Mucosal Immune Responses to the Introduction of Gut Flora in Mice and the Establishment of a Murine Model of Crohn’s Disease

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Received for publication, October 2, 2003

Gut flora plays a key role in the maturation of intestinal mucosal immune systems such as the expression of class II MHC antigens on intestinal epithelial cells, and the cell expansion and functional maturation of both IgA-producing B lymphocytes in the lamina propria and TCR αβ-expressing intraepithelial lymphocytes in the intestinal epithelium in mice. In normal mice, the mucosal immune responses evoked by colonization of gut flora attained levels found in mice reared under normal gut flora-bearing conditions. However, in SAMPI/Yit mice, recently established as a murine model of Crohn’s disease, transmural ileitis and cecitis developed following the introduction of commensal gut flora from normal mice, although no intestinal inflammation was observed under germfree conditions. These results suggested that commensal gut flora play critical roles in the development of Crohn’s disease-like intestinal inflammation in SAMPI/Yit mice. In this review, we focus on the specific interactions between the gut flora and mucosal immune systems that induce physiological or unphysiological mucosal immune responses.

Key words: Crohn’s disease; epithelial cell; intestinal microflora; inflammatory bowel disease

MUCOSAL IMMUNE RESPONSES TO COLONIZATION BY GUT FLORA UNDER PHYSIOLOGICAL CONDITIONS

We have already stated that introduction of gut flora from mice reared under specific-pathogen-free (SPF) condition into germfree (GF) Balb/c mice evoked various immune responses in the gut. For instance, class II major histocompatibility complex (MHC) antigens were induced to express themselves on small intestinal, but not large intestinal, epithelial cells on day 7 after colonization by gut flora (21). In contrast, class Ia MHC antigens (thymus leukemia antigen: TLA) were already expressed on small intestinal epithelial cells in the GF state and the expression remained constant during the colonization of the gut flora. We had also discovered the phenotypic and functional maturation of small intestinal intraepithelial lymphocytes (SI-IELs) on the introduction of gut flora into GF Balb/c mice (33). In brief, SI-IELs in GF Balb/c mice consisted predominantly of γδ TCR IELs. During contamination of gut flora, the population of CD8αα and CD8αβ T cell receptor (TCR) αβ SI-IELs which were the minor population in GF conditions markedly increased. Kawaguchi et al. discovered that cytolytic activity in TCRαβ SI-IELs also increased with colonization by gut flora (12). Moreover, the number of the IgA-producing B cells in the lamina propria also increased (6). When the mucosal characteristics evoked by the introduction of gut flora attained the levels found in normal gut flora-bearing mice, they were preserved at these levels.

LOCAL INTERFERON-γ RELEASED BY T CELLS MODULATES CLASS II MHC INDUCTION ON INTESTINAL EPITHELIAL CELLS

We examined the interferon-γ (IFN-γ)-dependency of the class II MHC induction on the small intestinal epithelial cells in ex-GF Balb/c mice. Intraperitoneal injection of a monoclonal antibody against IFN-γ into ex-GF mice blocked the expression of class II MHC antigens on the epithelial cells (20). Moreover, intraperitoneal injection of recombinant murine IFN-γ triggered an induction of class II MHC antigens in GF Balb/c mice in both the small and large bowel (Fig.1). These results suggested that the induction of class II MHC antigens on small intestinal epithelial cells during colonization by gut flora in ex-GF mice was regulated by IFN-γ. In support of this notion, we observed the up-regulation of class II transactivator gene expression, a trans-acting promoter for the class II MHC gene regulated by IFN-γ, during the colonization of the gut flora in ex-GF mice (20). Next, we examined the source of IFN-γ production during this period. It is known that
The helper type I (Th1) and CD8 T cell subpopulations, natural-killer cells and certain macrophage subpopulations produce IFN-γ (7, 10, 35). Therefore, we examined class II MHC induction in ex-GF T cell-deficient severe combined immune-deficient (scid) mice. The expression of class II MHC antigens on small intestinal epithelial cells was repressed in the scid model. This result suggested that the induction of the expression was regulated in a T cell-dependent manner. The question arises as to the anatomical sites for the generation of IFN-γ-producing cells during colonization of the gut flora in ex-GF mice, because the induction of class II MHC antigens on intestinal epithelial cells was restricted in the small bowel, but not in the large bowel, although systemic exposure to IFN-γ resulted in a widespread class II MHC induction on epithelial cells such as those of the large bowel (25, 29). Moreover, we could not detect any increase in the levels of IFN-γ in serum during the introduction of gut flora in ex-GF mice (21). Therefore, the local production of IFN-γ may contribute to the local induction of class II MHC antigens. To test this concept, we produced GF Balb/c mice bearing isolated small bowel segments and then introduced gut flora into these mice. The result was very clear. The epithelial cells in the gut flora associated with the small bowel expressed class II MHC antigens; however, those isolated bowel segments did not (Fig. 2). Consequently, the expression of class II MHC antigens in this period was modulated by a local immune response activated by the gut flora.

