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Commensal Bacteria Exacerbate Intestinal Inflammation but Are Not Essential for the Development of Murine Ileitis

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The pathogenesis of Crohn’s disease has been associated with a dysregulated response of the mucosal immune system against intraluminal Ags of bacterial origin. In this study, we have investigated the effects of germfree (GF) conditions in the SAMP1/YitFc murine model of Crohn’s disease-like ileitis. We show that the bacterial flora is not essential for ileitis induction, because GF SAMP1/YitFc mice develop chronic ileitis. However, compared with disease in specific pathogen-free (SPF) mice, ileitis in GF mice is significantly attenuated, and is associated with delayed lymphocytic infiltration and defective mucosal expression of Th2 cytokines. In addition, we demonstrate that stimulation with purified fecal Ags from SPF, but not GF mice leads to the generation of IL-4-secreting effector lymphocytes. This result suggests that commensal bacteria drive Th2 responses characteristic of the chronic phase of SAMP1/YitFc ileitis. Finally, adoptive transfer of CD4-positive cells from GF, but not SPF mice induces severe colitis in SCID recipients. These effects were associated with a decreased frequency of CD4⁺CD25⁺Foxp₃⁺ T cells in the mesenteric lymph nodes of GF mice compared with SPF mice, as well as a lower relative gene expression of Foxp3 in CD4⁺CD25⁺ T cells in GF mice. It is therefore apparent that, in the absence of live intraluminal bacteria, the regulatory component of the mucosal immune system is compromised. All together, our results indicate that in SAMP1/YitFc mice, bacterial flora exacerbates intestinal inflammation, but is not essential for the generation of the chronic ileitis that is characteristic of these mice. The Journal of Immunology, 2007, 178: 1809–1818.

The intestinal tract consists of the largest compartment of the immune system in close proximity to the most extensive and varied bacterial population found in the body (1). The diverse nature of the gut-associated immune system enables it to constantly respond to bacterial Ags and products from the commensal flora (2). This reactivity is believed to underlie the “activated” immunophenotype of gut mucosal lymphocytes, often referred to as “physiological” inflammation (3). At the same time, several control mechanisms prevent injury of the bowel under normal conditions by minimizing the reactivity of effector cells, including the function(s) of regulatory lymphocytes and cytokines (4–6). It has been shown that both the effector and regulatory compartments of the mucosal immune system require the presence of bacterial flora for their proper development (7, 8).

Under pathological conditions, a dysregulated mucosal immune response to intraluminal Ags in genetically predisposed individuals may lead to chronic intestinal inflammation, such as is observed in Crohn’s disease (CD) in humans. This dysregulation may result either in an overly aggressive effector response or may represent a primary defect in the regulatory component of the mucosal immune system. Irrespective of the underlying defect, converging lines of evidence support the notion that the intraluminal Ags that act as the trigger are derived from the commensal bacterial flora (9). CD is primarily localized to the intestinal compartments that contain the highest concentration of bacteria. Manipulation of the bacterial flora with antibiotics or probiotics is beneficial in certain subsets of patients (9). In addition, cellular and humoral responses against common bacterial Ags have been observed in patients with CD (10–12). More compelling evidence for an association between CD and the bacterial flora comes from studies in animal models of colitis (13). In fact, several studies have shown that colitis does not usually develop when animals are kept under germfree (GF) conditions (13). These observations have led to a general acceptance of the hypothesis of “no bacteria, no gut inflammation.”

We have extensively characterized the SAMP1/YitFc (SAMP) mouse, a unique model of spontaneous intestinal inflammation that has many similarities to CD (14, 15). This mouse originates from the SAMP1/Yit parental strain, initially described by Matsumoto et al. (16). After extensive brother-sister mating at the University of Virginia, novel phenotypic characteristics emerged that resulted in the designation of a separate SAMP1/YitFc strain (15).

