CD4+ T cells from C57BL/6 mice were stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of anti–IL-4 mAb and anti–IFN-γ mAb (neutral condition) with 100 ng/ml IL-6 or IL-6 plus 1 ng/ml TGF-β. At indicated times after stimulation, cells were stimulated with PMA/ionomycin, and intracellular staining for the indicated cytokines was performed. Shown are representative FACS profiles from three independent experiments.

Smad3 is involved in TGF-β–mediated inhibition of IL-21–producing CD4+ T cells

Smad2 and Smad3 are phosphorylated by TGF-β receptor and heterodimerize with Smad4 (23, 24). The activated Smad complex then translocates into the nucleus and regulates the transcription of target genes (23, 24). We then used the mice lacking Smad3 (Smad3$^{-/-}$ mice) to determine whether Smad3 is required for the TGF-β–mediated suppression of IL-21–producing CD4+ T cells. Purified CD4+ T cells from WT mice or asymptomatic Smad3$^{-/-}$ mice, in the latter of which lymph node CD4+ T cells exhibit a normal phenotype regarding the activation markers such as CD69 (unpublished data) (25), were stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of IL-6, anti–IL-4 mAb, anti–IFN-γ mAb, and anti–IL-2 mAb with or without TGF-β and the development of IL-21–producing CD4+ T cells and Th17 cells was evaluated by intracellular staining. As shown in Fig. 5, not only TGF-β–mediated induction of Th17 cell development but also TGF-β–mediated suppression of IL-21–producing CD4+ T cells were reduced in Smad3$^{-/-}$ CD4+ T cells compared with those in littermate WT mice. These results indicate that TGF-β signaling induces Th17 cell development, but inhibits the development of IL-21–producing CD4+ T cells, in part via Smad3-dependent pathways.

IL-2 does not inhibit the development of IL-21–producing CD4+ T cells

It has recently been reported that IL-2 signaling via STAT5 inhibits the development of Th17 cells (26). We next examined the role of IL-2 in the development of IL-21–producing CD4+ T cells. When naive CD4+ T cells were stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of IL-6, anti–IL-4 mAb, and anti–IFN-γ mAb, neither IL-2 nor anti–IL-2 mAb significantly affected the development of IL-21–producing CD4+ T cells (Fig. 6). On the other hand,

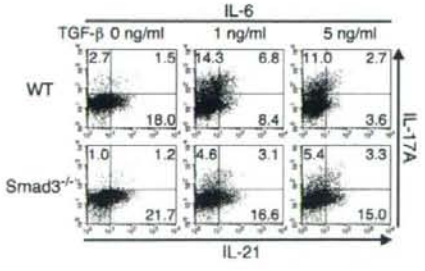

Figure 5. Smad3 is involved in TGF-β–mediated suppression of IL-21 production from CD4+ T cells. Purified CD4+ T cells from Smad3$^{-/-}$ mice or littermate WT mice were stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of anti–IL-4 mAb, anti–IFN-γ mAb, and anti–IL-2 mAb with 100 ng/ml IL-6 or IL-6 plus 1 or 5 ng/ml TGF-β for 3 d. Cells were then stimulated with PMA/ionomycin and intracellular staining for IL-21 versus IL-17A was performed. Shown are representative FACS profiles from three independent experiments.

under Th17-polarizing conditions, IL-2 significantly enhanced the development of IL-21–producing CD4+ T cells (Fig. 6). In contrast, IL-17A– and IL-17F–producing CD4+ T cells were decreased by IL-2 and were increased by anti–IL-2 mAb under Th17-polarizing conditions (Fig. 6), which is consistent with the previous study showing that the genetic deletion or antibody blockade of IL-2 promotes the differentiation of IL-17A–producing CD4+ T cells (26). These results suggest that, in contrast to the development of Th17 cells, IL-2 instead exerts an enhancing effect on the development of IL-21–producing CD4+ T cells under Th17-polarizing conditions.

IL-21 functions as an autocrine growth factor for IL-21–producing CD4+ T cells

It was recently reported that IL-21 functions as an autocrine growth factor for the development of Th17 cells (6–9). We determined the role of IL-21 on the development of IL-21–producing CD4+ T cells. IL-21, in the presence of the blocking antibodies to IL-4 and IFN-γ, induced the development of IL-21–producing CD4+ T cells from WT CD4+ T cells, but not from IL-21R–deficient (IL-21R$^{-/-}$) CD4+ T cells (Fig. 7 A). In addition, IL-21–induced development of IL-21–producing CD4+ T cells was inhibited by TGF-β (Fig. 7 A), like IL-6–induced development was (Fig. 3). Soluble IL-21R-Fc, which neutralizes IL-21 (10), also inhibited the development of IL-21–producing CD4+ T cells both in the presence of IL-6, anti–IL-4 mAb, and anti–IFN-γ mAb and under Th17-polarizing conditions by 20–30% (Fig. 7 B). These results suggest that IL-21 and -6 both induce the development of IL-21–producing CD4+ T cells, the effect of the latter of which is mediated in part by IL-21 production.

In contrast, the blockade of IL-21 signaling by soluble IL-21R-Fc resulted in the decreased development of Th17 cells (Fig. 7 B), which is consistent with previous reports (6–9). Together with our finding that a considerable number of IL-21–producing CD4+ T cells are negative for intracellular IL-17A and -17F under Th17-polarizing conditions (Fig. 2), these results suggest that IL-21 functions not only as an autocrine growth factor but also as a paracrine growth factor for the development of Th17 cells.

We also examined the effect of anti–IL-6 antibody on the differentiation of IL-21–producing CD4+ T cells from naive CD4+ T cells cultured under Th2-polarizing conditions. As shown in Fig. 7 C, a neutralizing antibody against IL-6 decreased the number of IL-21–producing CD4+ T cells by 50% without any significant change in the number of IL-4 single-positive cells. Moreover, although soluble IL-21R-Fc itself did not significantly decrease the number of IL-21–producing CD4+ T cells under Th2-polarizing conditions (unpublished data), IL-21R-Fc together with anti–IL-6 antibody significantly suppressed the differentiation of IL-21–producing CD4+ T cells under Th2-polarizing conditions to a greater extent than anti–IL-6 antibody alone (Fig. 7 C). These results suggest that during Th2 cell differentiation, IL-6 produced by Th2 cells promotes the differentiation of IL-21–producing CD4+ T cells from Th2 cells and non-Th2 CD4+ T cells.

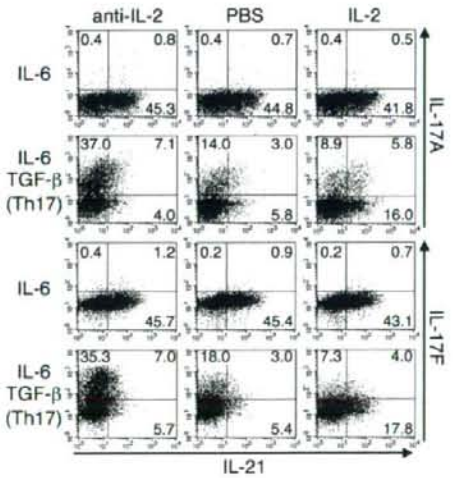

Figure 6. IL-2 does not inhibit the development of IL-21–producing CD4+ T cells. Naive CD4+ T cells from C57BL/6 mice were stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of anti–IL-4 mAb and anti–IFN-γ mAb with 100 ng/ml IL-6 or IL-6 plus 1 ng/ml TGF-β. Where indicated, either 10 ng/ml IL-2 or 10 μg/ml anti–IL-2 antibody was added to the culture. 4 d later, cells were stimulated with PMA/ionomycin and intracellular staining for the indicated cytokines was performed. Shown are representative FACS profiles from three independent experiments.

Expression of transcription factors in CD4+ T cells cultured in the presence of IL-6

We also examined the expression pattern of transcription factors in CD4+ T cells that were cultured in the presence of IL-6, anti–IL-4 mAb, and anti–IFN-γ mAb. Consistent with previous reports (5), CD4+ T cells cultured under Th17-polarizing conditions expressed RORγt, but not Foxp3, T-bet, or GATA3 (Fig. 8). In contrast, CD4+ T cells cultured in the presence of IL-6, anti–IL-4 mAb, and anti–IFN-γ mAb expressed a moderate level of RORγt, but not Foxp3, T-bet, or GATA3 (Fig. 8). As expected, Th1 cells expressed T-bet, Th2 cells expressed GATA3, and regulatory T (T reg) cells expressed Foxp3 (Fig. 8).