**γδ TCR IELs REGULATES CLASS II MHC INDUCTION ON INTESTINAL EPITHELIAL CELLS**

Earlier, we discussed a local immune activation in the gut that regulated IFN-γ production in T-lymphocytes and class II MHC expression in the small intestinal epithelial cells. In this section, I confirmed the kinds of T cells that regulate this phenomenon. A unique T cell subpopulation, which expresses γδ TCR, is located in the intestinal epithelial layer (1, 2). This subpopulation is the main counterpart of intestinal intraepithelial lymphocytes (IELs) in the small bowel, but not in the large bowel, in GF Balb/c mice (24). Moreover, these T cells exhibit cytolytic activity and produce Th1 type cytokines (9, 16). Therefore, we hypothesized that the activation of this T cell subpopulation contributes to class II MHC induction on small intestinal epithelial cells in ex-GF mice. To test this hypothesis, we examined class II MHC expression in ex-GF mice depleted of γδ TCR IELs. The induction of class II MHC antigens on the small intestinal epithelium was repressed in γδ TCR IEL-depleted ex-GF mice (20). Moreover, we also observed the down-regulation of class II MHC expression in the intestine in TCR δchain gene-deficient mice (14). In contrast, we observed over-expression of class II MHC antigens on the small intestinal epithelial cells in ex-GF TCR γδ gene transgenic mice (KN6): almost all the endogenous TCR in the IELs of these mice bear transgenic KN6 TCR (Vγ4/Vδ5), although the class II MHC antigens on epithelial cells were absent in GF KN6 mice (18). These results suggested that γδ TCR IELs play a critical role in the induction of
class II MHC expression in small intestinal epithelial cells in ex-GF mice.

**ACTIVATION OF γδ TCR IELs REGULATED MHC IN A NON-RESTRICTED MANNER**

In general, the activation of T cells determined the recognition of antigen-bearing MHC molecules via TCR. However, the mechanism behind the activation of γδ TCR IELs in the gut is unknown. To elucidate this mechanism, we examined whether the activation of γδ TCR IELs in the intestine during colonization by gut flora is a consequence of MHC-restriction. KN6 TCRs (Vγ4/Vδ5) recognize a novel TL22 gene product that is present in C57BL/6 (H-2b) mice. However, this gene product was absent in BALB/c (H-2d) mice. Then, we produced GF KN6 transgenic mice with a BALB/c background, and examined both class II MHC induction and the activation of γδ TCR IELs after contamination of gut flora in these mice. Unexpectedly, class II MHC antigens were also expressed on epithelial cells in H-2d KN6 transgenic mice at a level which could be observed in the H-2b background. Moreover, IFN-γ mRNA in γδ TCR IELs increased markedly after colonization of gut flora (18). Consequently, the activation of γδ TCR IELs might occur in an MHC non-restricted fashion that might be dependent on an alternative T cell signaling pathway modulated by the gut flora.

