Histol Histopathol (2004) 19: 907-914

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Review

Role of intestinal bacterial flora in oral tolerance induction

K. Tanaka and H. Ishikawa

Department of Infectious Diseases, Tokai University School of Medicine, Kanagawa, Japan

Summary. In healthy individuals, the immune responses against foods cannot be induced. This phenomenon is known as oral tolerance. We observed that the oral tolerance was impaired in germfree mice, and that Th2-dependent antibodies such as IgE could be thus induced by an orally given antigen. As a result, the germfree mouse was considered to be a good animal model for allergic disorder. When germfree mice were mono-associated with such bacteria as *E.coli* and *B. infantis*, then oral tolerance was restored in these gnotobiotes to a level similar to that observed in SPF mice. Thus, these bacterias seemed to be important in oral tolerance induction. In addition, the probiotics using these bacteria may be a useful material for the treatment of allergic disorders.

Key words: Oral tolerance, Bacterial flora, Th1/Th2 Balance, Food allergy, probiotics

Introduction

The intestinal tract, which is open to the external environment, has the following immunological features: 1) It eliminates pathological microorganism that have been orally invaded. 2) It allows indigenous flora to colonize in the tract. 3) It has a specially organized tissue, called gut-associated lymphoid tissue (GALT), that exists in it. 4) Antigenic epitopes in the food cannot usually be recognized. This phenomenon is known as oral tolerance.

Oral tolerance is defined as a systemic unresponsiveness to a previously ingested antigen (Ag) when encountered in the parenteral route (Strobel et al., 1998). Among the environmental factors that modulate oral tolerance, the intestinal bacterial flora in the gut is thought to be one important factor (Kirjavainen et al., 1999a). Around 10^{11} (/1g of feces) bacteria have been shown to colonize the gut of healthy individuals to make an intestinal bacterial flora. As a result, the intestinal bacterial flora continuously stimulates the host through GALT. In this review article, we discuss the relationship between the flora and the development of GALT, while also investigating the role of the intestinal flora in oral tolerance induction.

Offprint requests to: Kazuo Tanaka, M.D., Ph.D., Department of Infectious Diseases, Tokai University School of Medicine, Bohseidai, Isehara, Kanagawa 259-1193, Japan. Fax: 81-463-94-2976. e-mail: tanakaka@is.icc.u-tokai.ac.jp

Intestinal bacterial flora

Bacteria cannot colonize in the intestine of the fetus. However, the bacteria colonizes in the intestine so quickly that it can be detected even at 1 day after birth. In addition, non-pathogenic *E. coli* preferentially colonize the intestine of a 1 to 3-day-old newborn. Thereafter, Bifidobacterium quickly expands, and becomes a dominant bacterium at 5 days of age. Finally, anaerobic bacteria expands to form adult-type flora. In adults, intestinal bacterial flora can be divided into 3 groups (Table 1). The major bacteria in the flora of adults are anaerobic and non-pathogenic. The bacteria in this group (Bifidobacteria and Eubacteria etc.) maintain a symbiotic relationship to the host. In contrast, bacteria in the minor component, which includes *C. perfringens* and *P. aeruginosa*, are usually pathogenic, and also responsible for opportunistic infections.

The intestinal bacterial flora has several physiological benefits. First, it is useful to prevent intestinal infections by orally invading pathogens. The flora prevents intestinal infections by means of inhibiting both their attachment to the intestinal epithelium and their growth. Actually, in the mouse model, such bacteria as *Helicobacter pylori* or Vero-toxin producing *E. coli* cannot infect the mice bred under SPF conditions. However, these microorganisms can infect mice bred in germfree (GF) conditions, which lack the intestinal bacterial flora (Kabir et al., 1997; Shimizu et al., 1999). Secondly, the constitutive stimuli via the intestinal bacterial flora participate in the development of the immune system, especially GALT. Microbial pressure by the flora is considered to drive the immune

system towards a Th-1 like response (Björkstén., 1999).

GALT

The immune system of the gastro-intestinal tract demonstrates the dual functions; namely oral tolerance to food antigens (Ags) and immunity against invasive microorganisms. GALT, which develops in order to control the mucosal immune system, is composed of organized lymphoid tissue termed Peyer's patch (PP), epithelium, intra-epithelial lymphocytes (IEL), and lamina propria. PPs are the inductive sites for immune responses to orally encountered Ags (Griebel et al. 1996). PPs consist of macrophages, lymphocytes such as T or B cells and supporting stromal components including follicular dendritic cells. Furthermore, this tissue is covered with unique epithelial cells called M cells. M cells are components of the follicular-associated epithelium. The cells can take luminal Ags and pass them to the Ag-presenting cells (Owen et al., 1977; Spencer et al., 1986; Neutra et al., 1996). Considering that orally introduced Ags are primarily taken by PPs, PPs may thus play a crucial role in the mucosal immunity. On the other hand, IEL and the lymphoid cells in the lamina propria serve as effector cells. Effector cells, which include cytotoxic T lymphocytes (CTL), helper T cells, γ/δ T cells and IgA producing cells, are used to protect the mucosal surfaces. Maintaining animals under GF conditions after birth is well known to causes an obvious reduction in the overall size and cellularity of PPs (Pollard et al. 1970). Actually, as shown in Fig. 1, a microscopic examination revealed that the size of each PP from a GF mouse was smaller than that from SPF mouse. Moreover, PPs in GF mice were free from any distinct germinal center, while PPs in the SPF mice were larger and accompanied by definite germinal centers. Consequently, the total PP cell number in a whole intestine was about 8-times less in the GF mouse than that in the SPF mouse (Maeda et al., 2001). To specify the bacterial species affecting the organogenesis of PP, we previously made 4 mono-associated gnotobiotic mice; *Escherichia coli*, *Bifidobacterium infantis*, *Staphylococcus aureus*, and *Closridium perfringens*-associated mice. Next, the total numbers of PP were enumerated. The numbers of PP cells in E.coli-associated and B.infatis-associated gnotobiotic mice were significantly higher than those of the GF mice (Fig. 2A). Especially, *E. coli* was shown to be effective in the development of PP. Nevertheless, no germinal centers could be observed even in the PPs of