Similar to CD, disease in SAMP mice is localized to the terminal ileum, with histological features that include transmural, discontinuous and granulomatous inflammation, involving both active and chronic inflammatory elements. In addition, a subset of mice develops perianal manifestations. Ileitis in SAMP mice responds to administration of corticosteroids or Abs against TNF (17). The initiation of ileitis in SAMP involves an induction phase that precedes the histological injury and bears the characteristics of a Th1-mediated condition (18). In contrast, the maintenance phase that follows the development of chronic ileitis displays a mixed
Th1/Th2 phenotype. The role the bacterial flora plays in the pathogenesis of ileitis in SAMP mice has yet to be fully elucidated. Nevertheless, it appears that intestinal bacteria influence disease progression in SAMP mice, because treatment with broad-spectrum antibiotics prevents and ameliorates ileitis (19).

In this study, we report that bacterial flora is not essential for the establishment of disease in these mice. Nevertheless, commensal bacteria accelerate the progression and exacerbate the severity of ileitis. At the cellular level, we show that mucosal lymphocytes isolated from specific pathogen-free (SPF) mice proliferate and secrete cytokines in response to stimulation with fecal Ags (FA).

In addition, we demonstrate that bacterial flora may be critical for the development of mucosal Th2 responses. In contrast, SPF-derived FA were essential for the development of IL-4-secreting effectors lymphocytes. Finally, we provide evidence that regulatory pathways may be compromised in the absence of commensal flora, as evidenced by lower relative gene expression of Foxp3 in CD4+CD25+ T cells in GF mice compared with SPF mice, as well as a decreased frequency of CD4+CD25+Foxp3+ T cells in the mesenteric lymph node (MLNs) of GF mice.

Materials and Methods

Animals

SAMP and AKR mice were maintained under SPF conditions at the University of Virginia. SCID (C3HSmn.C-Ptkdscid/l) mice were purchased from The Jackson Laboratory. GF SAMP mice were generated and maintained at Taconic Farms as described previously (20). In brief, mice were aseptically derived from SAMP mothers by caesarian section and kept thereafter in a class 1 GF isolator. The isolator was tested each month in accordance with Taconic’s standard operating procedures for microbial monitoring of class 1 axenic animals. Sterility was confirmed by inoculation of fecal pellets into different culture media, including TSA with 5% sheep blood, Brucella agar (for anaerobes) and nutrient agar (for fungi). No aerobic or anaerobic bacterial contaminants have been found in the GF isolator since its build date the week of 24 February 2003. At the appropriate time points, GF mice were shipped in GF isolators to the animal facility at the University of Virginia. Upon arrival mice were kept overnight in the isolators. The following morning, mice were euthanized and their intestinal organs were removed, rinsed with PBS, fixed in 10% formalin solution, and processed for further studies. All protocols were approved by the Animal Care and Use Committee of the University of Virginia.

Adaptive transfer

CD4+ lymphocytes from the MLNs of SAMP mice (>13 wk old) were positively selected (purity >95%) on a CD4 column (Miltenyi Biotec). Adult (6–8 wk) MHC-matched SCID mice received 1 × 10^7 CD4+ cells per mouse by i.p. injection. Mice were tested weekly for weight loss and rectal prolapse. Five to 6 wk posttransfer, SCID mice were euthanized, and peritoneal exudates were collected for cellular and cytokine assays.

Intracellular cytokine staining

Cells were stimulated with FA for 2 days and then staining for intracellular cytokines was performed. Cells were harvested and submitted to short (6 h) stimulation with anti-CD3 (2 μg/ml; BD Biosciences), with or without the addition of FA in the cultures (primary stimulation). After 48 h, cells were recovered, washed, and expanded without stimulation in the presence of IL-2 (10 ng/ml). Cells were harvested and submitted to short (6 h) stimulation with anti-CD3 (2 μg/ml) and anti-CD28 (10 μg/ml; BD Biosciences), with or without the addition of FA in the cultures (primary stimulation). After 48 h, cells were harvested, washed, and expanded without stimulation in the presence of IL-2 (10 ng/ml). Cells were harvested and submitted to short (6 h) stimulation with anti-CD3 (2 μg/ml) and anti-CD28 (10 μg/ml), or PMA (50 ng/ml) and ionomycin (500 ng/ml) (secondary stimulation). The cells were used for intracellular staining and their supernatants were stored at −80°C.