The frequency of IL-21–producing cells is increased with the rounds of cell cycle progression

It has been shown that the production of cytokines such as IL-4 is correlatively increased in CD4+ T cells with cell divisions (27, 28). We next examined the correlation of IL-21 production and cell cycle progression of CD4+ T cells using a CFSE-labeling method. As shown in Fig. 9, the frequency of cell division was indistinguishable in CD4+ T cells cultured in the presence of IL-6, anti–IL-4 mAb, and anti–IFN-γ mAb and

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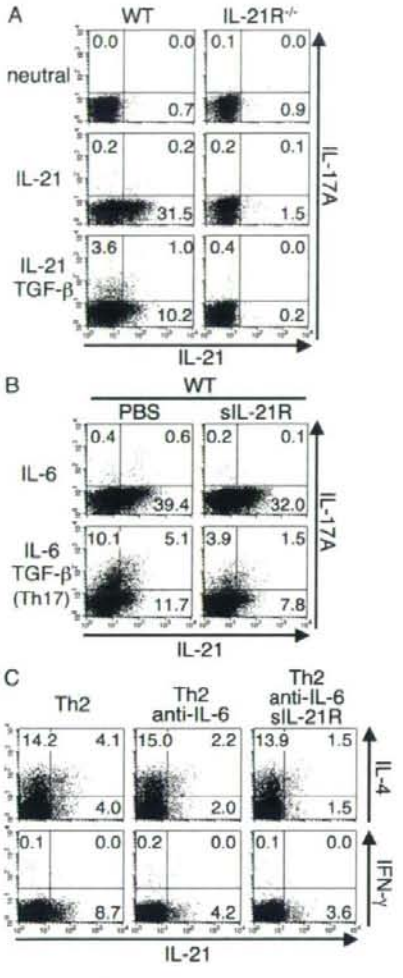

Figure 7. IL-21 induces the development of IL-21–producing CD4⁺ T cells. (A) Naive CD4⁺ T cells from IL-21R$^{-/-}$ mice or littermate WT mice on a BALB/c background were stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of anti–IL-4 mAb and anti–IFN-γ mAb (neutral condition) with 100 ng/ml IL-21 or 1 ng/ml IL-21 plus TGF-β. 4 d later, cells were stimulated with PMA/ionomycin and intracellular staining for IL-21 versus IL-17A was performed. Shown are representative FACS profiles from three independent experiments. (B) Naive CD4⁺ T cells from BALB/c mice were stimulated with anti-CD3 mAb/anti-CD28 mAb for 4 d in the presence of anti–IL-4 mAb and anti–IFN-γ mAb with IL-6 or IL-6 plus TGF-β. Where indicated, soluble IL-21R-Fc (10 μg/ml) was added. Cells were stimulated with PMA/ionomycin and intracellular staining for IL-21 versus IL-17A was performed. Shown are representative FACS profiles from three independent experiments. (C) Naive CD4⁺ T cells from C57BL/6 mice were stimulated with anti-CD3 mAb/anti-CD28 mAb under Th2-polarizing conditions for 5 d. Where indicated, a neutralizing antibody against 10 μg/ml IL-6 and/or soluble IL-21R-Fc were added. Cells were then stimulated with PMA/ionomycin and intracellular staining for the indicated cytokines was performed. Shown are representative FACS profiles from three independent experiments.

under Th17-polarizing conditions. IL-21–producing CD4⁺ T cells were observed in all rounds of cell cycle progression in the presence of IL-6, anti–IL-4 mAb, and anti–IFN-γ mAb, but the frequency of IL-21–producing cells was increased with the rounds of cell cycle progression (Fig. 9). Similarly, under Th17-polarizing conditions, IL-17A– and IL-17F–producing cells were observed in all rounds of cell cycle progression, but the frequency of them was also increased with the rounds of cell cycle progression (Fig. 9).

IL-21–producing CD4⁺ T cells do not lose the ability to produce IL-21 when they are restimulated under Th1- or Th2-polarizing conditions

Fully differentiated Th1 cells and Th2 cells exhibit a stable phenotype regarding the pattern of cytokine production (29, 30). Finally, we examined whether IL-21–producing CD4⁺ T cells exhibited a stable phenotype of IL-21 production. To address this issue, CD4⁺ T cells were stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of IL-6, anti–IL-4 mAb, and anti–IFN-γ mAb twice, at a 5-d interval, and IL-21 production was assessed by intracellular staining after restimulation with anti-CD3 mAb. As shown in Fig. 10 A (top), IL-21–producing CD4⁺ T cells did not lose their ability to produce IL-21 at 10 d and did not produce a significant amount of IFN-γ, IL-4, or IL-17A. This stable phenotype of IL-21–producing CD4⁺ T cells was observed at least for 20 d of culture (unpublished data). We also examined whether IL-21–producing CD4⁺ T cells exhibited a stable phenotype against Th1, Th2, or Th17 polarization. After CD4⁺ T cells were stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of IL-6, anti–IL-4 mAb, and anti–IFN-γ mAb for 5 d, these cells were restimulated under Th1-, Th2-, or Th17-polarizing conditions for 5 d. As shown in Fig. 10 A (bottom), IL-21–producing CD4⁺ T cells that were restimulated under Th1-polarizing conditions produced IL-21, but not IFN-γ, indicating that IL-21–producing CD4⁺ T cells are stable against Th1 polarization. In contrast, when IL-21–producing CD4⁺ T cells were restimulated under Th2-polarizing conditions, a considerable population of cells produced both IL-21 and -4 (Fig. 10 A), suggesting that IL-21–producing CD4⁺ T cells may have a potential for IL-4 production. When IL-21–producing CD4⁺ T cells were restimulated under Th17-polarizing conditions, IL-21–producing CD4⁺ T cells were decreased and IL-17A–producing cells were increased (Fig. 10 A), suggesting that IL-21–producing CD4⁺ T cells may also have a potential for the differentiation of Th17 cells. Collectively, these results indicate that IL-21–producing CD4⁺ T cells exhibit a stable phenotype of IL-21 production in the presence of IL-6, but may still have a potential for IL-4 and -17A production.

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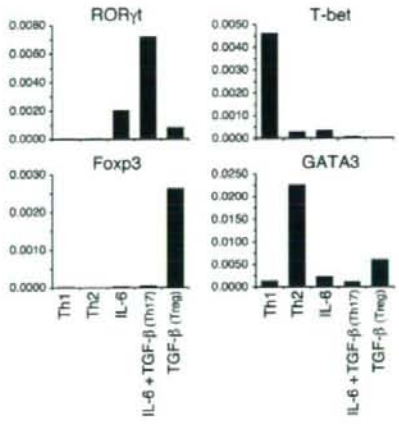

Figure 8. Expression of transcription factors in CD4⁺ T cells cultured in the presence of IL-6. Naive CD4⁺ T cells from C57BL/6 mice were stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of anti-IL-4 mAb and anti-IFN-γ mAb with IL-6, IL-6 plus TGF-β, or TGF-β alone for 60 h. As controls, naive CD4⁺ T cells were stimulated with anti-CD3 mAb/anti-CD28 mAb under Th1-polarizing conditions or Th2-polarizing conditions for 60 h. The expression of RORγt, Foxp3, T-bet, and GATA3 was assessed by real-time PCR. Data are representative of three independent experiments.

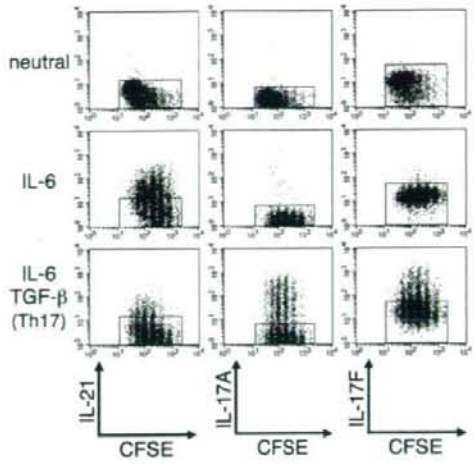

Figure 9. The frequency of IL-21–producing cells is increased with the rounds of cell cycle progression. Naive CD4⁺ T cells from C57BL/6 mice were labeled with CFSE, stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of anti-IL-4 mAb and anti-IFN-γ mAb (neutral condition) with IL-6 or IL-6 plus TGF-β for 4 d. Cells were stimulated with PMA/ionomycin and intracellular staining for IL-21, -17A, and -17F was performed. Shown are representative FACS profiles from three independent experiments.