Table 1. Components of intestinal bacterial flora.

	NUMBER OF BACTERIA (CFU/ 1 gram feces)	TYPICAL BACTERIAL GROUP
Major	10^9-10^{11}	*Bacteroides* *Peptostreptococcus* Bifidobacteria Eubacteria
Intermediate	10^5-10^8	*E. coli* Streptococci Lactobacilli Veillonella
Minor	0-10^4	*Clostridium perfringens* *Stapyloccocus aureus* *Pseudomonas aeruginosa*

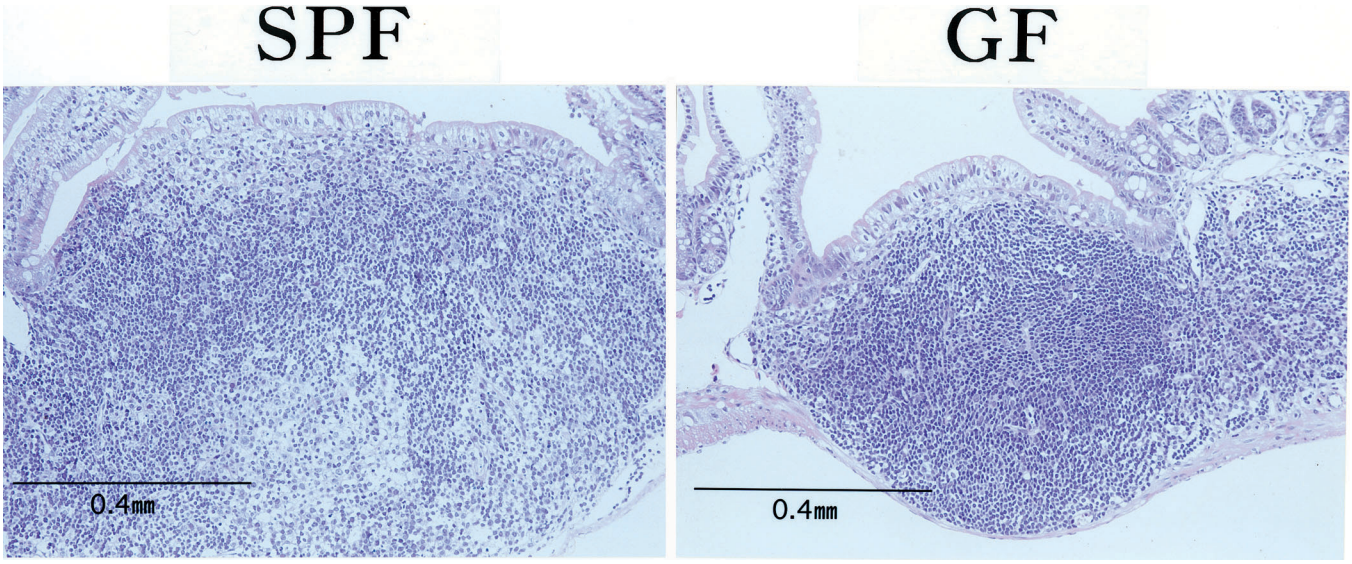

Fig. 1. Histological analyses of Peyer's patches (PP). PPs were obtained from germ-free (GF) and SPF mice. Note that PP in a SPF mouse is larger than that in a GF mouse. In addition, a germinal center was noted in SPF, but not in GF. HE staining.

such *E. coli* –associated mice.

In contrast, it has recently been reported that B cells in PPs can develop during fetal life in many animals even when the gut lumen remains sterile (Griebel et al., 1996; Adachi et al., 1997), thus suggesting that extraneous Ags are not essential for the initiation of either the colonization or expansion of B cells within the follicles. Actually, the PPs of a germfree mouse were filled with B cells to the similar extent observed in that of a SPF (Fig. 3, upper half). On the other hand, when compared with the PP of SPF mice, only a small number of T cells were noted in germfree mice (Fig. 3, lower half). These findings suggested that intestinal bacterial flora might play an important role in the colonization and expansion of T cells in PP, and the subsequent development of the tissue.

Interestingly, although the development of PP depends on the intestinal bacterial flora, the developments of mesenteric lymph nodes and spleens seemed to be independent of the flora. The sizes, total cell numbers and the basic structures of these organs in GF mice are also comparable to those in SPF mice (Fig. 4).

