Brief report

Probiotic effects on experimental graft-versus-host disease: let them eat yogurt

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Acute graft-versus-host disease (aGVHD) often limits feasibility and outcome of allogeneic bone marrow transplantation. Current pathophysiologic concepts of aGVHD involve conditioning regimens, donor-derived T cells, proinflammatory cytokines, and bacterial lipopolysaccharide (LPS) as a major trigger for aGVHD. LPS derives mostly from gram-negative bacteria and can enter circulation through the impaired mucosal barrier after the conditioning regimen. Probiotic microorganisms have been shown to alter the composition of the intestinal microflora and thereby mediate anti-inflammatory effects. We hypothesized that modifying the enteric flora using the probiotic microorganism *Lactobacillus rhamnosus* GG, would ameliorate aGVHD. Here we show that oral administration of *Lactobacillus rhamnosus* GG before and after transplantation results in improved survival and reduced aGVHD. Furthermore, subculturing of mesenteric lymph node tissue revealed a reduced translocation of enteric bacteria. Our findings suggest that alteration of the intestinal microflora plays an important role in the initiation of experimental aGVHD. (Blood. 2004;103: 4365-4367)

Introduction

Acute graft-versus-host disease (aGVHD) remains one of the major obstacles in allogeneic bone marrow transplantation (BMT). Despite the development of potent immunosuppressive drugs and reduction of conditioning regimens, a high percentage of patients develop aGVHD, resulting in a high mortality after transplantation. Bacterial lipopolysaccharide (LPS) is considered a major player in the development of aGVHD.[1-3] Hence, bowel decontamination using broad-spectrum antibiotics prior to transplantation has been introduced as standard practice to date.[4-6] In experimental studies, the proinflammatory potency of LPS varies from bacterial species to species. However, the clinical impact of different intestinal microorganisms on aGVHD is still unclear.

Chronic inflammatory bowel disease (IBD) appears to be the result of a genetically determined unbalanced immune response to ubiquitous luminal bacterial antigens.[7] Probiotic bacteria, mainly *lactobacilli* and *bifidobacteria,* are defined as living microbes, which confer benefits to the host. There is increasing evidence that probiotic therapy is effective in the treatment of IBD.[8] However, the mechanisms by which these bacteria mediate their effects are still unclear. It has been shown that probiotic bacteria have a temporary impact on the composition of the intestinal flora, but a direct influence on the immune system has also been suggested.[9-11] To address the impact of modulation of the bowel flora on aGVHD, we used a well-described murine transplantation model in which aGVHD is induced across a haploidentical major histocompatibility complex (MHC) mismatch. The model is characterized by severe damage of the bowel mucosa, high-serum LPS levels after transplantation, and strong release of proinflammatory cytokines.[12-14]

Study design

Mice, BMT, assessment of GVHD, and treatment protocols

C57BL/6 and B6D2F1 mice (8 to 12 weeks old) were purchased from Charles River Laboratories (Sulzbach, Germany). The BMT protocol has been described previously.[12,13] Mice received either *Lactobacillus rhamnosus* GG or ciprofloxacin (50 mg/kg body weight [BW]) alone in drinking water from day 7 prior to transplantation and throughout the posttransplantation period, or ciprofloxacin for 7 days, followed by *L rhamnosus* GG for the posttransplantation period, or autoclaved drinking water ad libitum. The severity of GVHD was assessed by a previously described scoring system.[1,13] Survival was assessed daily. Owing to German animal protection laws and local protocols by the committee, animals showing a GVHD score higher than 6 were killed and added to the Kaplan-Meier statistic the same day.

Flow cytometric analysis

Splenic cells were washed in ice-cold phosphate-buffered saline (PBS)/5% fetal calf serum (FCS) and incubated with 10% supernatant from clone 2.4G2 to block Fcγ receptors for 30 minutes. Fluorescein isothiocyanate (FITC)–labeled anti-CD3 antibody (clone 145-2c11) was incubated for 30 minutes on ice. Cells were washed twice in cold PBS/FCS and subsequently analyzed by flow cytometry (FACScan; Becton Dickinson, Heidelberg, Germany).

Cytokine analysis

Culture supernatant levels of tumor necrosis factor–α (TNF-α) and interferon-γ (IFN-γ) were determined by means of commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol.