Proliferation assay

APCs and effectector lymphocytes (10^3 of each) were cultured in triplicate for 72 h with the indicated stimulation. Cells were pulsed with [3H]thymidine (1 μCi/well) (MP Biomedicals) overnight and proliferation was estimated by measuring thymidine incorporation.

Cytokine measurement

The concentrations of TNF, IFN-γ, IL-4, IL-5, IL-13, and IL-10 in the supernatants were measured by multiplex cytokine array analysis. The Bioplex protein multianalyte assay was used and a mouse-plex assay was used according to the manufacturer’s recommendations (Bio-Rad). The samples were analyzed using the Bio-Plex 200 system (Bio-Rad Laboratories). A reference standard curve was generated by serial dilutions of a standard mixture of cytokines (BD Biosciences). Each cytokine was determined in triplicate.

Intracellular cytokine staining

Cells were stimulated, and then stained for intracellular cytokines using the Cytoset/CTyopein Plus kit (BD Biosciences) according to the manufacturer’s instructions. The concentrations of CD4+ lymphocytes expressing intracellular cytokines were determined by multiplex-color analyses, using a FACSAriaII system (BD Biosciences). All Abs were purchased from BD Pharmingen.

Flow cytometry

Isolated splenocytes or MLN cells in suspension (2 × 10^6 cells/ml) were labeled with 30-min incubation with the appropriate fluorochrome-tagged Abs against CD4 and CD25 (BD Pharmingen). Cells were washed and analyzed on a FACSCalibur (BD Biosciences) using the CellQuest software. Live cells were gated based on forward and side scatter.
with cold PBS to remove excess Ab. Before staining with Alexa Fluor 647 anti-Foxp3 Ab (BioLegend) at room temperature for 30 min, the cells were fixed and permeabilized with Fix/Perm solution (BioLegend) at room temperature for 20 min. Washing and staining were performed in Perm buffer (BioLegend). After gating for lymphocytes by forward and side scatter, the percentage of cells expressing Foxp3 was determined by three-color analysis using a FACSCalibur (BD Biosciences) and CellQuest software (BD Immunocytometry Systems).

Statistical analysis
Statistical analysis was performed using the Wilcoxon rank sum test for nonparametric data. An alpha level of 0.05 was considered significant (p < 0.05).

Results
Development of ileitis is attenuated in SAMP mice under GF conditions
To investigate the role played by bacterial flora in the development of ileitis in SAMP mice, a colony of GF SAMP mice was generated at Taconic Farms. Mice were aseptically derived from SAMP mothers by caesarian section and kept in a class 1 GF isolator. The isolator was tested each month in accordance with Taconic’s standard operating procedures for microbial monitoring of class 1 axenic isolators. Sterility was confirmed as described in detail in Materials and Methods. We then performed a time-course study during which we compared the histological appearance of the terminal ileum from AKR (negative control), SPF SAMP (positive control), and GF SAMP mice at various ages (Fig. 1A). In line with our previous observations (14, 15), SPF SAMP mice presented with severe ileitis by 10 wk of age, without significant fluctuation thereafter. To our surprise, GF SAMP mice also developed ileitis that was detected as early as 13 wk of age (Fig. 1A). The severity of ileitis was considerably lower in GF than in SPF mice at all time points. Indeed, the average TIS for GF mice at 10–20 wk and 20–30 wk was 45% of the TIS for SPF mice at the same time points (10–20 wk: 6.1 ± 1.2 vs 13.5 ± 1.4, p < 0.005; 20–30 wk: 6.2 ± 0.7 vs 13.9 ± 1.3, p < 0.005). After 30 wk of age, the average score for the GF group was 66% of the SPF group (>30 wk: 10.4 ± 2.5 vs 15.8 ± 2, NS).