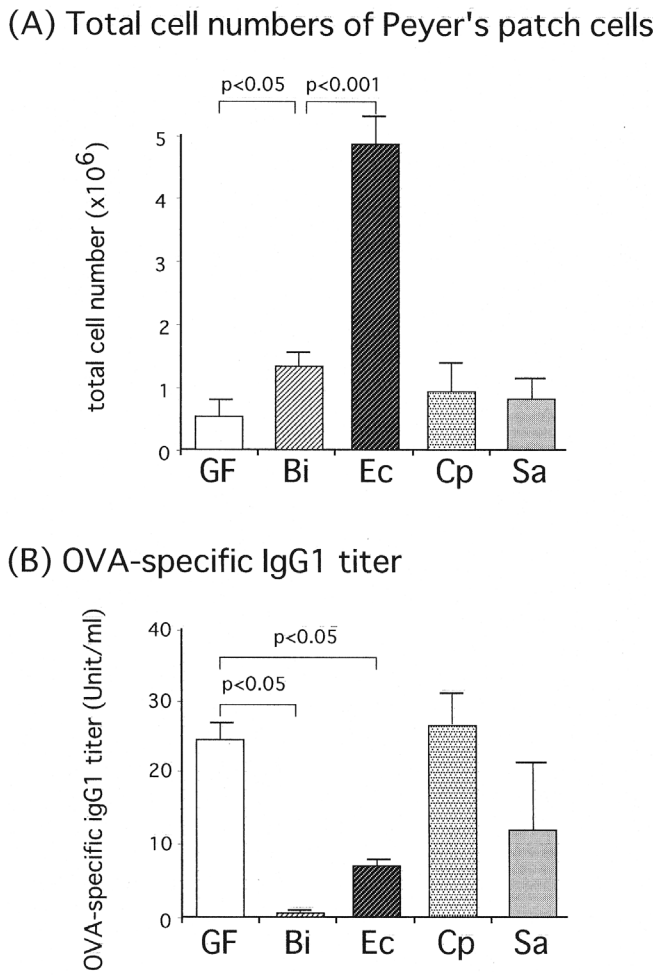

Fig. 2. Enumeration of the PP cells in untreated mice **(A)** and ovalubumin (OVA)-specific IgG1 titer in mice that underwent oral tolerance induction **(B).** Samples were obtained from GF mice or mono-associated gnotobiotes. To produce gnotobiotic mice, the parent mice were orally administrated with *Bifidobacterium infantis* (Bi), *Esherichia coli* (Ec), *Clostridium perfringens* (Cp) or *Staphylococcus aureus* (Sa) in the neonatal stage. **A.** The total number of PPs obtained from an untreated mouse was counted (n=5, mean±SD). A statistical analysis was done by Student's t-test. **B.** The serum level of OVA specific IgG1 in mice, that underwent the oral ingestion of OVA and thereafter were challenges ip with OVA, was measured by ELISA (n=5, mean±SD).

Oral tolerance

Oral tolerance is defined as systemic unresponsiveness to previously ingested Ags when encountered in the parenteral route (Strobel et al., 1998). The breakdown of oral tolerance is a key factor of food allergy. Oral tolerance was first reported by Wells in 1911 (Wells, 1911). As a result, this phenomenon has been long recognized as the way to induce peripheral immune tolerance. Oral tolerance is acquired during the maturation of the host's immune system. Three basic mechanisms explain the antigen-specific oral tolerance; clonal deletion, clonal anergy and active suppression (Weiner et al., 1994). As for clonal deletion, Chen et al. reported that ovalbumin (OVA)-reactive T cells in PPs of OVA-transgenic mice were deleted by the oral administration of high-doses of OVA (Chen et al., 1995). As for clonal anergy, Asai et al. demonstrated that the hyporesponsive state of the CD4 T cells was maintained by a selective impairment in the TCR-induced calcium/NFAT signaling pathway in the mouse model of OVA-specific oral tolerance (Asai et al., 2002). Although the existence of an active suppression has been well recognized for a long time, the precise mechanism for such suppression has recently been demonstrated. Much evidence indicates that CD4+ T regulatory (Tr) cells play a important role for active suppression. At least 3 different types of Tr has been reported; $CD25^{+}CD4^{+}$ T cells, Tr1, and Th3 cells (Levings et al., 2002). These cell types have been independently reported in various experimental systems, and appear predominantly to be distinguishable on their cytokine production profile. CD25+CD4+ T cells, which are reported to be produced by the normal thymus, are naturally anergic and suppressive (Sakaguchi et al., 2001). Although $CD25^{+}CD4^{+}$ T cells may play a role in controlling tumor immunity and transplantation tolerance, these cells seem to play a critical role in preventing autoimmunity. Actually, Zhang et al. reported that $CD25^{+}CD4^{+}$ T were activated by oral antigen administration (Zhang et al., 2001). Tr1 cells were originally defined as the anergic cells induced by IL-10 (Groux et al., 1996). It is now known that Tr1 cells are differentiated from CD4 T cells by the presence of IL-10, and these cells also have a suppressive effect on naive and memory T cells via the production of IL-10 and TGF-ß (Roncarolo et al., 2001). Tsuji and her colleagues demonstrated that IL-10-secreting Payer's

patch cells were responsible for the active suppression of T-cells mediated responses in a low dose of oral tolerance (Tsuji et al., 2001). Th3 was originally defined in studies of oral tolerance (Chen et al., 1994). Th3 exerts its effect by secreting TGF-ß, which has a suppressive effect on Th1 and other immune cells (Weiner et al., 2001).