DISCUSSION

In this study, we investigated the differentiation and characteristics of IL-21–producing CD4⁺ T cells. By the newly developed intracellular staining of IL-21, we found that although IL-21–producing CD4⁺ T cells developed preferentially under Th17-polarizing conditions, a considerable number of IL-21–producing CD4⁺ T cells were negative for intracellular IL-17A and -17F (Fig. 2). We also found that IL-6 strongly induced the development of IL-21–producing CD4⁺ T cells without the induction of IL-4, IFN-γ, IL-17A, and IL-17F production (Fig. 3). Furthermore, TGF-β inhibited the IL-6–induced development of IL-21–producing CD4⁺ T cells in a dose-dependent manner (Fig. 3). In contrast, consistent with previous reports (20–22), TGF-β together with IL-6 induced the development of Th17 cells (Fig. 3). IL-21 itself also induced the development of IL-21–producing CD4⁺ T cells from WT CD4⁺ T cells, but not from IL-21R$^{-/-}$ CD4⁺ T cells (Fig. 7). In addition, IL-21–induced development of IL-21–producing CD4⁺ T cells was inhibited by TGF-β (Fig. 7), like IL-6–induced development was (Fig. 3). Finally, CD4⁺ T cells cultured in the presence of IL-6 expressed a moderate level of RORγt, but not Foxp3, T-bet, or GATA3 (Fig. 8), and exhibited a stable phenotype of IL-21 production (Fig. 10). Collectively, these results suggest that IL-21–producing CD4⁺ T cells exhibit distinct characteristics from Th17 cells, that IL-21–producing CD4⁺ T cells develop preferentially in an IL-6–rich environment devoid of TGF-β, and that IL-21 functions as an autocrine growth factor for IL-21–producing CD4⁺ T cells.

IL-21 was originally reported to be a product of activated CD4⁺ T cells (10). Subsequently, it has been shown that among CD4⁺ T cells, Th2 cells but not Th1 cells produce IL-21 (13). More recently, it has been demonstrated that Th17 cells produce higher levels of IL-21 than Th2 cells and that IL-21 functions as an autocrine growth factor for Th17 cells (6–9). However, all of these findings were based on the analysis on a mixed population of CD4⁺ T cells because there has been no single-cell analysis for IL-21 production available as yet. In this study, we have established the intracellular cytokine staining of IL-21 and have shown that a considerable number of IL-21–producing CD4⁺ T cells under Th17-polarizing conditions are negative for intracellular IL-17A and -17F (Figs. 2 and 3), providing a new insight into the cellular source of IL-21 in activated CD4⁺ T cells.

We show that IL-6 and -21 both potently induce the development of IL-21–producing CD4⁺ T cells (Figs. 3 and 7). In addition, IL-6–induced development of IL-21–producing CD4⁺ T cells is mediated in part by IL-21 production because IL-6–induced development of IL-21–producing CD4⁺ T cells was modestly, but reproducibly, decreased by the neutralization of IL-21 (Fig. 7). In a preliminary study, we also found that IL-21 enhanced the survival of IL-21–producing CD4⁺ T cells (unpublished data). These results suggest that IL-21 may function as an autocrine growth factor for IL-21–producing CD4⁺ T cells. In contrast, because IL-21 induces Th17 cell development (Fig. 7 A) (6–9), and because a considerable number of IL-21–producing CD4⁺ T cells

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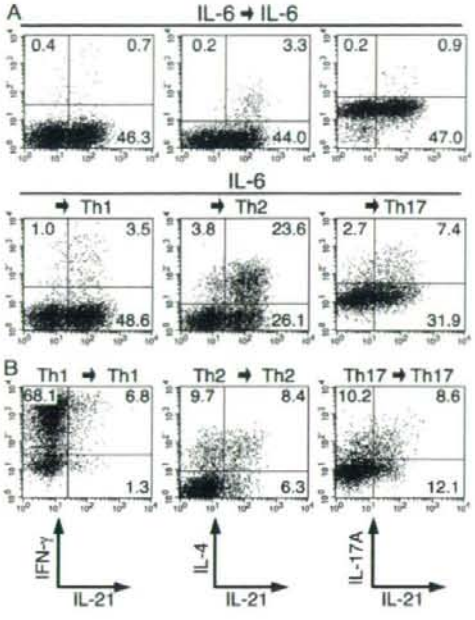

Figure 10. IL-21–producing CD4⁺ T cells do not lose their ability to produce IL-21 when they are restimulated under Th1- or Th2-polarizing conditions. (A) Purified CD4⁺ T cells from C57BL/6 mice were stimulated with anti-CD3 mAb/anti-CD28 mAb in the presence of anti-IL-4 mAb and anti-IFN-γ mAb with IL-6 for 5 d. Cells were restimulated for another 5 d under the same condition (top) or Th1-, Th2-, or Th17-polarizing conditions (bottom). On day 10, cells were stimulated with anti-CD3 mAb and intracellular staining for indicated cytokines was performed. Shown are representative FACS profiles from three independent experiments. (B) As positive controls, CD4⁺ T cells from C57BL/6 mice were stimulated with anti-CD3 mAb/anti-CD28 mAb under Th1-, Th2-, or Th17-polarizing conditions for 5 d. Cells were restimulated under the same conditions for another 5 d. On day 10, cells were stimulated with anti-CD3 mAb and intracellular staining for indicated cytokines was performed.

under Th17-polarizing conditions are negative for IL-17A and -17F (Figs. 2 and 3), it is suggested that IL-21 functions on Th17 cells not only as an autocrine growth factor but also as a paracrine growth factor.

Our results indicate that the developmental characteristic of IL-21–producing CD4⁺ T cells is different from that of Th17 cells, although both T cells require IL-6 and/or IL-21 for their development (Figs. 3 and 7) (6–9). We demonstrate that TGF-β inhibits IL-6– and IL-21–induced development of IL-21–producing CD4⁺ T cells (Figs. 3, 4, and 7). In contrast, both IL-6 and TGF-β are required for the development of Th17 cells (Fig. 3) (20–22). On the other hand, it has been shown that TGF-β induces, but IL-6 inhibits, the development of Foxp3-expressing regulatory T cells (20, 31). In addition, IL-2 signaling inhibits the development of Th17 cells, but not of IL-21–producing CD4⁺ T cells (Fig. 6). These findings suggest that the development of IL-21–producing CD4⁺ T cells, Th17 cells, and T reg cells is reciprocally regulated by the balance among IL-6 plus IL-21, TGF-β, and IL-2 in an environment.

Molecular mechanisms underlying the development of IL-21–producing CD4⁺ T cells remain to be elucidated. We show that IL-6 and -21, both of which use STAT3 as a signaling molecule (11, 12, 32), induce the development of IL-21–producing CD4⁺ T cells (Figs. 3 and 7). It has also been reported that under Th17-polarizing conditions, IL-21 production is induced by a STAT3-dependent mechanism, but not a RORγt-dependent mechanism (6, 7), suggesting that STAT3 is critical for the development of IL-21–producing CD4⁺ T cells. Because IL-6 up-regulates NFAT transcriptional activity by increasing the levels of NFATc2 (33), and because NFATc2 activates IL-21 promoter (34), it is also plausible that IL-6–mediated activation of NFATc2 is involved in the induction of IL-21 gene expression. Because Foxp3, which is induced by TGF-β–mediated signaling (31, 35), interacts with NFAT and down-regulates NFAT activity (36–38), TGF-β may suppress the development of IL-21–producing CD4⁺ T cells (Fig. 3) by producing Foxp3–NFATc2 complexes.

Recently, it has been shown that IL-22 is expressed in CD4⁺ T cells under Th17-polarizing conditions (6, 39, 40) and that it mediates IL-23–induced dermal inflammation and hyperplasia of the epidermis in psoriasis (40). Interestingly, similar to IL-21, IL-22 is induced by IL-6 but is inhibited by TGF-β (40). In contrast, IL-17A and -17F are expressed in the presence of IL-6 and TGF-β (Fig. 3) (41, 42). However, at present, it is still unknown whether IL-21–producing CD4⁺ T cells do produce IL-22 together. The detailed analysis of autoimmune disease models in IL-21–deficient mice, IL-17A/IL-17F double-deficient mice, and IL-17A/IL-17F/IL-21 triple-deficient mice may provide evidence for a nonredundant role of IL-21–producing CD4⁺ T cells and IL-17A/IL-17F–producing CD4⁺ T cells in the pathogenesis of autoimmune diseases.

Regarding the phenotypic stability of IL-21–producing CD4⁺ T cells, we found that IL-21–producing CD4⁺ T cells could produce IL-21 for at least 20 d of culture (Fig. 10 and not depicted). We also found that IL-21–producing CD4⁺ T cells did not lose their ability to produce IL-21 even when they were restimulated under Th1- or Th2-polarizing conditions (Fig. 10). Interestingly, IL-21–producing CD4⁺ T cells still had a potential to produce IL-4 when IL-21–producing CD4⁺ T cells were restimulated under Th2-polarizing conditions, whereas IL-21–producing CD4⁺ T cells did not produce IFN-γ even when IL-21–producing CD4⁺ T cells were restimulated under Th1-polarizing conditions (Fig. 10). These results indicate that IL-21–producing CD4⁺ T cells exhibit a stable phenotype of IL-21 production and the silencing of IFN-γ production. This is consistent with our previous finding that IL-21 inhibits IFN-γ production in developing Th1 cells through the repression of Eomesodermin

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expression (43). In contrast, when IL-21–producing CD4+ T cells were restimulated under Th17-polarizing conditions, their ability to produce IL-21 was decreased and IL-17A–producing cells were increased (Fig. 10). Therefore, IL-21–producing CD4+ T cells still have a potential for IL-4 and -17A production.