The distribution of GF and SPF mice according to the severity of ileitis is depicted in Fig. 1B. Because ileitis in SAMP mice is first detected around 10 wk of age, the data are only presented for mice of that age and older. From these data, it can be seen that under SPF conditions the penetrance of ileitis in SAMP mice is 100%. Moreover, the majority of mice present with severe ileitis, because 70% have a TIS of >13. However, when SAMP mice were maintained under GF conditions, we observed several differences. First, no ileitis was observed in a substantial subset of mice (8 of 25, 32%), even at 40 wk of age (Fig. 1B).
Second, approximately half of the mice with histologically evident disease (8 of 17) developed only mild ileitis. Nevertheless, 36% of all the GF mice examined (9 of 25) developed disease of comparable severity to the SPF mice (moderate or severe).

We also studied the relative contribution of the active (polymorphonuclear) and chronic (lymphocytes and monocytes) inflammatory elements to the histological injury. In SPF mice, both active and chronic inflammation develop rapidly and are fully established by 10 wk of age (data not shown). In contrast, in GF mice the polymorphonuclear component appears to develop faster, with an active inflammatory index that is 46% of that in SPF mice at 10–20 wk, 57% by 20–30 wk, and reaches 63% of the severity in SPF mice by 30 wk. In comparison, the chronic inflammatory...
The histological characteristics of ileitis in GF SAMP mice were similar to those previously described in SPF mice (14) (Fig. 1C). No inflammatory changes were detected in the jejunum of either GF or SPF SAMP mice (Fig. 1C). Mild inflammation was occasionally seen in the colon of GF SAMP mice.

Ags derived from the bacterial flora are essential for the generation of mucosal Th2 responses

We have recently shown that the chronic phase of ileitis in SAMP mice is characterized by a marked up-regulation of mucosal Th2 responses, including higher ileal expression of IL-5 and IL-13, and an elevated ratio of GATA3:T-bet (18). We hypothesized that the bacterial flora provides the immunostimulatory signals for the up-regulation of mucosal Th2-type responses in SAMP mice. To test our hypothesis, we first compared the expression of the mRNAs for IL-5 and IL-13 in the terminal ileum of GF and SPF SAMP mice (Fig. 2A). Th2 responses were dramatically suppressed in GF mice, with significant decreases in the ileal expression of the mRNAs for IL-5 (25% of the average expression in age-matched SPF controls; \( p < 0.0005 \)) and IL-13 (26%; \( p < 0.005 \)). This defect in the up-regulation of Th2 responses under GF conditions was confirmed by the significant decrease in the GATA3:T-bet ratio in GF SAMP mice compared with SPF controls (\( p < 0.001 \)). In contrast, no considerable differences were seen in the expression of Th1 cytokines, such as IFN-\( \gamma \) (data not shown).

These data indicate that, in the absence of bacterial flora, the mucosal immune system is unable to mount a vigorous Th2 effector response in SAMP mice. This inability may underlie the decreased severity of inflammation under GF conditions. We therefore hypothesized that Ags derived from fecal material containing commensal bacteria (i.e., from SPF mice) may be able to induce Th2 responses. To test our hypothesis, we conducted long-term cultures of lymphocytes from SPF SAMP mice with or without stimulation with SPF-FA. We used splenocytes as the effector population, because they contain high numbers of naive lymphocytes and do not produce significant amounts of Th2-type cytokines when nonspecifically stimulated (data not shown). A primary stimulation (with or without SPF-FA) was applied, which was followed by expansion with IL-2. Finally, cells were subjected to a short stimulation with anti-CD3/anti-CD28 or PMA/ionomycin. The type and number of cytokine-secreting lymphocytes that were generated in the primary cultures were detected by intracellular cytokine staining.