Although these 3 types of CD4+ Tr are considered to play a role in oral tolerance via direct cell-to-cell interactions or the secretions of immunosuppressive cytokines, the relationship among the 3 types of Tr remains still unclear.

Intestinal bacterial flora and oral tolerance

Epidemiological data and animal experiments have suggested that a disorder of intestinal bacterial flora is closely related to allergy development. In epidemiological data, large amount of antibiotic medication during infancy has been shown to possibly destroy the intestinal bacterial flora, thus leading to subsequent allergic disorders (Wickens et al., 1999; Droste et al., 200). Oyama and others clarified this phenomenon in animal experiments (Oyama et al., 2001). When intestinal bacteria are transiently removed by antibiotic medication in the weaning mice, the mice clearly show the symptoms of allergy accompanied with elevated serum levels of total IgE. On the other hand, such elevated IgE levels could not be detected when 52-wk-old mice were given the same antibiotic medication.

To clarify the role of intestinal bacterial flora in oral tolerance induction, we tried to induce oral tolerance in SPF and GF mice (Sudo et al., 1997). Six-wk-old SPF or GF BALB/c mice were immunized ip with 1μg of ovalbumin (OVA) every 2 weeks starting at 0 week. To induce oral tolerance, the mice were orally given 5mg/day of OVA for 4 consecutive days starting from day –7 to day –4. At 7 weeks after starting the

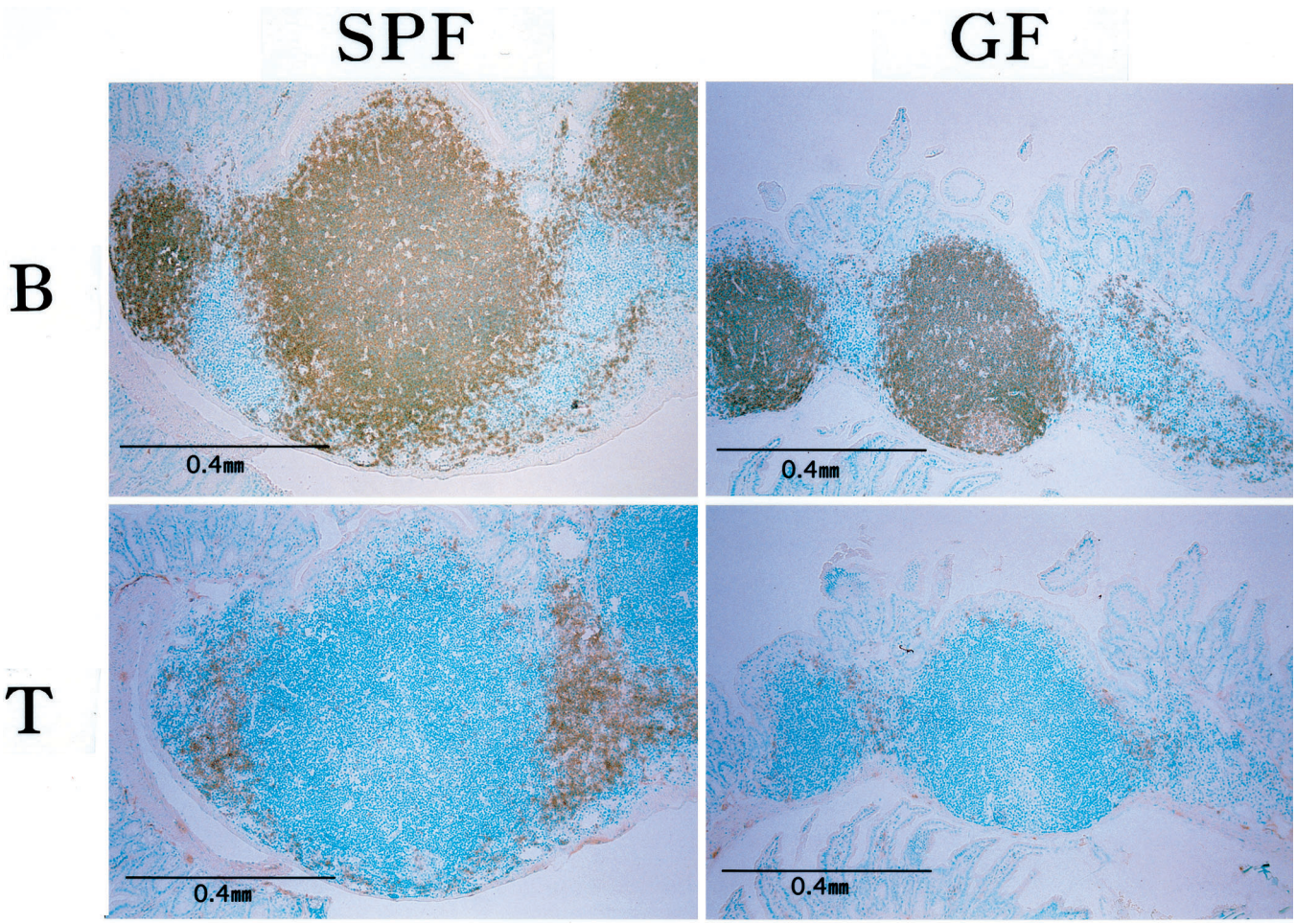

Fig. 3 Histochemical analyses of Peyer's patches (PP). The B and T cells in PPs of SPF and GF mice were examined by an immunohistochemical analysis. The samples were frozen in OCT compound, and 6-μm-thick acetone-fixed sections were stained with anti-Thy-1 or anti-CD45R/B220 for T and B cells, respectively. Note that only a small number of T cells was noted in a GF mouse in comparison to a SPF mouse.

