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Effects of Orally Administered Viable *Lactobacillus rhamnosus* GG and *Propionibacterium freudenreichii* subsp. *shermanii* JS on Mouse Lymphocyte Proliferation

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Immunomodulation by probiotics is a subject of growing interest, but the knowledge of dose response and time profile relationships is minimal. In this study we examined the effects of *Lactobacillus rhamnosus* GG (LGG) and *Propionibacterium freudenreichii* subsp. *shermanii* JS (PJS) on the proliferative activity of murine lymphocytes ex vivo. Dose dependency was assessed by treating animals perorally with a low or a high dose (i.e., 10^9 or 10^{12} viable bacteria/kg of body weight) for 7 days. The lower dose levels of each strain appeared to enhance T-cell proliferation at the optimal concanavalin A (ConA) concentration (by 69 to 84%) and B-cell proliferation at the optimal and supraoptimal concentrations of lipopolysaccharide (by 57 to 82%). B-cell proliferation was also enhanced by the high LGG dose (by 32 to 39%) but was accompanied by a marginal decrease in T-cell proliferation (by 8%) at the optimal ConA concentration. The time profiles of the immune responses were assessed after daily treatment with the higher dose for 3, 7, and 14 days. A significant decrease in basal lymphoproliferation (by 32 to 42%) was observed with PJS treatment after the 3- and 7-day periods; however, this activity returned to control levels after 14 days of treatment, which also resulted in significantly enhanced T-cell proliferation at optimal and supraoptimal ConA concentrations (by 24 to 80%). The 14-day LGG treatment also enhanced the latter activity (by 119%). In conclusion, LGG and PJS have specific dose- and duration-dependent immunomodulatory effects on the proliferative activity of B and T lymphocytes and may also reduce lymphocyte sensitivity to the cytotoxic effects of lectin mitogens.

Immunomodulation in humans and animals via the oral route can be desirable because of the convenience and low cost of such treatments. Oral immunization normally requires large quantities of antigen and may also induce systemic tolerance to the orally administered soluble protein antigens. Microorganisms are known to generally induce greater mucosal immunity than soluble antigens and therefore have greater potential for immunomodulation (23).

Among the most potent and most-studied bacterial immunomodulators are cutaneous propionibacteria, which are part of the normal flora of the human skin and thus possess low antigenicity and rarely cause serious side effects (7, 19). Since 1964, propionibacteria have been used in humans for immunostimulation in oncology and the treatment of infectious diseases (8). Conflicting findings have resulted from these studies, suggesting a need for further clinical investigations (7, 8, 19). Cutaneous propionibacteria are also known to be involved in opportunistic infections and may carry virulence factors such as hyaluronidase (7). In addition, much of the available information on the immunological effects of propionibacteria has been based on results obtained via parenteral administration and cannot be extrapolated to predict effects after oral administration. Parenteral administration has been shown to induce side effects such as hepato- and splenomegaly, tissue injury in the liver and skin, polyarthritis, and pyrogenicity (2, 3, 11, 13). Therefore, the potential side effects of cutaneous propionibacteria are limiting the possible therapeutic applications of these strains.

Issues of safety have recently focused particular interest on the use of probiotics, i.e., those microbial feed or food supplements which have a beneficial influence on the host (18). Various immune responses to probiotics have been reported, and the immunomodulatory properties of these microorganisms have been proposed for several potential applications. These include prevention of infectious diarrhea, regulation of bowel movements, enhancement of the immune system, treatment of hypersensitivity reactions, tumor suppression, and as a vehicle for vaccine delivery (13, 16). At present, the paucity of knowledge concerning the direct comparisons between the immunomodulatory effects of various probiotic strains and different dosage schedules is limiting the number of controlled clinical trials with these microorganisms (13, 14).

In this study, we assessed the immunomodulatory effects of orally administered viable dietary bacteria on the in vitro proliferative activity of murine splenic lymphocytes and examined the influence of various dosage regimens. Lymphocytes were cultured without stimulants (spontaneous lymphoproliferation) and in cultures with concanavalin A (ConA), lipopolysaccharide (LPS), or allogeneic immune cells. These bacterial strains were *Lactobacillus rhamnosus* GG (LGG) and *Propionibacterium freudenreichii* subsp. *shermanii* JS (PJS). LGG is commonly used in fermented milks and drinks and is one of the best-studied probiotic bacteria (20). PJS is a propionibacterium strain used in cheese production and thus, unlike its cutaneous counterparts, has a long safe history in human nutrition, although little information is available on its health effects (7).