It has recently been shown that a subset of CD4+ T cells, termed follicular B helper T cells (TFH cells), produce a large amount of IL-21 (14). TFH cells are distinguishable from other CD4+ T cell populations by several criteria, including its location in B cell follicles and the expression of chemokine receptor CXCR5, and provide a helper function to B cells (14). In preliminary experiments, we found that CD4+ T cells cultured in the presence of IL-6 were negative for CXCR5 expression (unpublished data). Therefore, although further studies are required, it is suggested that IL-21–producing CD4+ T cells described in the present study seem to be different from TFH cells. The comparative analysis of their helper function to B cells, as well as the regulatory mechanism of IL-21 production, is required to further address the relationship between these populations.

We found that TGF-β–mediated suppression of IL-21 production was reduced in Smad3$^{-/-}$ CD4+ T cells compared with that in WT CD4+ T cells (Fig. 5). We also found that TGF-β–mediated induction of Th17 cells was reduced in Smad3$^{-/-}$ CD4+ T cells (Fig. 5). These results indicate that TGF-β–Smad3 signaling is involved not only in the induction of Th17 cell development but also in the suppression of IL-21–producing CD4+ T cells. Interestingly, however, the inhibitory effects of TGF-β were still observed in Smad3$^{-/-}$ CD4+ T cells (Fig. 5). This is consistent with a previous study showing that TGF-β exhibits partial effects on Smad3$^{-/-}$ CD4+ T cells (44). Because the molecular functions of Smad3 are somewhat overlapping to Smad2 (23, 24), it is suggested that TGF-β exhibits partial effects on Smad3$^{-/-}$ CD4+ T cells by using Smad2 as a partner of Smad4.

In conclusion, we have shown that IL-6 and -21 preferentially induce the differentiation of IL-21–producing CD4+ T cells and that the differentiation of IL-21–producing CD4+ T cells and Th17 cells is differently regulated by TGF-β and IL-2. Although further studies are required to address the physiological importance of IL-21–producing CD4+ T cells in vivo, our results should add a new insight into the regulatory mechanism of helper T cell differentiation and the pathogenesis of autoimmune diseases.

MATERIALS AND METHODS

Mice. BALB/c mice and C57BL/6 mice were purchased from Charles River Laboratories. IL-21R–deficient (IL-21R$^{-/-}$) mice (45) on a BALB/c background and Smad3$^{-/-}$ mice (46) on a C57BL/6 background (provided by S. Honjo, M. Fujimoto, K. Kobayashi, and A. Sakamoto, Chiba University, Chiba, Japan) were described previously. All mice were housed in microisolator cages under specific pathogen-free conditions and animal procedures used in this study were approved by the Chiba University Animal Care and Use Committee.

Reagents. Antibodies to CD3 (145-2C11), CD25 (PC61), CD28 (37.51), CD44 (IM7), IL-4 (11B11), IL-6 (MP5-20F3), and IFN-γ (XMG1.2) were purchased from BD Biosciences. Murine IL-2, -4, -6, and -12 were purchased from PeproTech. Human TGF-β, murine IL-21, murine IL-23, IL-21R–Fc chimera, and anti–IL-2 antibody (JES6-1A12) were purchased from R&D Systems.

Cell isolation. CD44^{low} CD25^{-} CD4+ naive T cells were isolated from lymph nodes of mice, as previously described (40), with minor modifications. In brief, CD4+ T cells were collected from lymph nodes of mice using CD4+ T cell isolation kit according to the manufacturer's instructions (Miltenyi Biotec). Purified CD4+ T cells were stained with anti-CD44 mAb and anti-CD25 mAb, and CD44^{low} CD25^{-} cells were sorted on a FACSAria cell sorter (BD Biosciences). In the experiments shown in Fig. 1 A, Fig. 5, and Fig. 10, CD4+ T cells were purified from lymph nodes of mice by EasySep mouse CD4+ T cell enrichment kit (StemCell Technologies, Inc.) using an automated cell separator RoboSep (StemCell Technologies, Inc.). In both cases, the resultant cells were >98% pure CD44^{low} CD25^{-} CD4+ T cells or CD4+ T cells, respectively, by FACS analysis.

Cell culture. Naive CD4+ T cells or purified CD4+ T cells were stimulated with 1 μg/ml plate-bound anti-CD3 mAb at 0.5–10^{6} cells/ml in the presence of 1 μg/ml anti-CD28 mAb in a 48-well plate. Where indicated, cells were cultured under either Th1-polarizing conditions (10 ng/ml IL-2, 1 ng/ml IL-12, and 10 μg/ml anti–IL-4 mAb), Th2-polarizing conditions (10 ng/ml IL-2 and -4 and 10 μg/ml anti–IFN-γ mAb), or Th17-polarizing conditions (100 ng/ml IL-6, and 1 ng/ml TGF-β, anti–IL-4 mAb, and anti–IFN-γ mAb). When cells were cultured for >4 d, 20 ng/ml IL-23 was added under Th17-polarizing conditions. Where indicated, 100 ng/ml IL-6, 0.2–5 ng/ml TGF-β, 10 ng/ml IL-2, 100 ng/ml IL-21, 10 μg/ml anti–IL-2 mAb, 10 μg/ml anti–IL-6 mAb, or 10 μg/ml IL-21R–Fc was added to the culture.

Retrovirus transduction. Bicistronic retrovirus vector pMX-IRES-GFP was a gift from T. Kitamura (Tokyo University, Tokyo, Japan). The IL-21 cDNA was amplified by PCR and cloned into pMX-IRES-GFP (IL-21-IRES-GFP). The production of retroviruses and infection to Ba/F3 cells were performed as previously described (43).

Intracellular cytokine analysis for IL-21. Cultured cells were washed and restimulated with 20 ng/ml PMA (Calbiochem) plus 1 μg/ml ionomycin (Calbiochem) or plate-bound anti-CD3 mAb at 37°C for 5 h in the presence of 2 μM monensin (Sigma-Aldrich). Cells were fixed, permeabilized with Perm/Wash buffer (BD Biosciences), and incubated with IL-21R/Fc chimera for 30 min at 4°C. Cells were then washed with Perm/Wash buffer and stained with PE-conjugated affinity-purified F(ab')2 fragment of goat anti–human Fcγ antibody (anti-Fc PE; Jackson ImmunoResearch Laboratories) for 30 min at 4°C. Cells were washed twice and stained with anti-CD4 FITC and anti–IL-4 allophycocyanin (BD Biosciences), anti–IFN-γ allophycocyanin (BD Biosciences), anti–IL-17A Alexa Fluor 647 (eBioscience), or anti–IL-17F Alexa Fluor 647 (eBioscience). Cytokine profile on CD4+ cells was analyzed on a FACSCalibur using CellQuest Pro software (BD Biosciences). Intracellular cytokine staining for IL-4 versus IFN-γ was performed using anti–IL-4 PE (BVD4-1D11; BD Biosciences) and anti–IFN-γ allophycocyanin (XMG1.2, BD Biosciences) as previously described (43).

Real-time PCR analysis. Total cellular RNA was extracted with TRIzol solution (Invitrogen). Reverse transcription was performed using an iScript cDNA synthesis kit (BioRad Laboratories). Primers and TaqMan probes for T-bet, GATA3, RORγt, Foxp3, and β-actin have been previously described (5, 6, 41, 43). Quantitative PCR was performed with an ABI PRISM 7300 sequence detection system (Applied Biosystems). The levels of T-bet, GATA3, RORγt, or Foxp3 mRNA were normalized to the levels of β-actin mRNA.

ELISA. The amount of IL-4 and IFN-γ in the culture supernatant was measured by ELISA kits from BD Bioscience. The amount of IL-17A and -21 in the culture supernatant was measured by ELISA kits from R&D Systems.

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The assays were performed in duplicate according to the manufacturer's instruction. The minimum significant values of these assays were 8 pg/ml of IL-4, 30 pg/ml of IFN-γ, 17 pg/ml of IL-17A, and 63 pg/ml of IL-21.

CFSE-labeling of CD4⁺ T cells. Naive CD4⁺ T cells were labeled with 1 μM CFSE (Invitrogen) for 10 min at 37°C according to the manufacturer's instruction.