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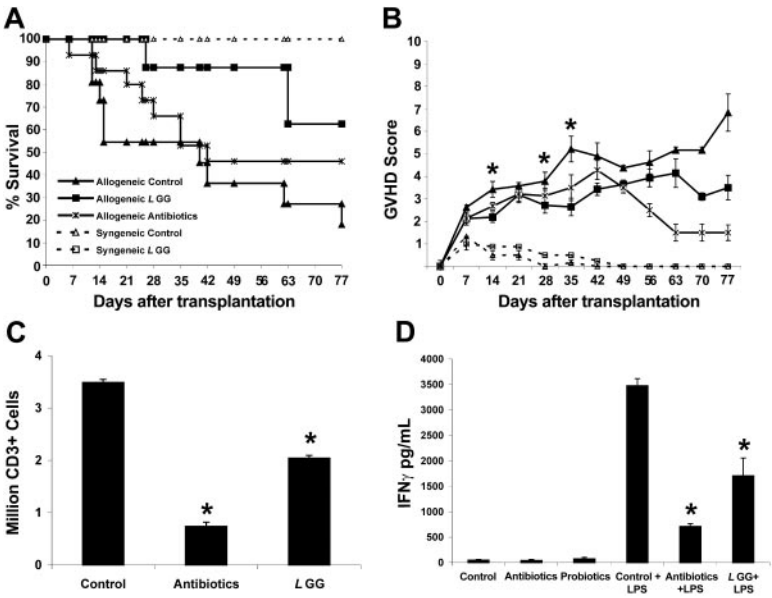

Figure 1. Survival, GVHD, and cytokines. (A-B) Survival (A) and GVHD scoring (B) after experimental allogeneic bone marrow transplantation. Animals received transplants after 2 × 600 cGy total body irradiation and were scored for GVHD as described.[1,2] Animals received autoclaved water containing probiotic lactobacteria (*L rhamnosus* GG) or autoclaved water alone. Animals receiving probiotics displayed reduced mortality during the early phase after transplantation and thereafter (12 to 14 animals per group, $P < .01$). At the same time, GVHD was significantly reduced in animals receiving *L rhamnosus* GG after transplantation (*$P < .02$, control versus *L rhamnosus* GG). (C) Total numbers of $CD3^+$ spleen cells were also significantly reduced in mice receiving *L rhamnosus* GG (*$P < .02$ compared with control treated mice, Mann-Whitney test). (D) Cytokine release by splenic cells at day 13 after allogeneic bone marrow transplantation. Cells from 4 individual mice were plated at 200 000 cells per well. Some cells were stimulated with 100 ng LPS per milliliter. Culture supernatants were harvested after 24 hours of culture and immediately analyzed for cytokines. Upon LPS stimulation, splenic cells from animals treated with *L rhamnosus* GG displayed a lower release of IFN-γ (*$P = .02$, control plus LPS versus *L rhamnosus* GG plus LPS, and antibiotics plus LPS versus control plus LPS).

Histologic grading of bowel inflammation

Intestinal tissue was cut into 5-μm sections and stained with hematoxylin and eosin (H&E). The sections were scored in blinded fashion by 2 investigators (H. Rath and M.S.) for histologic evidence of inflammation (influx of inflammatory cells, mucosal edema, destruction of crypt architecture, ulcerations, and abscesses), as described previously.[15]

Microbiological analysis of bacterial translocation

Mesenteric lymph nodes from mice that had received transplants were removed aseptically and homogenized in 200 μL sterile saline 0.9%. Then, 100 μL was cultured aerobically on blood agar and deMan-Rogosa-Sharp agar plates (Difco, Detroit, MI) for 24 hours in room air supplemented with 10% CO_2; colony-forming units were counted and numbers adjusted to weight.