**γδ TCR IELs IS THE PRIMARY RESPONDER AGAINST GUT FLORA-COLONIZATION**

The functions and fate of γδ TCR IELs are yet to be defined. We also investigated whether γδ TCR IELs regulate epithelial mitotic activity in the small intestine in mice. γδ TCR IELs may contribute to the maintenance of physiological and immunological function in intestinal epithelial cells after epithelial injuries due to infectious disease or inflammation. Supporting this concept, recovery from intestinal inflammation was delayed under γδ TCR T cell-deficient conditions in mice (4). Moreover, the numbers and functions of extrathymic-derived IELs, including γδ TCR IELs, were repressed in SAMP1/Yit mice established as an animal model of Crohn’s disease. Further analysis should clarify the true functions of intestinal γδ TCR IELs.

**ESTABLISHMENT OF SAMP1/YIT MICE**

The senescence accelerated mouse strain (SAM) strains are a series of murine strains that spontaneously exhibit signs of the senescence (31). The original SAM strain was kindly provided to us by Dr. T. Takeda of Kyoto University in 1988. We discovered an ulcerative skin disease in some colonies of the original SAM strain. For the establishment of a new strain, SAMP1 mice showing spontaneous skin ulceration were selected and mated. Mice showing skin lesions were selected in the F1 generation of this strain and brother-sister matings performed over 20 times so as to establish the SAMP1/Yit strain. This strain showed no amyloidosis and no shortened life span, unlike the original SAMP1. In addition, inflammatory disease in the ileum and cecum was observed in this new strain as described below. The inflammation of the gut in this new strain was associated with the presence of skin lesions. No differences were detected the standard genetic markers between the Yit strain and the original mice in the chromosomes tested: Chr 1 (Idh1, Pep 3, Akp 1, Apo a2), Chr 2 (Hsc), Chr 3 (Car 2), Chr 4 (Mup 1, Gpd 1), Chr 5 (Pgm-1), Chr 6 (Ldr 1), Chr 7 (Gpi 1, Hbb), Chr 8 (Es 1, Es 2), Chr 9 (Tfy 1, Trf), Chr 11 (Es 3) and Chr 17 (H-2K, H-2D).

**INTESTINAL PATHOLOGY OF SAMP1/YIT MICE**

Observations under a stereomicroscope showed that there were many longitudinal or round lesions in the ileal mucosa, which were clearly distinct from the normal mucosa (data not shown). Characteristics of the histopathology in the ileum of the SAMP1/Yit strain included a discontinuous, patchy and transmural intestinal inflammation with many inflammatory cells. The intestinal tract showed stenosis due to thickening of the intestinal walls. Crypt necrosis and granulomas were often detected in the intestinal walls (Fig. 3). Immunohistochemical analyses showed that the inflammatory infiltrates in LP consisted of macrophages, CD11c-positive dendritic cells, neutrophils and CD3ε-positive T lymphocytes (19). The histopathological characteristics of SAMP1/Yit resembled those of CD. Due to the severe intestinal inflammation, the tissue weight of the ileum was drastically increased. Crypt elongation with increased mitotic activity was pronounced in the epithelial layer during disease development. Intestinal inflammation could not be detected in the upper part of the small intestine (duodenum and upper part of the jejunum). Mast cell infiltration was pronounced in the muscle layer, but not in the lamina propria. Colonic inflammation is rare in the SPF SAMP1/Yit strain, however inflammation in the rectum was often detected. It should also be noted that nitric oxide metabolism was up-regulated not only in the epithelial cells but also in the lamina propria macrophages. In addition, myeloperoxidase activity in the mucosa of the intes-
tine was markedly increased (19). The lesions in the cecum were characterized by hyperplasia of the crypt epithelial cells and cellular infiltration in the LP, as observed for the pathology in the ileum.