When nonspecific stimulation was applied, the default pathway in both primary and secondary cultures was a Th1-type response, with large numbers of IFN-\( \gamma \)-positive, but not of IL-4-positive
lymphocytes (Fig. 2B). In sharp contrast, when SPF-FA were added to the primary cultures, we detected a distinct population of IL-4-positive lymphocytes in addition to the IFN-γ-positive population, indicating a mixed Th1/Th2 secondary response (Fig. 2B). Primary stimulation with FA resulted in a significant increase in the number of IL-4-positive cells compared with primary stimulation in the absence of FA, irrespective of the nature of the secondary stimulation (15 ± 1 vs 5 ± 2% for anti-CD3/anti-CD28 and 8 ± 1 vs 3 ± 1% for PMA/ionomycin) (Fig. 2C). To further confirm that Th2 responses are induced by FA, we measured the

**FIGURE 4.** Severity of ileitis induced in SCID mice by the transfer of lymphocytes from SAMP mice. A, H&E-stained sections depict the histological characteristics of the terminal ileum of SCID mice that were injected with 1 × 10⁵ CD4⁺ cells from the MLNs of SPF or GF SAMP donors. Severe ileitis is observed in recipients of SPF donors, whereas ileitis was either not observed or was mild in recipients of GF donors. B, Indices for villus distortion, active and chronic inflammation, and the TIS were calculated as described in Materials and Methods. Data are expressed as the mean ± SEM. n = 9 for recipients of SPF donor cells and n = 6 for recipients of GF donor cells.

**FIGURE 5.** Severity of colitis induced in SCID mice by the transfer of CD4⁺ lymphocytes from SPF or GF SAMP mice. A–C, SCID mice that had received donor cells from GF SAMP mice showed frequent rectal prolapse, significantly decreased weight loss, and shortened and thickened coli. D, H&E-stained sections depict the histological characteristics of the colon of SCID mice that were injected with 1 × 10⁵ CD4⁺ cells from the MLN of SPF or GF SAMP donors. Severe colitis is observed in recipients of GF donors, whereas mild colitis was induced in SCID mice that had received cells from SPF donors. E, Indices for transmural inflammation, active and chronic inflammation, and the TIS were calculated as described in Materials and Methods. Data are expressed as the mean ± SEM. n = 9 for recipients of SPF donor cells and n = 6 for recipients of GF donor cells.
Interestingly, stimulation of CD4⁺B response to SPF-FA but not GF-FA (Fig. 3).Addition to proliferation, stimulation of mucosal CD4⁺ recognize Ags that originate from live intraluminal bacteria. In vitro studies, freshly isolated MLN lymphocytes secreted a mixed pattern of Th1/Th2 cytokines when stimulated with FA. The most profound increases were in IFN-γ (Fig. 2A). Mucosal lymphocytes from SAMP mice show immunoreactivity against Ags from commensal bacteria. We then hypothesized that immunoreactivity against FA takes place in SAMP mice in vivo. To test our hypothesis, we studied the responses of freshly isolated mucosal (i.e., MLN) CD4⁺ cells to FA presented by APCs. We also investigated the responses to FA from GF mice (i.e., in the absence of commensal bacteria). Our study showed that CD4⁺ cells from SAMP mice proliferated in response to SPF-FA but not GF-FA (Fig. 3A), indicating that they recognize Ags that originate from live intraluminal bacteria. In addition to proliferation, stimulation of mucosal CD4⁺ cells with SPF-FA led to increased cytokine secretion. In line with our in vitro studies, freshly isolated MLN lymphocytes secreted a mixed pattern of Th1/Th2 cytokines when stimulated with FA. The most profound increases were in IFN-γ (202% increase; p = 0.041), IL-5 (441% increase; p = 0.0043), and IL-13 (191% increase; p = 0.065) (Fig. 3B). Interestingly, stimulation of CD4⁺ cells with GF-FA also resulted in some degree of cytokine production in our studies, but to a lesser extent compared with SPF mice. Adaptive transfer of GF CD4⁺ lymphocytes induces severe colitis in SCID recipient mice

We have previously demonstrated that injection of CD4⁺ lymphocytes from the MLNs of SAMP into MHC class II-matched SCID mice induces severe ileitis but mild colitis in the recipient mice (14). In this study, we used this adoptive transfer system to directly test the effects of GF conditions on the immunological properties of the mucosal lymphocytes. We transferred CD4⁺ cells from either SPF or GF SAMP donors and compared the effects on the induction of intestinal inflammation in SCID recipients.