immunization, serum was obtained to measure the Ab (OVA-specific IgG1, IgG2a and IgE) titers. As shown in Fig. 5, in SPF mice, oral tolerance was induced both in Th1 (IgG2a production) and Th2 (IgG1 and IgE productions)-meditated immune responses. On the other hand, in GF mice, oral tolerance was induced in Th1 responses, but not in Th2 responses. Taken together that allergic responses are mediated Th2 cells and IgE production plays a key role in allergy, GF mice seem to be an animal model of food allergy. We next tried to specify the bacteria affecting the oral tolerance of Th2 mediated immune responses, using 4 gnotobiotes mentioned above. Oral tolerance was induced as the same protocol as that in SPF or GF mice. Five weeks after starting the immunization, IgG1 titers were measured by ELISA. As shown in Fig. 2B, the OVA-specific IgG1 titers in *B. infantis* and *E. coli*–associated mice were significantly lower than that in GF mice, thus suggesting that oral tolerance was retrieved in these gnotobiotes. On the other hand, the OVA-specific IgG1 titers in *C. perfringens* and *S. aureus*-associated mice were comparable to those in GF mice, thus suggesting that these two bacteria are not involved in the induction of oral tolerance.

Two possibilities were raised regarding the reasons why oral tolerance against OVA could not be induced in Th2-type cells from GF mice.

One possibility is that PP is necessary for oral tolerance induction. If this is true, then the poorly developed PP in GF mice might be responsible for the defect in oral tolerance induction in these mice. However, it remains controversial as to whether PP is essential for oral tolerance induction. In order to examine this issue, several PP-defective mice, including GF mice, have been used. Fujihashi et al. demonstrated that oral tolerance against OVA could be not be induced in PP "null" mice, which had been generated by treating female mice late in gestation with soluble lymphotoxin (LT) ß-immunoglobulin fusion protein (Fujihashi et al., 2001). Nonetheless, oral tolerance to a hapten could be noted in these mice. In addition, several groups observed the oral tolerance induction in the mice treated with the same way (Spahn et al., 2001, 2002), and oral tolerance could be induced in B-cell-deficient mice, which have only rudimentary Peyer's patches (Alpan et al., 2001). Moreover, it has previously been reported that a surgical resection of all PPs did not affect oral tolerance induction (Enders et al. 1986). Finally, oral tolerance could be induced in Th1-immune response in our model of oral tolerance using GF mice (Fig. 5). Although it is true that PP plays a role in oral tolerance induction, other tissues such as mesenteric lymph nodes (MLN) may be a critical check point for oral tolerance induction (Mowat et al., 2003). In fact, the development of MLNs in GF micw is comparable to those in SPF (Fig. 4).

Another possibility is that Th1/Th2 balance is Th2 dominant in GF mice. Th1-type immunity is well known to work against bacterial and viral infections. It is thus likely that continuous stimulation by intestinal bacterial flora stimulates Th1 cells, and that the flora counterbalances the Th2 activity. As a result, the Th1/Th2 balance is maintained in SPF animals (Fig. 6). In contrast, such microbial pressure toward Th1 immune response is missing in GF mice, it is thus thought that Th2 cells consequently become dominant in GF mice. Actually, we previously observed IL-4 (Th2 cytokine) production in vitro to be significantly higher in the spleen cells from GF mice than in those from SPF mice,