Data analysis. Data are summarized as the mean ± the SD. The statistical analysis of the results was performed by the unpaired Student's *t* test. P values <0.05 were considered significant.

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特集❶ | アレルギー疾患の代替医療

展望

Overview

アレルギー疾患に対する代替医療の実態と有効性の評価

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1. はじめに

代替医療の定義は必ずしも統一されておらず，広く保険診療以外の医療をすべて含めることもあるが「通常多くの医師が医療施設において施行したり指導する医療以外の医療で，その多くのものは作用機序が科学的には検証されていないもの」と考えられている．国民の30%以上が罹患しているとされるアレルギー疾患ではさまざまな代替医療が用いられているが，国内での代替医療に関する実態調査は少なく，有効性に関する報告も鍼などで経験的な評価に基づくものが中心である．米国では国民の40～60%が何らかの代替医療を受けているとされ，代替医療に年間200億ドル以上が費やされており，有効性，問題点，経済効果，民族・文化の影響など科学的な代替医療の研究のため代替医療研究の国立機関（National Institute of Health's Nation Center for Complimentary and Alternative Medicine：NCCAM）が設立され検討を行っている．

今回，昨年度から厚生労働省科学研究補助金事業として始まったアレルギー疾患における代替医療の班研究の取り組みも含めて，これまでの国内の代替医療の実態調査を中心に有効性に関する評価，今後の展望について報告する．

2. 代替医療の実態調査

今回の調査にあたって代替医療調査用のアンケート用紙を作成した．項目として年齢，性，代替医療の経験の有無，最も長く実施した代替医療の経験，副作用，代替医療の情報入手先，医師への申告と対応，代替医療の費用などを含んでいる．できるだけバイアスを防ぐため，医療機関を受診したアレルギー疾患患者全員を対象に調査を医療機関に依頼するように申し合わ

表　代替医療のアンケート調査

代替医療の実態調査：計11,364名（2007年末）実施	
1. 成人アレルギー疾患での医療機関受診者の調査	
・千葉県内	
・アレルギー疾患の違いによる比較	940名
2. 小児アレルギー疾患で受診患児の調査	
千葉県と北海道との比較	3,400名
3. アレルギー性鼻炎での受診患者に対する調査	
・国内の地域による違い（千葉，秋田，山梨，岡山，鹿児島）	3,280名
・同一地域での6年前との比較（山梨）	1,329名
4. 地域一般成人住民対象のアレルギー性鼻炎検診	950名
5. インターネット調査	2,770名
6. 市民公開講座受講者の調査	135名

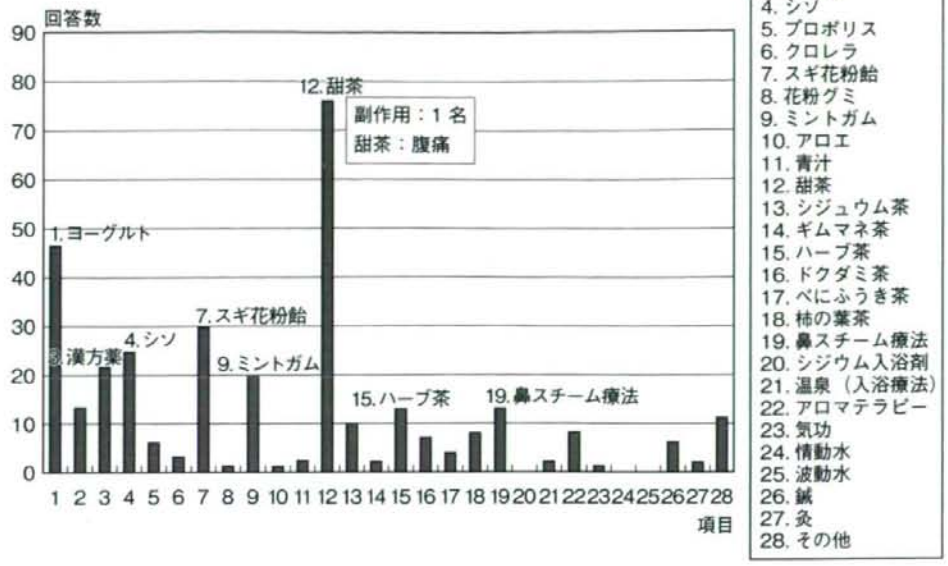

図 千葉県のアレルギー性鼻炎患者の代替医療の内容

せて行った．

千葉県においては喘息，アトピー性皮膚炎，アレルギー性鼻炎について成人患者，小児患者の実態を明らかにするため，内科，小児科，皮膚科，耳鼻科にアレルギー疾患で通院中の患者を対象に調査を行った．アレルギー性鼻炎については，千葉，秋田，東京，岡山，鹿児島，北海道での調査から，地域による内容，医療機関の違いなどについて検討を行った．小児の喘息，アトピー性皮膚炎，アレルギー性鼻炎については北海道，千葉で広く調査を行い，地域差，疾患による違いを検討した．また，2000年に厚生労働省班研究で，代替医療の調査を行った山梨県では，同様の医療機関，調査内容で再調査を行い，代替医療の内容や頻度について比較を行った．さらに千葉県では一般住民や小学生を対象とした検診から医療機関に通院していないアレルギー疾患患者についても調査を行った．さらに，インターネットを用いたWeb調査も行った．2007年末の段階で**表**に示すように10,000人以上を対象に調査が終了し，その後も調査を続け，特にアレルギー性鼻炎に関しては三重県，福井県，関西地区でも新たに検討を行っている．

解析を終了した2007年末までの調査をまとめると，代替医療はアレルギー疾患成人患者の20～40%，小児患者の10～20%で経験が見られ，内容は多彩で年齢や疾患により違いが見られた．アレルギー性鼻炎では，甜茶，ヨーグルト（**図**），喘息ではヨーグルト，漢方，アトピー性皮膚炎では温泉入浴療法が多くなった．地域差は大きくはないが，温泉入浴療法は温泉が多い北海道や秋田県で多く，クマ油など地域特産品との関連も見られた．大学病院などのアレルギー治療専門病院の患者，インターネットでの調査に回答するアレルギー疾患患者では代替医療の受療頻度が高く，患者の高い意識を反映していることが考えられた．また，住民検診での

調査からは，医療機関を受診していない有病者が代替医療を受ける割合も，またその効果に対する評価も高く，医療機関を受診しない理由とも考えられた．

副作用発現は少なく重篤なものは今回の調査では見られなかったが，温泉入浴療法では副作用の評価がしやすいためか症状悪化例が散見された．臨床効果については，なし，不明が多く，プラセボ効果が大きいウェイトを占めることが示唆された．ただ，安全で安いという点が代替医療を行った理由との回答が多かったが，10万円以上負担する者も10%以上見られ，必ずしも安価とは考えられなかった．

3．有効性の評価

代替医療の有効性はアンケート調査から見ても患者の評価は低く，標準治療の一般的に示されている有効率には及ばない．代替医療の臨床での有効性に関する科学的評価も多くは行われていない．しかし，甜茶や乳酸菌などの食品についてはさまざまな生物活性を有することが *in vitro* の検討や動物実験で報告されている．甜茶に含まれるポリフェノールについてはヒスタミンなど化学伝達物質の遊離抑制作用あるいは抗炎症作用を有し，乳酸菌はさまざまな免疫調整作用が多数報告されている．食品として安全性が高く，価格も比較的安価なこれらの食品の効果が期待され，臨床試験の報告も見られる．Kalliomaki らは[1] Lactobcillus rhamnosus GG (LGG) をアトピー素因を持つ妊婦とその乳児に投与したところ，アトピー性皮膚炎の発症率がプラセボ投与に比較して有意に低いとLancet に報告し，Viljanen らは[2] LGG を食物アレルギーの乳児に投与し，高い改善率を報告している．しかし，一方で否定的検討結果の報告も見られる[3]．スギ花粉症に対しても高い有効性を示す国内でのランダム化検討の結果の報告が見られるが[4)5)]，臨床試験に不可欠なconcealment の保証，試験のメーカーからの独立性などの課題も指摘されている．ただ，有症者の症状改善効果は，標準治療には及ばないが，食品としての安全性と安価であるといったことから，早期治療介入の一手段としては期待されている．また，ミントやアロマ療法はアンケート調査でも比較的広く使われている代替医療であり，アレルギー疾患治療を直接の目的として行われているとは限らず，精神的ストレスの改善，精神安定を目的に使用され，その結果アレルギー疾患症状の改善も副次的に期待されて用いられていることも少なくない．ストレスとアレルギー症状との関連は重要な検討課題であり，これら代替医療の有効性も含めた評価は意義があると考えられる．