**ACTIVATION OF IL-6/STAT-3 SIGNALING PATHWAYS IN SAMPI/Yit MICE**

We have already described how interleukin (IL)-6 and its signal transducing molecules, signal transducer and activator of transcription (Stat)-3, were activated in IBD in humans and an animal model. In SAMPI/Yit mice, the activation of Stat-1, Stat-3 and Stat-6 was confirmed (Fig. 4). The phosphorylation of Stat-3 molecules was pronounced in relation to the increasing amount of IL-6 mRNA in the lamina propria lymphocytes. By immunohistochemistry, phosphorylation of Stat-3 proteins was detected in the nuclei of intestinal epithelial cells and lamina propria lymphocytes (30).

**THE GF SAMPI/YIT STRAIN DID NOT DEVELOP IBD**

There is much evidence that apathogenic intestinal microflora regulate the development of the intestinal immune system in mice. To determine the effect of enteric bacteria on the development of IBD in our mouse strain, we generated the GF SAMPI/Yit strain and analyzed its intestinal histology. In GF mice, the intestinal inflammatory disease seen under SPF conditions could not be detected (19). The intestinal architecture of the GF littermates was quite normal, as in other strains of GF mice. To determine the effect of enteric bacteria on the development of the CD-like disease in our mouse strain, we conventionalized 5-week-old GF SAMPI/Yit strain mice with a fecal suspension from SPF AKR/J mice. At 15 weeks after conventionalization, we detected intestinal inflammation in the conventionalized SAMPI/Yit strain. In addition, the tissue weight of the distal part of the small intestine was greater than that in the control age-matched AKR/J and GF SAMPI/Yit strain mice, due to the intestinal inflammatory disease. These results were comparable with those for the effect of intestinal flora on the development of intestinal inflammation in other experimental IBD models.

**ROLES OF CD4⁺ CD8⁻ T CELLS IN THE PATHOGENESIS OF IBD IN THE SAMPI/YIT STRAIN**

We described in the above section that many CD3ε-positive T cells infiltrated the mucosa of the lesions at the onset of the CD-like disease development in SAMPI/Yit mice. We hypothesized that the T cells might have an effector function in the development of CD-like disease in our mouse strain. To test this notion, we transferred MACS-sorted CD4⁺ CD8⁻ T cells or CD8⁺ CD8⁻ T cells derived from SAMPI/Yit mice into histocompatible scid mutant mice. Intestinal inflammation could be detected in the ileum in scid mice in-
cells also secrete large amounts of IFN-γ and TNF-α, and less IL-10 on stimulation with immobilized anti-CD3ε antibody. Therefore, the aberrant cytokine secretion by CD4+ αβ T cells is responsible for the development of CD-like intestinal inflammation in the SAMP1/Yit strain.

ENTERIC BACTERIA ARE IMPORTANT FOR ACTIVATION OF THE EFFECOR FUNCTION OF CD4+ αβ T CELLS

We have detected an effector function for the control of CD-like disease in the CD4+ αβ T cell fraction in our mouse strain. To determine the specific interaction between the activation of the effector function for IBD in CD4+ αβ T cell and enteric bacteria, we introduced CD4+ αβ T cells derived from SAMP1/Yit mice into GF or SPF scid mutant mice. We detected CD-like disease in SPF scid mice injected with CD4+ T cells from SAMP1/Yit (23). However, there was no evidence of CD-like disease in GF scid mutant mice injected with CD4+ αβ T cells from SAMP1/Yit mice (23). These results suggested that the presence of the enteric bacteria is critical for the activation of CD-like disease inducing CD4+ αβ T cells in SAMP1/Yit strain mice.