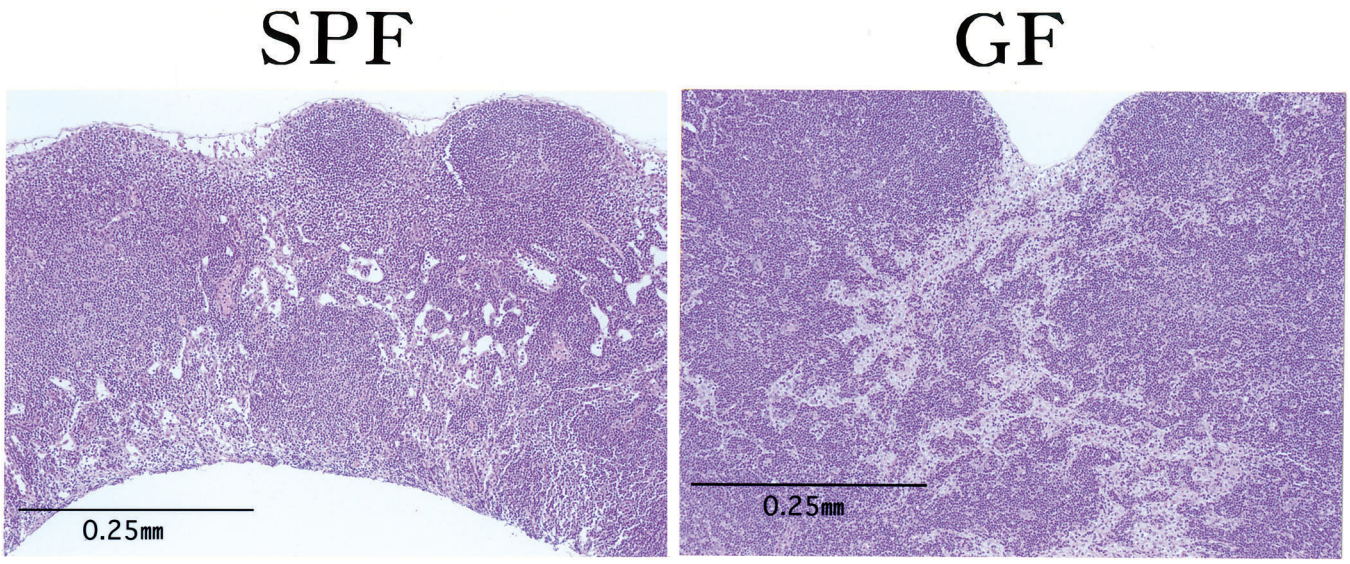

Fig. 4. Microscopy images of mesenteric lymph nodes (MLNs). MLNs were obtained from germ-free (GF) and SPF mice. No significant difference was seen between the MLNs from SPF and GF mice in the histological analysis of HE staining.

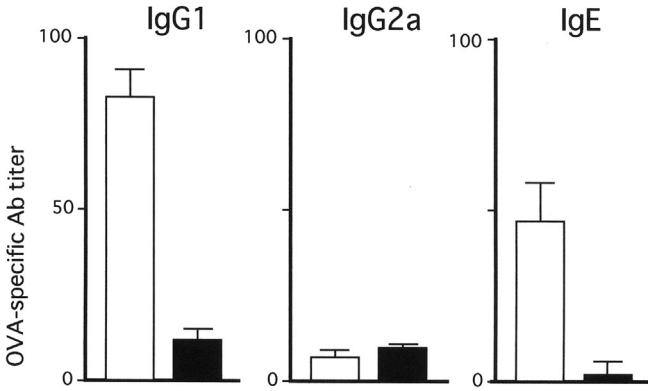

Fig. 5. OVA-specific antibody titers in the serum of mice that underwent oral tolerance induction. To induce oral tolerance, GF (open column) or SPF (closed column) mice at 6 wks of age were orally given 5 mg/day of OVA for 4 consecutive days from day -7 to day -4 before the initiation of immunization on day 0. Then the mice ip injected with 1 μg of OVA 4 times at 0, 2, 4, and 6 wk. The mice were killed at 7 wk to collect the blood specimens. OVA-specific antibody titers were measured by fluorescent ELISA. The antibody titers of GF or SPF mice, which were only received ip challenges of OVA4 times at 0, 2, 4, and 6 wk, were designated as 100 unit/ml. Note that oral tolerance was noted in IgG1 (Th2 dependent), IgG2a (Th1 dependent) and IgE (Th2 dependent) productions in SPF mice. In contrast, in GF mice, oral tolerance was induced in the Th1 responses, but not in the Th2 responses.

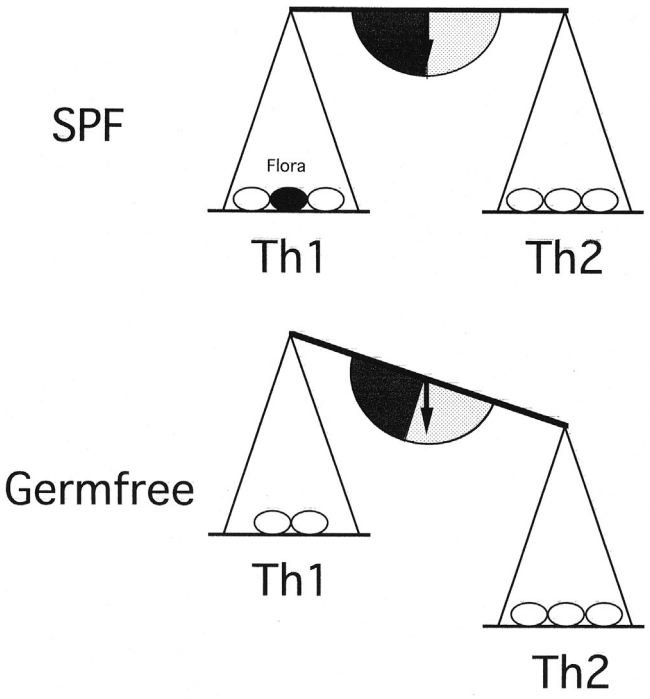

Fig. 6. A Schematic drawing demonstrating the role of the intestinal bacterial flora on the Th1/Th2 balance of the host. The flora acts as "microbial pressure" which may drive the immune system towards the Th1 responses. As a result, in GF mice, Th1/Th2 balances skewed to Th2 responses.

when the cells, which had been obtained from mice in vivo immunized with OVA, were challenged in vitro with Ag (Sudo et al., 1997).

Several factors in the flora are considered to play a role in maintaining Th1 immunity. Among them, a specific bacterial CpG motif may be one of the candidates. The bacterial CpG motif was first reported by Japanese researchers as an anti-tumor factor (Tokunaga et al., 1984), and recent results have demonstrated that the bacterial CpG motif plays a major role in maintaining and augmenting Th1 responses (Chu et al., 1997; Jakob et al., 1998, 1999; Zimmermann et al., 1998; Chu et al., 2000; Horner et al., 2000).