The capacity of CD4⁺ lymphocytes from the MLNs of GF SAMP mice to induce ileitis in SCID recipients was significantly decreased (Fig. 4A). SCID mice that received cells from GF donors displayed decreased indices for both active (0.3 ± 0.2 vs 2.7 ± 0.8 in SPF transferred cells) and chronic inflammation (0.5 ± 0.5 vs 2.1 ± 0.8) (Fig. 4B). In fact, there were signs of active inflammation in only two of six (26%) SCID recipients of GF donors, whereas only one of six (13%) had chronic inflammatory cells in the small intestinal lamina propria. Overall, the TIS was significantly lower in the group that received cells from GF donors (4.8 ± 0.7 vs 9 ± 1.4, 50% decrease; p = 0.0082) (Fig. 4B).

In contrast to their inability to induce substantial ileitis, CD4⁺ cells from GF mice induced severe colitis in the recipient mice. By 5 wk after the transfer, all mice that had received cells from GF donors showed clinical signs of severe colitis. In particular, all recipients of GF donor cells (100%) had marked rectal prolapse, in comparison with only three of nine mice (33%) that received cells from SPF donors (Fig. 5A). Mice that received cells from GF donors suffered from significantly greater weight loss, a marker of severe colonic inflammation, compared with the SPF recipient group (Fig. 5B). There was also increased incidence of shortening and thickening of the large bowel in the recipients of GF donor cells (Fig. 5C). At the histological level, SCID mice that received cells from GF mice showed increases in all parameters of inflammation (transmural infiltration, GF recipients: 3.3 ± 0.3, SPF: 1.6 ± 0.4, p < 0.05; active inflammation, GF: 4.4 ± 0.3, SPF: 2.7 ± 0.7, p = 0.09; chronic inflammation, GF: 4.4 ± 0.3, SPF: 2.7 ± 0.5, p < 0.05) (Fig. 5D). Overall, the TIS was significantly higher in the group that...
Regulatory mucosal pathways are defective in GF SAMP mice

The colitis that develops in the adoptive transfer model has traditionally been linked to an imbalance between pathogenic pro-inflammatory factors and disease-preventing regulatory elements. The latter include regulatory lymphocytes, as well as cytokines such as IL-10 and TGF-β1. We therefore hypothesized that the induction of severe colitis after adoptive transfer of CD4+ cells from GF donors could result from a defect in the T regulatory (Treg) cell population and their ability to secrete regulatory cytokines. To test this hypothesis, we first compared the secretion of IL-10 after stimulation with FA originating from GF or SPF mice. Our results (Fig. 6) clearly show that stimulation with SPF-FA induced secretion of IL-10 (814 ± 248 pg/ml) at levels that were significantly higher than both the levels at baseline (101 ± 41 pg/ml; p < 0.05) and the levels secreted upon stimulation with GF-FA (187 ± 90 pg/ml; p < 0.05). In fact, stimulation with GF-FA did not induce any significant increase in IL-10 secretion over baseline.

Secondly, we analyzed cells from the spleens and MLNs of AKR, and GF and SPF SAMP mice for the Treg cell markers CD25+ and the Foxp3 protein. The data presented in Fig. 7 show that a lower proportion of CD4+CD25+ cells in the MLNs of GF mice express intracellular Foxp3 protein (74.4%) compared with the MLNs of AKR (89.4%) and SPF SAMP mice (93.4%; Fig. 7A). In addition, CD4+CD25+ cells isolated from the MLNs of GF mice showed a significantly lower relative expression of Foxp3 mRNA compared with the MLNs of SPF mice (p < 0.01; Fig. 8A). These effects appear to be specific to the MLN compartment, because no differences were observed in the proportion of CD4+CD25+Foxp3 cells isolated from splenocyte populations of GF SAMP (89.7%) compared with AKR (84%) and SPF SAMP mice (89.3%; Fig. 7B). Similarly, no differences in Foxp3 mRNA expression were observed in CD4+ splenocytes obtained from GF and SPF SAMP mice (Fig. 8B).