4．おわりに

アレルギー疾患の増加とともに，さまざまな代替医療を利用する患者の増加も見られている．

しかし，アンケート調査からの結果も代替医療の多くは効果が乏しく，また必ずしも安価ではない．今後，代替医療の科学的評価も進めてこれらの情報を広く患者に提供することが必要である．医師の対応としては，患者に現在の標準的治療の有用性を十分に説明することが何より重要であろう．

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原　著

小学生のヨーグルト・乳酸菌飲料摂取とアレルギー感作・アレルギー疾患との関係

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【背景】プロバイオティクスのアレルギー疾患予防効果の可能性が期待されている．ヨーグルト・乳酸菌飲料を摂取している小児ではアレルギー感作やアレルギー疾患発症が少ないという報告があるものの，評価はいまだ確立していない．
【方法】都市部の小学生472名を対象に，ヨーグルト・乳酸菌飲料の摂取の有無と摂取量，納豆の摂取状況，アレルギー疾患の有無についてアンケートによる調査を行った．血清の総IgEと6種の特異IgEを測定した．
【結果】対象者をヨーグルトや乳酸菌飲料の1週間あたりの摂取量で4群に分け，摂取量とIgE値，アレルギー疾患有病率との相関を見ると，ダニ特異IgE値とカモガヤ特異IgEの陽性率は多量摂取群で高い傾向があった．背景因子で補正したオッズ比（OR）とその95%信頼区間（CI）はダニで2.20，1.11-4.40，カモガヤで2.14，1.07-4.30であった．卵白特異IgE値陽性率は少量（OR：5.08；CI：1.68-15.37），中等量（OR：6.45；CI：2.21-18.89），多量摂取群（OR：3.50；CI：1.15-10.63）のいずれも非摂取群に比べ有意に高かった．喘息の罹患率は中等量摂取群が無摂取群より低かった（OR：0.21；CI：0.05-0.83）．他のアレルギー疾患の罹患率へのヨーグルト・乳酸菌飲料摂取量の影響は認められなかった．以上の結果は，ヨーグルト・乳酸菌飲料の摂取が単純にアレルギー感作とアレルギー疾患発症を予防するという仮説を積極的に支持するものではなかった．納豆の摂取は，アスペルギルス特異IgEとの相関を示したが，アレルギー疾患との相関は無かった．
【結論】ヨーグルト・乳酸菌飲料の摂取量は，特異IgE産生と喘息の発症との相関を示した．しかし，摂取量を増やせばアレルギー感作とアレルギー疾患発症を予防する効果が大きくなるという単純な関係ではないことが示唆された．

Key words: allergy —— epidemiology —— fermented milk —— IgE —— yogurt

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Abbreviation: ISAAC "international study of asthma and allergy in childhood"
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はじめに

プロバイオティクスは，宿主に健康増進効果を示す生きた微生物ないしそれを含む食品を指す[1][2]．アレルギー疾患におけるプロバイオティクスの効果で最初に注目を集めたのは，Majamaaらの *Lactobacillus rhamnosus* GG (LGG) 菌の投与で牛乳アレルギーが改善したという1997年の論文[3]である．その後，アトピー素因を持つ妊婦および生後6カ月までの乳児にLGG菌を投与した二重盲検の研究で，LGG菌投与群でアトピー性皮膚炎の発症が有意に低いとの結果が発表され[4][5]，アレルギー疾患の予防効果への期待が高まっている．アレルギー疾患以外でもプロバイオティクスの効果は広く認められ，これまで多くの研究が報告されてきた[6][7]．

特定の菌の投与ではなく，日常摂取する食品に注目して行われた疫学調査では，乳酸菌で発酵させた野菜を多く摂取して抗生物質の投与を避けた生活をしている小児がアレルギー疾患の発症が少なく，腸内の腸球菌，乳酸菌が多いという報告がなされた[8][9]．さらに，最近のヨーロッパの大規模な研究では，農家の自家製牛乳の摂取は喘息とアレルギー性鼻炎・結膜炎の発症率と，花粉，食物などへの感作率を低下させているという報告もされている[10]．日本においても，榎本らは中学生を対象に，ヨーグルトや乳酸菌飲料，納豆の摂取状況と血清総IgE値，特異的IgE値，アレルギー疾患の状況を調べ，ヨーグルト，乳酸菌飲料の摂取歴のある者では，総IgE値が低値であり，疾患の発症も少なかったと報告している[11]．特定の菌の投与ではなく日常的なプロバイオティクス食品で十分なアレルギー疾患の予防効果があるとすれば，抗アレルギーを目指す栄養指導にとって，大きな意味を持つと考えられる．

我々は，ヨーグルトなどの発酵食品摂取にアレルギー感作やアレルギー疾患発症の予防効果があるかどうかを明らかにする目的で，ヨーグルト・乳酸菌飲料や納豆の摂取量とアレルギー感作やアレルギー疾患有病率との相関を都市部の小学生を対象に検討した．

対象および方法

1) 対象

千葉県千葉市の中心部に位置する小学校の1年生から6年生を対象とした．まず全校児童(843名)の保護者に研究への協力を呼びかけ，詳細な研究の説明の後，528名から文書による同意を得た．2006年7月にアンケートを配布した．同年11月までに472名からの有効な回答を得た．2006年7月に411名の児童について血清IgE測定のための採血を行った．本研究は，千葉大学医学部生命倫理委員会の承認を得て行われた．

2) 方法

現在のアレルギー疾患の有無に関する質問はISAACに準じて行った．すなわち，アトピー性皮膚炎有りについては，「あなたのお子さんは，今までに6カ月以上，出たり消えたりするかゆみを伴った皮疹がありましたか」，「このかゆみを伴った皮疹は最近12カ月のあいだの何れかの時期にありましたか」，「あなたのお子さんは，医者にアトピー性皮膚炎と診断されたことがありますか」の質問の全てに「はい」と答えた場合とした．喘息有りは，「あなたのお子さんは，今までに，胸がゼーゼーまたはヒューヒューしたことがありますか」「最近12カ月のあいだに，あなたのお子さんは胸がゼーゼーまたはヒューヒューしたことがありますか」，「あなたのお子さんは，今までに医者に喘息（ぜんそく）と診断されたことがありますか」の質問の全てに「はい」と答えた場合とした．アレルギー性鼻炎有りは，「あなたのお子さんは，今までカゼやインフルエンザにかかっていない時に，くしゃみや鼻みず，鼻づまりの症状が起こったことがありますか」，「最近12カ月のあいだで，カゼやインフルエンザにかかっていない時に，くしゃみや鼻みず，鼻づまりの症状が起こったことがありますか」の全てにはいと答え，かつ，「今までに医者に花粉症と診断されたことがありますか」または「今までに医者にアレルギー性鼻炎と診断されたことがありますか」に「はい」と答えた場合とした．食物アレルギー有りは，「これまでお子さんが特定のものを食べて，2時間以内に皮膚に変化が起こったり，体調が悪くなったり，病気になったりしたことがありますか（食中毒によるものは除いてく

Table 1 Questionnaire

Q1. Do your child eat (or drink) fermented milk foods such as yogurt and/or fermented milk drinking?
1. Yes 2. No (If No, go to Q4)
Q2. The amounts and frequency of eating.
About _____times/week, about _____g (ml)/time
Q3. When have your child begun eating?
1. Before 3-year old 2. After 3-year old but before entering elementary school
3. After entering elementary school 4. uncertain
Q4. Do your child eat Natto (fermented soy beans)?
1. Frequently (more than 2 times/week) 2. Occasionally (more than one time/month)
3. Almost not

Table 2 Characteristics of the subjects

			Sex difference p value
Total Number		472	
Age (months)			
Mean ± standard deviation (SD)		111.1 ± 19.9	
Range		76 – 147	
Sex ratio (male : female)		1.00 : 1.01	
Total IgE (IU/ml) (mean ± SD)	male	254 ± 340	0.053[a]
	female	241 ± 469	
Rate of positive specific IgE (> 0.34IU/ml)			
D. Pteronyssinus	male	59.0%	**0.026**[b]
	female	47.5%	
Felis domesticus	male	17.9%	**0.026**[b]
	female	9.9%	
Alternaria alternate	male	9.7%	0.908[b]
	female	9.4%	
Egg white	male	23.1%	0.876[b]
	female	23.8%	
Cryptomeria japonica	male	53.8%	0.062[b]
	female	44.2%	
Dactylis glomerata	male	29.2%	**0.008**[b]
	female	17.7%	
Prevalence of allergic disorders			
Asthma	male	14.1%	**0.008**[b]
	female	6.6%	
Atopic dermatitis	male	11.5%	0.508[b]
	female	9.7%	
Allergic rhinitis	male	42.1%	**0.014**[b]
	female	31.2%	
Food allergy	male	3.0%	0.812[b]
	female	3.4%	

[a] Kruskal-Wallis test.
[b] Chi-square test.