Probiotics for the management of food allergy

The above results suggest that the perturbation of intestinal bacterial flora may induce Th2-dominant immunity, which is one of the risk factors of allergy development. Normalization of intestinal bacterial flora in allergic patients is thus suggested as a useful alternative for controlling allergies. Therefore, probiotics may potentially be the novel therapeutic approach for the treatment of allergies. Probiotics are designated as the bacteria or bacterial components, which have a beneficial effect on the health (Dugas et al., 1999). Usually, the bacteria in the genera of lactobacilli and bifidobacteria are used as probiotics, due to their safety, acid and bile stability, and colonizing ability in the intestine (Kirjavainen et al., 1999b).

As shown in Fig. 2B, oral tolerance in Th2 immune cells were noted in *B. infantis*-associated gnotobiotes. Our results therefore also support the hypothesis that *B. infantis* may be a potentially effective probiotic for the management of food allergies.

In a previous work using *B. infantis*-associated mice, mice associated with bacteria in the neonatal period could obtain a level of oral tolerance similar to that observed in SPF mice, whereas no oral tolerance could not observed in the mice associated with *B. infantis* at 5 wks of age (Sudo et al., 1997). As a result, we consider that allergic individuals should be treated with probiotics as early as possible after birth.

The results in Fig.2 suggested that non-pathogenic *E. coli* may also be a candidate of probiotics. Although *E. coli* has not yet been so widely used as a probiotic microorganism as lactobacilli or bifidobacteria, this bacterium activated the GALT (Fig. 2A). This effect of E.coli would is therefore considered to be preferential for the probiotic use of the bacteria.

References

Adachi S., Yoshida H., Kataoka H. and Nishikawa S. (1997). Three distinctive steps in Peyer's patch formation of murine embryo. Int. Immunol. 9, 507-14.

Alpan O., Rudomen G. and Matzinger P. (2001). The role of dendritic cells, B cells, and M cells in gut-oriented immune responses. J. Immunol. 166, 4843-4852.

Asai K., Hachimura S., Kimura M., Toraya T., Yamashita M., Nakayama T. and Kaminogawa S (2002). T cell hyporesponsiveness induced by oral administration of ovalbumin is associated with impaired NFAT nuclear translocation and p27kip1 degradation. J. Immunol. 169, 4723-4731.

Björkstén B. Allergy priming early in life. (1999). Lancet 353, 167-168.

Chen Y., Kuchroo V.K., Inobe J., Hafler D.A. and Weiner H.L. (1994). Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. Science 265, 1237-1240.

Chen Y., Inobe J., Marks R., Gonnella P., Kuchroo V.K. and Weiner H.L. (1995). Peripheral deletion of antigen-reactive T cells in oral tolerance. Nature 376, 177-180. Erratum: Nature (1995) 377, 257.

Chu R.S., Targoni O.S., Krieg A.M., Lehmann P.V. and Harding C.V. (1997). CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. J. Exp. Med. 186, 1623-1631.

Chu R.S., Askew D. and Harding C.V. (2000). CpG DNA switches on Th1 immunity and modulates antigen-presenting cell function. Curr Top. Microbiol. Immunol. 247, 199-210.

Droste J.H., Wieringa M.H., Weyler J.J., Nelen V.J., Vermeire P.A. and Van Bever H.P. (2000). Does the use of antibiotics in early childhood increase the risk of asthma and allergic disease? Clin. Exp. Allergy 30, 1547-1553.

Dugas B., Mercenier A., Lenoir-Wijnkoop I., Arnaud C., Dugas N. and Postaire E. (1999). Immunity and probiotics. Immunol. Today 20, 387-390.

Enders G., Gottwald T. and Brendel W. (1986). Induction of oral tolerance in rats without Peyer's patches. Immunology 58, 311-314.

Fujihashi K., Dohi T., Rennert P.D., Yamamoto M., Koga T., Kiyono H. and McGhee J.R. (2001). Peyer's patches are required for oral tolerance to proteins. Proc. Natl. Acad. Sci. USA 98, 3310-3315.

Griebel P.J. and Hein W.R. (1996). Expanding the role of Peyer's patches in B-cell ontogeny. Immunol. Today 17, 30-39.

Groux H., Bigler M., de Vries J.E. and Roncarolo M.G. (1996). Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. J. Exp. Med. 184, 19-29.

Horner A.A. and Raz E. (2000). Immunostimulatory-sequence DNA is an effective mucosal adjuvant. Curr. Top. Microbiol. Immunol. 247, 185-198.

Jakob T., Walker P.S., Krieg A.M., Udey M.C. and Vogel J.C. (1998). Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. J. Immunol. 161, 3042-3049.

Jakob T., Walker P.S., Krieg A.M., von Stebut E., Udey M.C. and Vogel J.C. (1999). Bacterial DNA and CpG-containing oligodeoxynucleotides activate cutaneous dendritic cells and induce IL-12 production: implications for the augmentation of Th1 responses. Int. Arch. Allergy Immunol. 118, 457-461.

Kabir A.M., Aiba Y., Takagi A., Kamiya S., Miwa T. and Koga Y. (1997). Prevention of Helicobacter pylori infection by lactobacilli in a gnotobiotic murine model. Gut 41., 9-55.

Kirjavainen P.V. and Gibson G.R. (1999a). Healthy gut microflora and allergy: factors influencing development of the microbiota. Ann. Med. 31, 288-292.