Discussion

The currently accepted hypothesis in mucosal immunology is that commensal bacteria are essential for the development of chronic intestinal inflammation. This concept is primarily supported by the consistent absence of intestinal inflammation in animal models of colitis when the bacterial flora is completely removed (13, 21). In contrast, this study demonstrates that chronic ileitis can develop in SAMP mice under GF conditions. Indeed, the ileitis that developed in GF SAMP mice was histologically similar to the disease seen in SPF animals. This indicates that the development of intestinal inflammation under GF conditions is a true characteristic of this mouse strain, and not a nonspecific effect of the GF status. This is further supported by the absence of ileitis in normal C57BL/6 mice that were raised GF in the same facility (data not shown), and the fact that no significant inflammatory changes were observed in other intestinal regions of GF SAMP mice. Nevertheless, our studies confirm the important role played by the commensal flora in triggering and exacerbating chronic intestinal inflammation in this model.

The differences observed in SAMP mice compared with other models of colitis that do not develop disease in GF conditions were unexpected. Nonetheless, this phenomenon may be explained by the specific immunogenetic characteristics of the SAMP mouse (22). First, SAMP mice present with terminal ileitis rather than colitis. The ileum contains the most developed compartment of the mucosal immune system, and may therefore display a lower threshold for immunological activation compared with the colon, where stimulation by live bacteria appears to be indispensable for the expression of colitis. Second, experimental colitis results from single causative factors, such as administration of chemicals, deletion or insertion of specific genes, or manipulation of particular cell types. Such single pathway manipulations may easily be inactive in the absence of the immunostimulation provided by the bacterial flora. In contrast, ileitis in SAMP mice is truly spontaneous and represents a polygenic trait (23). In addition, there are multiple and complex immunological abnormalities, which may vary in their dependence on the presence of commensal flora. Indeed, several of the findings from the current study support this hypothesis. Active neutrophilic inflammation develops earlier in GF SAMP mice, whereas the chronic lymphocytic response was significantly delayed. In addition, there was selective suppression of the intestinal Th2 cytokines IL-5 and IL-13, whereas no effect was seen on others, i.e., IFN-γ. Finally, we have recently shown that SAMP mice display an epithelial cell defect that occurs before the development of histological injury (24). Because this defect is also present in GF mice (25), it therefore occurs independently of colonization by commensal flora.

Our findings differ from the original description of the Japanese SAMP1/Yit strain, which did not appear to develop ileitis under GF conditions (16). It is possible that the relative independence of ileitis from the bacterial flora may be among the novel phenotypic features of the SAMP1 substrain at the University of Virginia. The presence of perianal manifestations in the United States but not the Japanese SAMP1/Yit mice is a good example of major phenotypic changes that can occur even between closely related substrains. A second explanation could be that the GF Japanese mice were not observed for a sufficient length of time to detect the presence of ileitis. Given the fact that disease in the Japanese strain develops later than in the SAMP1 substrain, and that ileitis in GF mice is
later in onset and slower to progress, it is possible that inflammation might also have been observed if the Japanese strain raised under GF conditions had been permitted to reach an older age.