IN VITRO CELL PROLIFERATIVE RESPONSE OF CD4+ αβ T CELLS FROM IBD SAMP1/YIT MICE TO ENTERIC BACTERIA

To examine the possibility that enteric bacteria activate IBD-inducing CD4+ αβ T cells, we analyzed the cell proliferative response of CD4+ αβ T cells to sonicated cecal bacteria in vitro. CD4+ αβ T cells were prepared from SAMP1/Yit mice or control age-matched AKR/J mice. A cecal bacterial fraction was prepared from both strains. CD4+ αβ T cells in the two strains were co-cultured for 48 hours with or without the sonicated cecal bacterial fraction and the cell proliferative response was analyzed by measuring the [3H]-thymidine incorporation during the last 18 hours of cultivation. The proliferative response to the sonicated cecal bacteria was higher in CD4+ αβ T cells from SAMP1/Yit strain mice than in those from AKR/J mice, although cell proliferative responses against universal mitogen such as the anti-TCR β chain are comparable between the two groups (23). Moreover, CD4+ αβ T cells did not proliferate in response to the sonicated cecal content preparation derived from GF mice or to the medium only. Among the cultivable enteric bacteria, several strains of Bacteroides induced marked T cell proliferative responses to CD4+ αβ T cells derived from the SAMP1/Yit strain. An IL-10-dependent unrespon-
siveness of intestinal mucosal T lymphocytes toward enteric bacteria had been reported by Khoo et al. (13). However, Duchmann et al. showed that lamina propria cells derived from active IBD patients, both with UC and CD, but not inactive IBD patients or non-IBD human subjects, markedly proliferated in response to enteric bacteria (8). Moreover, in the cases of other experimental IBD models, abrogation of immune tolerance toward resident enteric bacteria has been reported (5). The problem is whether this immune abrogation is a result or a cause of IBD. Detailed kinetics data on the appearance of the reactivity of CD4+ αβ T cells may solve this problem. Our and other results suggested that the mechanism underlying the pathogenesis of IBD involves the abnormal response of CD4+ αβ T cells to conventional enteric bacteria, especially Bacteroides species. The mechanisms underlying the interaction between CD-like disease-inducible CD4+ αβ T cells and enteric bacteria remain unknown. We have already noted that the development of the gut immune system, such as the acquisition of the cytolytic function of αβ TCR intraepithelial lymphocytes, maturation of IgA-containing B cells, expression of class II MHC antigens on epithelial cells, etc., was closely related with the presence of apathogenic intestinal microbes. Among these apathogenic microbes, chlorof orm-resistant flora, which mainly consist of clostridia and segmented filamentous bacteria (Basal flora: BF), are responsible for normalization of the gut immune system (26, 32). We have reported that the intestinal inflammation of BF-contaminated Balb/c mice induced by TNBS was less severe than that of SPF mice. These results were interesting, because our study indicated that BF is enough to mature gut immune systems during normalization; however, the stimuli produced by BF are not enough to cause gut inflammation (24). In other words, the stimuli that accelerate intestinal inflammation and those that mature intestinal immune systems are different. To test this possibility, we constructed various gnotobiotic scid mice and induced gut inflammation by injection with CD4+ αβ T cells from SAMP1/Yit. Small intestinal inflammation was mild in CD4+ αβ T cell-transferred BF-associated scid (BF scid) mice as with our previous results in hapten-induced colitis models. Next we introduced Bacteroides spp. that induce higher or low-proliferative responses to CD4+ αβ T cells of SAMP1/Yit mice into BF scid (HB-BF scid and LB-BF scid, respectively) and induced gut inflammation. We also constructed GB scid mice contaminated with Bacteroides spp. that induced a strong proliferative response to SAMP1 CD4+ αβ T cells only (HB-scid). CD-like gut inflammation could be observed both in HB-BF scid and in LB-BF scid, and histological disease scores were comparable between these two groups.
The scores are similar to those of SPF scid injected with CD4+ αβ T cells of SAMP1/Yit. In contrast to the histological disease scores, the lethality of these two groups is different. Induction of gut inflammation was highly lethal in HB-BF scid, but not in LB-BF scid. Interestingly, we could not detect any intestinal inflammation in recipients after the injection of SAMP1 CD4+ αβ T cells in HB-scid. Taken together, these results suggested that the BF flora initiate gut inflammation and Bacteroides determines the severity of the inflammation in SAMP1/Yit mice. Surprisingly, Bacteroides itself had no ability to initiate gut inflammation. So, the step-wise activation of gut immune systems by the stimuli produced in different enteric bacteria resulted in Crohn’s disease-like gut inflammation in SAMP1/Yit mice (Fig. 5). Moreover, abrogation of the tolerance of CD4+ αβ T cells to Bacteroides spp. determined the severity of the disease. The mechanism underlying the interaction between the activation of CD4+ αβ T cells and Bacteroides was unclear. What kinds of bacterial components in Bacteroides are responsible for the induction of CD4+ αβ T cell proliferation in SAMP1/Yit mice? Our preliminary data indicated that on elimination of LPS from the Bacteroides cell preparation, the activity of CD4+ αβ T cells against the bacterial components was unchanged (Matsumoto et al., unpublished data). Onderdonk et al. also suggested that intraperitoneal injection with Bacteroides-derived outer membrane protein components, but not LPS ones, accelerated polysaccharide-induced colitis in guinea pigs (27). Taken together, the non-LPS fraction of Bacteroides cell components may determine the acceleration of CD-like gut inflammation in SAMP1/Yit mice.