Kirjavainen P.V., Apostolou E., Salminen S.J. and Isolauri E. (1999b). New aspects of probiotics--a novel approach in the management of food allergy. Allergy 54, 909-915.

Levings M.K., Sangregorio R., Sartirana C., Moschin A.L., Battaglia M., Orban P.C. and Roncarolo M.G. (2002). Human CD25+CD4+ T suppressor cell clones produce transforming growth factor beta, but not interleukin10, and are distinct from type 1 T regulatory cells. J. Exp. Med. 196, 1335-1346.

Maeda Y., Noda S., Tanaka K., Sawamura S., Aiba Y., Ishikawa H., Hasegawa H., Kawabe N., Miyasaka,M. and Koga Y. (2001). The failure of oral tolerance induction is functionally coupled to the absence of T cells in Peyer's patches under germfree conditions. Immunobiology 204, 442-457.

Mowat A.M. (2003). Anatomical basis of tolerance and immunity to intestinal antigens. Nat. Rev. Immunol. 3, 331-341.

Neutra M.R., Frey A. and Kraehenbuhl J.P. (1996). Epithelial M cells: gateways for mucosal infection and immunization. Cell 86, 345-348.

Owen R.L. (1977) Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: an ultrastructural study. Gastroenterology 72, 440-451.

Oyama N., Sudo N., Sogawa H. and Kubo C. (2001). Antibiotic use during infancy promotes a shift in the T(H)1/T(H)2 balance toward T(H)2-dominant immunity in mice. J. Allergy Clin. Immunol. 107, 153-159.

Pollard M. and Sharon N. (1970). Responses of the Payer's patches in germ-free mice to antigenic stimulation. Infect. Immun. 2, 96-100.

Roncarolo M.G., Bacchetta R., Bordignon C., Narula S. and Levings M.K. (2001). Type 1 T regulatory cells. Immunol. Rev. 182, 68-79.

Sakaguchi S., Sakaguchi N., Shimizu J., Yamazaki S., Sakihama T., Itoh M., Kuniyasu Y., Nomura T., Toda M. and Takahashi T. (2001) Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. Immunol. Rev. 182, 18-32.

Shimizu K., Tanaka K., Akatsuka A., Endoh M. and Koga Y. (1999). Induction of glomerular lesions in the kidneys of mice infected with vero toxin-producing *Escherichia coli* by lipopolysaccharide injection. J. Infect. Dis. 180, 1374-1377.

Spahn T.W., Fontana A., Faria A.M., Slavin A.J., Eugster H.P., Zhang X., Koni P.A., Ruddle N.H., Flavell R.A., Rennert P.D. and Weiner H.L. (2001). Induction of oral tolerance to cellular immune responses in the absence of Peyer's patches. Eur. J. Immunol. 31, 1278-1287.

Spahn T.W., Weiner H.L., Rennert P.D., Lugering N., Fontana A., Domschke W. and Kucharzik T. (2002). Mesenteric lymph nodes are critical for the induction of high-dose oral tolerance in the absence of Peyer's patches. Eur. J. Immunol. 32, 1109-1113.

Spencer J., Finn T. and Isaacson P.G. (1986). Human Peyer's patches: an immunohistochemical study. Gut 27, 405-410.

Strobel S. and Mowat A.M. (1998). Immune responses to dietary antigens: oral tolerance. Immunol. Today 19, 173-181.

Sudo N., Sawamura S., Tanaka K., Aiba Y., Kubo C. and Koga Y. (1997). The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. J. Immunol. 159, 1739-1745.

Tokunaga T., Yamamoto H., Shimada S., Abe H., Fukuda T., Fujisawa Y., Furutani Y., Yano O., Kataoka T., Sudo T., Makiguchi N. and Suganuma T. (1984). Antitumor activity of deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. I. Isolation, physicochemical characterization, and antitumor activity. J. Natl. Cancer Inst. 72, 955-962.

Tsuji N.M., Mizumachi K. and Kurisaki J. (2001). Interleukin-10-

secreting Peyer's patch cells are responsible for active suppression in low-dose oral tolerance. Immunology 103, 458-364.

Weiner H.L. (2001). Oral tolerance: immune mechanisms and the generation of Th3- type TGF-beta-secreting regulatory cells. Microbes Infect. 2001 3, 947-954.

Weiner H.L., Friedman A., Miller A., Khoury S.J., al-Sabbagh A., Santos L., Sayegh M., Nussenblatt R.B., Trentham D.E. and Hafler DA. (1994). Oral tolerance: immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. Annu. Rev. Immunol. 12, 809-837.

Wells H. (1911). Studies on the chemistry of anaphylaxis. III. Experiments with isolated proteins, especially those of hen's egg. J.I J. Infect. Dis. 9, 147-151

Wickens K., Pearce N., Crane J. and Beasley R. (1999). Antibiotic use in early childhood and the development of asthma. Clin. Exp. Allergy 29, 766-771.

Zhang X., Izikson L., Liu L. and Weiner H.L. (2001). Activation of CD25(+)CD4(+) regulatory T cells by oral antigen administration. J. Immunol. 167, 4245-4253.

Zimmermann S., Egeter O., Hausmann S., Lipford G.B., Rocken M., Wagner H. and Heeg K. (1998). CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine leishmaniasis. J. Immunol. 160, 3627-3630.

Accepted February 3, 2004