Two hypotheses may explain the presence of ileitis in GF SAMP mice. One possibility is that disease is caused by bacterial-independent, autoimmune reactivity against small intestinal Ags. The acceleration of disease under SPF conditions could then result from a generalized nonspecific immune stimulation in the presence of colonization by bacterial flora. An alternative explanation is that the sterile diet fed to GF mice may nonetheless contain dead bacteria and bacterial proteins, in addition to food- and epithelium-derived Ags (26). Indeed, cytokine production was detected against fecal extracts from GF mice, although to a significantly lower level than against SPF extracts. Therefore, the low amounts of antigenic material present in sterilized food pellets in combination with the significant defect in intestinal permeability characteristic of SAMP mice (25) may be sufficient to induce the attenuated immune response that results in low-grade intestinal inflammation in GF SAMP mice. Nevertheless, severe disease occurs consistently only in the presence of an intact bacterial flora that provides large quantities and variable specificities of Ags. Our laboratory is currently engaged in actively pursuing both these hypotheses.

Our results emphasize the importance of the commensal flora in triggering and exacerbating chronic intestinal inflammation. Bacterial-dependent and -independent mechanisms therefore coexist during the course of ileitis in SAMP mice. First, the presence of intraluminal bacteria under SPF conditions significantly accelerates the inflammatory process and results in even severe disease. Second, mucosal lymphocytes from the MLNs of SAMP mice proliferate and produce cytokines after stimulation with SPF-FA. Such immunobacterial interactions are expected in vivo, because SAMP mice display an early epithelial cell defect (24, 25) that allows for a constant cross-talk between commensal bacteria and the gut immune system, which is in line with previous studies from both humans and mice (7, 10, 27). Third, our results indicate that different components of the mucosal immune system have unequal dependence on the presence of commensal bacteria. Th2 effector pathways clearly require the presence of intact flora, because IL-5, IL-13, and GATA3 are not up-regulated in the mucosa of GF SAMP mice. In addition, IL-4-secreting lymphocytes were only generated in response to stimulation with SPF-FA. Therefore, the regulatory mechanisms appear to be dependent on the presence of bacterial flora.

Our results provide additional information regarding the development of the gut-associated immune system. Indeed, data presented in this study point to the presence of a default Th1 pathway within the intestinal mucosa that exists irrespective of the presence of bacteria. Upon colonization, this default pathway may be of critical importance for the rapid and effective clearance of pathogenic microorganisms. Moreover, Th2 and regulatory responses may primarily function to prevent an uncontrolled expansion of Th1 responses, and a bystander injury to the bowel. Because such an expansion would occur in the constant presence of immunostimulatory flora, it is not surprising that the regulatory mechanisms are also driven by Ags of bacterial origin. Our observation of a significant decrease in Tregs and IL-10 production from MLNs of GF mice strongly supports this concept.

It is generally accepted that the development of CD requires breakdown of the mechanisms that are responsible for tolerance against intestinal flora. Mucosal lymphocytes from patients with CD showed increased reactivity against antigenic material that originated from the commensal flora (10–12, 30). Patients with CD could be divided into two separate groups depending on whether or not they had circulating anti-bacterial Abs (31). This difference in immunophenotype appears to have clinical and therapeutic implications. In one study, patients with serum reactivity against multiple bacterial Ags suffered from more aggressive disease with frequent localization to the small intestine, a fibrotic and perforating phenotype, and increased need for surgery (30, 32). Subsequent studies also reported that patients in the “high reactivity” group responded well to antimicrobial therapy but less so to steroids, whereas the opposite situation was observed for the group with no bacterial reactivity (33). These studies indicate that there may be bacterial-dependent and bacterial-independent pathways in the human condition as well. Given that perspective, the data presented in this study raise the possibility that the SAMP murine model of ileitis may be representative of a specific subgroup of patients with CD localized to the ileum, in whom bacterial-dependent mechanisms are less critical for the development of chronic intestinal inflammation.

In conclusion, we describe for the first time the development of spontaneous, chronic small intestinal inflammation in the complete absence of colonization by commensal flora. This model offers a unique opportunity to study and dissect bacterial-dependent and bacterial-independent components of chronic intestinal inflammation, as well as providing a context to address local (ileum vs colon) issues controlling homing, regulation, and microbial impact on disease susceptibility.
ROLE OF COMMENSAL FLORA IN CHRONIC ILEITIS

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