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MATERIALS AND METHODS

Animals. Male Swiss albino and C3H/HeJ mice (Monash University, Clayton, Victoria, Australia), 8 to 10 weeks old, were housed in plastic cages in an air-conditioned room and given free access to food and water. This study was performed under the approval of RMIT Animal Experimentation Ethics Committee Project no. 9608.

Bacterial strains and culture conditions. The bacterial strains used in this study were obtained from Valio, Ltd. (Helsinki, Finland). Mice received either a low or a high dose, i.e., 10^9 or 10^{12} viable bacteria/kg (body weight)/day of either LGG or PJS. For the lower dose, bacteria were grown with an inoculum. LGG was grown in de Man, Rogosa, and Sharpe broth (MRS; Oxoid, Hampshire, United Kingdom) for approximately 24 h at 37°C, and PJS was grown in YEL broth (12) in an anaerobic chamber for 3 to 4 days. After cultivation, bacteria were harvested by centrifugation (LGG, 2,000 × *g* for 5 min; PJS, 2,500 × *g* for 10 min) and washed three times with sterile saline. To achieve the higher dose, a suspension of lyophilized bacteria was used. For assessment of the number of viable bacteria, a sample of each suspension was analyzed by flow cytometry (Coulter Elite) as previously described (22). SYTOX (a fluorescent green nucleic acid dye staining the DNA of cells with compromised membranes) and a mixture containing equal amounts of SYTO 9 and propidium iodide (LIVE/DEAD *Bac*Light bacterial viability kit; Molecular Probes, Eugene, Oreg.) were used to evaluate the viability of LGG and PJS samples, respectively. The bacterial suspensions were made to the desired concentration with sterile saline and administered orally (at 1 ml/kg) to mice by using a stainless steel gavage needle (22 gauge by 38 mm, with a ball diameter of 1.025 mm) and a 1-ml syringe. Control mice were treated in a similar manner but received only sterile saline.

Splenocytes. Mice were killed by cervical dislocation. Spleens were removed and placed in media containing RPMI 1640 (ICN Biomedicals, Inc., Aurora, Ohio) with HEPES buffer (20 mM), sodium bicarbonate (24 mM), and gentamicin (100 μg/ml; Sigma Chemical Co., St. Louis, Mo.). The splenocytes were isolated by injecting the medium into the spleen, after which the spleens were teased apart. The splenic cell suspension was filtered through nylon mesh, and the cells were pelleted by centrifugation (200 × *g* for 5 min at 4°C). To remove erythrocytes, splenocytes were incubated for 3 min in lysing buffer (155 mM ammonium chloride, 10 mM potassium bicarbonate, 127 μM EDTA) and then washed twice (200 × *g* for 5 min at 4°C). For the mitogenesis assay, the viable lymphocytes in 3 ml were diluted with fetal calf serum (FCS) (CSL Biosciences, Parkville, Victoria, Australia)-enriched medium to 10^6 cells/ml. Trypan blue exclusion was used to determine the viability of the immune cells.

Lymphocyte proliferation assay. The immune cell suspensions were incubated at 37°C (with 5% CO_2 in air) in 96-well round-bottom microtiter plates (Greiner, Frickenhausen, Austria) with either the T-cell mitogen ConA or the B-cell mitogen LPS. Splenic lymphocytes were incubated with suboptimal, optimal, and supraoptimal (cytotoxic) concentrations of mitogen (i.e., 2.5, 5.0, or 10.0 μg of ConA per ml or 12.5, 25.0, or 50.0 μg of LPS per ml). The basal proliferation rate was detected by incubating the splenocyte suspension alone without mitogen, and all tests were performed in triplicate. To quantitate lymphocyte proliferation, tritiated thymidine (5′-^{3}H; Amersham International, Amersham, United Kingdom) was added to all the wells (0.5 μCi/well) after 24 h, and this was followed by a further 48-h incubation. After the 72-h incubation period, the cells were harvested onto glass filter mats (Flow Laboratories Australasia, North Ryde, New South Wales, Australia) by using a semiautomatic 12-well cell harvester (Skatron, Lier, Norway). Radioactivity incorporated into the newly dividing cells was measured with a liquid scintillation counter (LKB Wallac, Turku, Finland) by using ACS-II scintillation fluid (Amersham International). Tritiated thymidine incorporation was determined as disintegrations per minute (dpm) of incorporated tritium.