ださい)」,「医師から食物アレルギーと診断されたことがありますか」,「現在も食物アレルギーがありますか」の全てに「はい」と答えた場合とした.

ヨーグルト,乳酸菌飲料,納豆の摂取状況についてのアンケートは Table 1 に示す内容で行った.比較のため,榎本らの行った調査と類似の内容とした[11].背景因子としては,性別,月齢,兄弟数,生まれ順,両親または兄弟のアレルギー疾患(喘息,アレルギー性鼻炎,アトピー性皮膚炎,食物アレルギー)の有無,現在と2歳以前の住居地(商店街,住宅街,農地に囲まれている,工場に囲まれている,林や森に囲まれている),現在と2歳以前の家の構造(木造か鉄筋か,窓枠が木かアルミか,一戸建てか集合住宅か),ペットの飼育,同居者の喫煙,2歳までの保育所通園,離乳食開始時期について調査した.

末梢血については血清を分離し,血清総 IgE 値,6種類の特異 IgE 値(ヤケヒョウヒダニ *Dermatophagoides pteronyssinus*,スギ *Cryptomeria japonica*,カモガヤ *Dactylis glomerata*,アルテルナリア *Alternaria alternata*,ネコのフケ *Felis domesticus*,卵白 *Egg white*)についてキャップ IgE アッセイ試薬(ファルマシア)を用いて測定した.特異 IgE は 0.35IU/ml 以上(クラス1以上)を陽性とした.血清 IgE 値の測定は SRL 社に依頼した.

ヨーグルト・乳酸菌飲料の1週間あたり摂取量は,1回摂取量(ml)×週あたり摂取回数として計算した.

3) 統計解析

ヨーグルト・乳酸菌飲料摂食量群と総 IgE 値の相関は Kruskal-Wallis (KW) 検定で評価した.摂食量群と特異 IgE 値の陽性/陰性,疾患の有無との相関は,交絡因子となる可能性のある背景因子を加えて,ロジェスティック回帰式を用いて解析した.年齢と IgE 値との相関は Spearman の ρ にて検討した.統計値の計算には Windows 版の SPSS 15.0J を用いた.

結 果

1) 調査対象者の背景

アンケート調査ないし採血から得られた今回の対象者の背景を Table 2 に示した.

参加者はほぼ男女同数で,総 IgE 値についての男女での有意差は無かった(KW 検定 $p=0.053$).

特異 IgE 値のクラス1以上(>0.34IU/ml)を陽性とした場合の陽性率は,ヤケヒョウヒダニが最も高く,男児 59.0%,女児 47.5% で,次にスギが高く,男児 53.8%,女児 44.2% であった.ヤケヒョウヒダニ,ネコのフケ,カモガヤで男女差を認め,アルテルナリア,卵白,スギでは男女差を認めなかった.月齢は,総 IgE 値,アルテルナリア特異 IgE,卵白特異 IgE 値と弱い相関を示した.それぞれの IgE 値に対する相関係数は,0.103($p=0.047$),0.125 ($p=0.016$),−0.206 ($p<0.001$) であった.

アレルギー疾患の有病率では喘息が男児 14.1%,女児 6.6% と男児が高い傾向を示した($p=0.008$).アレルギー性鼻炎は男児 42.1%,女児 31.2% と,やはり男児に多い傾向がみられた($p=0.014$).アトピー性皮膚炎の有病率は約 10%,食物アレルギーについては約 3% で,男女差は認められなかった.

2) ヨーグルト・乳酸菌飲料の摂取と IgE 値の相関

ヨーグルト・乳酸菌飲料の摂取についてのアンケートの回答を得られた 472 名のうち,ヨーグルト・乳酸菌飲料を摂取しないと答えた者は,男児 40 名,女児 39 名であった.ヨーグルト・乳酸菌飲料を摂取すると答えた児童は,男児 195 名,女児 198 名であった.摂取歴の有無と総 IgE 値ないし6種の特異 IgE 値との相関を見ると,卵白特異 IgE 値陽性率が摂取群で高い傾向を示した.月齢補正後のオッズ比 (OR) は 3.88,その 95% 信頼区間 (CI) は,1.58–9.51 であった.総 IgE 値,その他の特異 IgE 値では有意差がなかった.

1回摂取量と週あたり摂取回数の質問に答えた児童は,368 名であった.摂取量の多少によってほぼ同じ例数の3群(少量摂取群,中等量摂取群,大量摂取群)に分け,非摂取群に比して,IgE 値の差があるかどうかを検討することとした.摂取量群間でみられた IgE の差の交絡因子による影響を検討するため,方法に述べた背景因子がヨーグルト摂取量4群間で差があるかどうか検討した.有意差が認められたものは,月齢(分散分析 $p=0.008$),性別(χ^2検定 $p=0.009$),現在と2歳までの家の造り(χ^2検定 $p=0.046$, $p=0.004$),5カ月ま

Table 3 Association of consumption of fermented milk foods with serum IgE levels

Fermented milk foods (ml/week)		NO	～300	301～540	541～	*P* value [a]
Number of subjects		79	124	131	113	
Total IgE (IU/ml)	mean ± SD	209 ± 303[b]	232 ± 460	246 ± 440	291 ± 401	0.119
Specific IgE						
D. pteronyssimus	Positive[b] rate	46.9%	51.6%	47.6%	64.9%	
	OR	reference	1.23	1.09	**2.20** #	
	(CI)		(0.61－2.48)	(0.56－2.12)	(1.11－4.40)	
Felis domesticus	Positive rate	14.1%	11.0%	11.4%	20.6%	
	OR	reference	0.77	0.80	1.58	
	(CI)		(0.27－2.25)	(0.29－2.17)	(0.62－4.05)	
Alternaria alternata	Positive rate	9.4%	6.6%	10.5%	10.3%	
	OR	reference	1.06	1.36	1.17	
	(CI)		(0.28－3.94)	(0.42－4.35)	(0.36－3.80)	
Egg white	Positive rate	9.4%	29.7%	30.5%	18.6%	
	OR	reference	**5.08** #	**6.45** #	**3.50** #	
	(CI)		(1.68－15.37)	(2.21－18.89)	(1.15－10.63)	
Cryptomeria japonica	Positive rate	45.3%	46.2%	44.8%	61.9%	
	OR	reference	1.27	1.08	**2.14** #	
	(CI)		(0.62－2.59)	(0.55－2.13)	(1.07－4.30)	
Dactylis glomerata	Positive rate	17.2%	23.1%	25.7%	27.8%	
	OR	reference	2.24	2.16	2.07	
	(CI)		(0.91－5.53)	(0.91－5.11)	(0.87－4.92)	

OR: odds ratio, adjusted for age, sex, weaning age, current and past house types.
CI: 95% confidence interval of odds ratio.
[a] Kruskal-Wallis test.
[b] > 0.34IU/ml.
# $P < 0.05$.

での離乳開始（χ^2検定 $p=0.015$）の5つの因子であった．これらの因子は，交絡因子となる可能性があるためロジェスティック回帰式に投入し，ヨーグルト非摂取群に対するヨーグルト・乳酸菌飲料摂取ありの3群のORとCIを求めた（**Table 3**）．卵白特異IgE陽性率の少量，中等量，大量摂取群におけるORとCI（括弧内）は5.08（1.68–15.37），6.45（2.21–18.89），3.50（1.15–10.63）であり，全ての群で，非摂取群に比し有意に上昇していた．ダニ特異IgE値陽性率は大量摂取群で高い傾向がみられた（OR：2.20；CI：1.11–4.40）．同様にスギ特異IgEの陽性率も大量摂取群で高い傾向があった（OR：2.14；CI：1.07–4.30）．総IgE値は摂取量の多い群ほど高い平均値を示していたがKW検定のp値は0.119であり有意差はなかった．

3) ヨーグルト・乳酸菌飲料の摂取とアレルギー疾患有病率との相関

ヨーグルトの摂取量の有無で2群に分けた場合の4種のアレルギー疾患（アトピー性皮膚炎，喘息，アレルギー性鼻炎，食物アレルギー）の有病率には有意差がなかった．

IgE値との相関と同様に摂取量の多少による4群に分け，非摂取群を参照群とした疾患発症に対する交絡因子修正後ORを求めると，喘息と有病率は中等量摂取群で有意に低い傾向を示した（OR：0.21；CI：0.05–0.83）（**Table 4**）．その他の疾患では，非摂取群と摂取3群間で有病率に有意の

Table 4 Odds ratio and its 95% confidence interval of the consumption group for allergic diseases

Fermented milk foods (ml/week)	NO	～300 OR (CI)	301～540 OR (CI)	541～ OR (CI)
Atopic dermatitis	reference	0.83 (0.31－2.26)	0.98 (0.38－2.49)	0.98 (0.37－2.57)
Asthma	reference	0.79 (0.29－2.19)	**0.21# (0.05－0.83)**	1.83 (0.73－4.58)
Allergic rhinitis	reference	1.03 (0.54－1.96)	0.87 (0.46－1.63)	1.55 (0.83－2.91)
Food allergy	reference	0.36 (0.05－2.45)	0.72 (0.15－3.46)	1.48 (0.32－6.73)

OR: odds ratio, adjusted for age, sex, age of weaning, current and past house types.
CI: 95% confidence interval of odds ratio.
#$p < 0.05$.