Results and discussion

Probiotic microorganisms have recently received much scientific attention owing to reports of beneficial effects in the treatment of various chronic intestinal inflammatory conditions.[8] Intestinal manifestation of aGVHD is common and may even be considered the first onset of aGVHD.[16] Therefore, the aim of this study was to analyze the impact of alteration of the intestinal microflora by the probiotic microorganism *L rhamnosus* GG on the initiation of experimental aGVHD. Treatment of recipient mice with *L rhamnosus* GG significantly reduced mortality ($P < .01$) after transplantation (Figure 1A). The reduction of mortality was more pronounced in the early phase after transplantation between days 7 and 14. Animals treated with ciprofloxacin from day −14 until the end of the experiments showed a delay in early mortality, but eventually were not significantly different from animals receiving autoclaved drinking water alone. Furthermore, animals that had had transplants and were receiving *L rhamnosus* GG displayed a significantly reduced GVHD score (Figure 1B). GVHD of ciprofloxacin-treated recipients was also reduced on days 14, 28, and 35, but this difference was not significant. Sequential administration of ciprofloxacin from days −14 to −7 followed by *L rhamnosus* GG did not show a synergistic effect (data not shown). Combination of both was not performed owing to sensitivity of *L rhamnosus* GG against ciprofloxacin. B6D2F1 mice receiving syngeneic transplants did not show any mortality in any experiment performed in this study. The GVHD scoring system used focuses on 5 parameters: fur, mobility, posture, skin integrity, and weight loss. Analyzing the subgroups, we observed that reduction in weight loss accounted for most of the GVHD score improvement but also that skin integrity was improved in animals receiving *L rhamnosus* GG. Spleens from control treated mice contained more $CD3^+$ cells on day 13 when compared with animals receiving *L rhamnosus* GG (3.49 ± 0.06 versus 2.04 ± 0.05 million cells), indicating a stronger T-cell proliferation in control-treated mice (Figure 1C). This was confirmed by a lower spontaneous release of IFN-γ by cultured splenocytes from *L rhamnosus* GG–treated mice after LPS stimulation (Figure 1D). Since LPS is known to trigger aGVHD, we analyzed bacterial translocation into mesenteric lymph nodes (MLNs) as a sign of an affected mucosal barrier function due to intestinal inflammation. The reduction in early mortality and aGVHD in animals treated with *L rhamnosus* GG was accompanied by a lower concentration of translocated microorganisms into MLN (Figure 2A) (number of colonies, 25.0 ± 8.1 versus 6.2 ± 1.5, $P = .07$). However, the reduction of bacterial translocation did not result in reduced serum LPS levels (data not shown), indicating that reduction in bacterial translocation rather than lower LPS levels

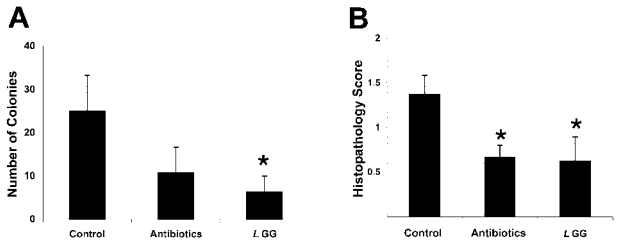

Figure 2. Bacterial translocation and gut histopathology. (A) Number of colonies from mesenteric lymph nodes at day 13 after allogeneic transplantation. Lymph nodes were harvested from 4 mice receiving allogeneic transplants, homogenized, and plated on solid bacteria growth media. Number of colonies were counted after 24 hours (*$P = .07$, control versus *L rhamnosus* GG, Mann-Whitney test). (B) Histopathology score of terminal ileum at day 22 after allogeneic transplantation. Animals receiving antibiotics (4 animals per group) (*$P < .04$, Mann-Whitney test) or *L rhamnosus* GG (*$P < .1$, Mann-Whitney test) displayed reduction in histopathology when compared with animals receiving autoclaved drinking water only.

accounted for the effects. Our observations were further supported by reduced bowel inflammation. Histologic inflammation in the terminal ileum, a major site of GVHD, was reduced in animals receiving *L rhamnosus* GG and was comparable to that of animals receiving ciprofloxacin, as indicated by a lesser influx of inflammatory cells and no evidence of ulcerations and abscesses. Protection of the mucosal barrier was previously demonstrated to result in a lower incidence of GVHD and therefore reduced mortality after transplantation.[12,14] The authors concluded that lower LPS serum levels account for the reduced GVHD since LPS represents an enormous stimulus in the development of GVHD. We did not observe lower LPS levels, but did notice reduced penetration of bacteria to MLN. This would support the hypothetic role of direct T-cell stimulation in MLNs and local toxin production.[6,17,18] To our knowledge, this is the first report to show that lower bacterial penetration affects GVHD and survival postexperimental transplantation.

From our results, we can speculate that intestinal microflora, direct interaction of bacteria with the adjacent lymphoid tissue, and, especially, an intact intestinal barrier seem to play major roles in the development of aGVHD.[12,13,16] Further experimental, but also clinical, studies are necessary to explore and confirm any beneficial effects of probiotics after transplantation.

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