SAMP1/YIT MICE ARE A MODEL SYSTEM FOR RESEARCH INTO NEW BIOLOGICAL OR CHEMICAL THERAPEUTICS FOR CROHN’S DISEASE

There have been many clinical or basic attempts to develop a treatment for CD using biological agents targeting cytokines or cell adhesion molecules. Also reported was a therapeutic approach to the treatment of CD that targeted intestinal microflora such as the use of probiotics and prebiotics. Kosiewicz et al., found that treatment with anti-TNF-α antibody ameliorated intestinal inflammation in the SAMP1/Yit strain (15). It was also reported that the administration of antibodies against ICAM-1 and VCAM-1 or α4 integrin and ICAM-1 ameliorated disease in SAMP1/Yit mice (3). It was already reported that anti-TNF-α therapy and antisense oligonucleotide treatment against ICAM-1 produced positive clinical outcome in CD patients (11, 34). These results suggested that the SAMP1/Yit strain was a useful model for developing new biological or chemical therapeutics in CD.

PROBIOTICS IMPROVED THE INTESTINAL INFLAMMATION IN SAMP1/YIT MICE

We have stated that probiotics suppress the intestinal inflammation in SAMP1/Yit mice (22). Other studies revealed the same effect of probiotics in various IBD models (28). Moreover, a clinical trial of several probiotics in patients with IBD also showed positive results. However, the mechanism of the effect of probiotics is unclear. It may be due to the regulation of intestinal conditions or the regulation of the mucosal immune system. We recently obtained evidence in support of the latter hypothesis. We have already discussed the activation of the IL-6/Stat-3 pathway in the intestinal mucosa in IBD above. We discovered that Lactobacillus casei strain Shirota (LcS) and a polysaccharide-peptidoglycan complex (PSPG) that is a major constituent of the cell wall in gram-positive bacteria inhibit IL-6 production in LPS-stimulated lamina propria lymphocytes isolated from IBD mice and peripheral blood mononuclear cells from IBD patients. Moreover, ingestion of heat-killed LcS in SAMP1/Yit mice ameliorated intestinal inflammation (17). Therefore, immunomodulatory effects of the cellular components of certain probiotics could guide the host mucosal immune response toward anti-inflammation and may lead to the improvement of intestinal inflammation.

Acknowledgments. We thank Drs. Y. Umesaki and M. Nanno (Yakult Central Institute), and Drs. T. Hibi (Keio University School of Medicine) and K. Mitsuyama (Kurume University School of Medicine) for advice on the preparation of this manuscript. We also thank Misses H. Setoyama, N. Watanabe and T. Hara for their expert technical assistance. Mesdames H. Funabashi and Y. Okebe are also acknowledged for the breeding of SAMP1/Yit mice.

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