差はなかった.

4) ヨーグルト・乳酸菌飲料の摂取開始時期の影響

ヨーグルト・乳酸菌飲料の摂取開始時期については, 381 名からの有効な回答が得られた. 3 歳以前, 3 歳から小学校入学前, 小学校入学後からの開始時期と答えた者は, それぞれ 317 名, 46 名, 18 名であった. 小学校入学後の群が少ないため, 3 歳以前群と 3 歳以降群の 2 群と IgE 値ないし疾患の有無との相関を検討した.

IgE 値については, ネコのフケに対する特異 IgE 値は 3 歳以前開始群で陽性率 12.2%, 3 歳以降群 26.5% で有意差が認められた (χ^2乗検定 $p=0.0092$). 交絡因子となる可能性のある背景因子(性別, 月齢, 現在の家の造り, 離乳開始時期, 保育所通園) で補正した OR (CI) は 2.78 (1.23-6.29) であった. 喘息, アレルギー性鼻炎, アトピー性皮膚炎, 食物アレルギーの有病率と開始時期との有意の相関は認められなかった.

5) 納豆の摂取と IgE 値

納豆の摂取については, しばしば食べる (週 2 回以上), たまに食べる (月 1 回以上), ほとんど食べないといった 3 群間で各 IgE 値ないしアレルギー疾患発症との相関を検討した.

アルテルナリア特異 IgE 値は「ほとんど食べない」群で有意に陽性率が高かった (χ^2検定 $p=0.0025$). 交絡因子の可能性のある背景因子(性別, 月齢, 離乳開始時期) で補正した OR (CI) は 3.24 (1.28-8.20) であった. 総 IgE 値, その他の特異 IgE 値との有意の相関はなかった.

アレルギー疾患の有病率と納豆の摂取状況との間に有意な相関は認められなかった.

考 案

本研究では, ヨーグルト・乳酸菌飲料の摂取量を 4 群に分けて検討した場合, 総 IgE 値が摂取量の多い群ほど高めの傾向を示していた. この変化は KW 検定では統計学的有意とはならなかったが, log (総 IgE) を従属変数, 摂取量 4 群を説明変数とする直線回帰分析では有意 ($p=0.016$) であり, 正のトレンドありとも解釈できる結果であった. また, 卵白特異 IgE 値については, 非摂取群に比べいずれの摂取群でも高い陽性率を示し, ダニ特異 IgE 値, スギ特異 IgE 値は大量摂取群で有意に高い陽性率を示していた. アレルギー疾患の有病率へのヨーグルト摂取量の影響の検討では, 喘息への中等量群の OR が有意に低かったが, 大量摂取群の OR はむしろ高い値を示し, 他のアレルギー疾患有病率への有意な影響は認められなかった. 本研究の結果からは, ヨーグルト・乳酸菌飲料の摂取量を増やせば, 単純に, IgE 値を指標としたアレルギー感作やアレルギー疾患の発症が予防できるという証左は得られなかった.

本研究におけるヨーグルト・乳酸菌飲料と納豆の摂取に関しての質問項目は, 2006 年に報告された榎本らの論文とほぼ同じ内容となっている. 榎本らの報告では, ヨーグルト・乳酸菌飲料の摂取の「有り」と「ほとんど無し」の 2 群での総 IgE 値比較で,「有り」群が有意に低値であった ($p=0.001$). 本研究では, これと同様なグループ分けで

の総 IgE 値との有意差も認められなかった．本研究のサンプル数は摂取無し 79 例，摂取有り 392 例で，統計学的検出力は，榎本らの研究（摂取無し 37 例，摂取有り 97 例）よりも高いと考えられ，ヨーグルト・乳酸菌飲料の摂取の有無と総 IgE 値が単純な相関を示しているとすれば再現された可能性が非常に高いはずであるが，現実にはそうならなかった．この差異の理由として，以下のようなことが考えられる．第 1 に対象年齢が異なっていたこと．すなわち本研究の対象は小学生 1 年生から 6 年生，榎本らの研究は中学 1 年生である．第 2 に，住居環境の違いである．本研究では千葉市の中心部でマンションに住んでいる家庭が多い都会の住居環境であるのに対し，榎本らのそれは，和歌山県の日高郡ということで，千葉市の環境よりは人口密度の低い地域を対象としている．第 3 には，ヨーグルト・乳酸菌飲料以外の西日本と東日本の食習慣の違いが影響していたかもしれない．第 4 に，ヨーグルト・乳酸菌飲料の具体的な内容が大きく異なっていた可能性もある．同じ乳製品や発酵乳でも，商品になっているものと自家製のものでは効果が異なっていたという報告[10]もあり，今回のアンケート調査の「ヨーグルト・乳酸菌飲料」という用語は曖昧であり，今後の検討を要する．最後に，下記に述べるように，プロバイオティクスによるアレルギー抑制効果に関する知識の有無が両研究の行われた地域で異なっていた可能性もある．

本研究におけるアレルギー疾患の有病率へのヨーグルト摂取量の影響の検討では，喘息への中等量群の OR が有意に低く，大量摂取群の OR はむしろ高い値を示す結果となった．このように，摂取量と有病率が単純なトレンドを示さなかった結果の解釈としていくつかの可能性が考えられる．1 つは，ヨーグルト・乳酸菌飲料の摂取は喘息の発症に抑制的に働く適切な量があり，大量摂取した場合は逆効果になるかもしれないという可能性である．プロバイオティクスの投与にてアレルギー感作やアレルギー罹患率が減少したとの報告が多いが[10)12)-16)]，アレルゲン感作を促進したとの報告もみられる[17]．プロバイオティクスの菌の投与ではなく，発酵食品等の摂取状況を検討する研究では，菌の影響以外にも食品に含まれる蛋白を初めとするさまざまな成分の影響も考えられ，今回みられたように，摂取量とアレルギー感作・疾患との非直線的関係となる可能性も考えられる．もう 1 つの解釈は，ヨーグルト・乳酸菌飲料の摂取は，小量・中等量摂取群の喘息発症の変化にみられるように，喘息発症抑制効果があるが，今回の研究協力者に含まれた喘息の患者にすでにプロバイオティクスにアレルギー抑制効果があるという知識があるため，大量にヨーグルト・乳酸菌飲料を摂取しており，大量消費群に多く患者が含まれてしまったという可能性である．

納豆の摂取状況はアルテルナリア特異 IgE 値と有意の相関を示したが，アレルギー疾患の発症との有意の相関は認められなかった．納豆に関しては，榎本らの研究と今回の研究でいずれも特異 IgE との相関が認められているが，アレルギー疾患発症への影響は検出されなかった．現時点では，ヨーグルト・乳酸菌の摂取に比して，アレルギー疾患の予防効果は小さいと考えられる．

本研究では，ヨーグルト・乳酸菌飲料の摂取はアレルギー感作に対して促進的であるが，中等量摂取群での喘息発症を抑制しているように見える結果を示し，アレルギー感作とアレルギー疾患発症へ影響の乖離が認められている．しかし，感作と疾患発症の乖離は本研究に特有なものではなく，Kalliomaki らの研究でも，LGG 菌の投与で 2 歳，4 歳の時点でアトピー性皮膚炎の発症率の低下がみられるが，IgE 値の差が認められていない[4)5)]．また，Van de Water の研究でも IgE の変化が軽微であるのにかかわらず，アレルギー症状の軽減が観察されている[13]．これらの結果から，プロバイオティクスの効果のメカニズムには IgE 抗体の産生抑制のみではなく，効果相の抑制が関与している可能性がある．また，動物実験レベルでは，摂取乳酸菌等の菌種の違いによって樹状細胞での各種サイトカイン産生パターンの違いや[18)19)]，特異 IgG1・IgG2 の血清レベルへの影響が認められており[20]，プロバイオティクスの効果のメカニズムの理解には更なる研究が必要であろ

う．いずれにしろ，本研究のような横断的な研究では，原因と結果の検討は困難であり，それらを明らかにするために，今後，ヨーグルト・乳酸菌飲料の種類を特定し，摂取量のより正確な定量法等を考慮した上での介入研究が必要であろう．

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