MLR assay. The splenic immune cells of Swiss mice were used as the responders, and their mixed-lymphocyte response (MLR) activity to stimulator splenocytes obtained from C3H/HeJ mice was measured. To prepare a nonproliferating stimulator population with minimal damage to the surface antigens, the C3H splenocytes were incubated with mitomycin C (40 μg of splenocytes per ml at 10^7 cells/ml) (Sigma Chemical Co.) in a shaking water bath (37°C, in the dark). The cell suspension was then washed three times with media (200 × *g*, 5 min, 22°C), after which the pellet was resuspended and the viability of the isolated cells was assessed by trypan blue exclusion. The viable lymphocytes were diluted with FCS-enriched medium to a concentration of 3×10^6 cells/ml. The MLR was induced by incubating (at 37°C with 5% CO_2 in air) 100 μl of responder cells (at 10^6 cells/ml) in 96-well U-bottom microtiter plates with 100 μl of stimulator cells (at 3×10^6 cells/ml). The incubation procedure and subsequent steps were performed as previously described for the mitogenesis assay.

Statistical analyses. The results are presented as relative proliferation indices (i.e., percent mean dpm for mice in the same treatment group/mean dpm for control mice tested simultaneously). The statistical significance ($P < 0.05$ and $0.1 > P > 0.05$) of the experimental data was tested by using a two-tailed *t* test comparing treatment groups with control animals. The statistical significance of the replicate experiments were evaluated by using nested analysis of variance (Abacus Concept, Inc., Berkeley, Calif.).

TABLE 1. Somatic indices (SI) and splenic lymphocyte yields (SLY) of mice treated with LGG or PJS at a low peroral dose (10^9 viable bacteria/kg [body weight]/day) for 7 days or a high peroral dose (10^{12} viable bacteria/kg [body weight]/day) for 3, 7, or 14 days

Bacterial strain	Dose (viable bacteria/kg [body wt]/day)/ treatment duration (days)	SI, % control (SEM for 4–8 mice)	SLY, % control (SEM for 4–8 mice)
L. rhamosus GG (LGG)	10^9/7	83 (6)	98 (10)
	10^{12}/3	95 (2)	91 (9)
	10^{12}/7	91 (8)	74 (2)
	10^{12}/14	154 (24)	120 (32)
P. freudenreichii subsp. *shermanii* JS (PJS)	10^9/7	83 (9)	83 (15)
	10^{12}/3	116 (11)	104 (15)
	10^{12}/7	119 (7)	139 (13)
	10^{12}/14	121 (26)	81 (24)

RESULTS

Spleen parameters. The somatic indices (spleen-to-body-weight ratios) and the splenic lymphocyte yields are presented in Table 1. Neither of these two parameters were significantly affected by any of the treatments studied.

Dose-response relationship. The effects of either a low or high oral dose of bacteria (10^9 or 10^{12} bacteria/kg [body weight]/day for 7 consecutive days) on splenic lymphoproliferation are shown in Table 2. Treatment with both PJS doses caused significant ($P < 0.05$) decreases in the spontaneous proliferative activity of splenic immune cells (by 32 to 42%), whereas this basal lymphoproliferation was not altered by LGG treatments. The lower dose of both LGG and PJS appeared to enhance T-cell proliferation at optimal concentrations of ConA (by 69 to 84%; $P < 0.1$) and increase B-cell proliferation at the optimal and supraoptimal concentrations of LPS (by 57 to 82%). B-cell proliferation at similar LPS concentrations was also enhanced by the high LGG dose (by 32 to 39%), but this was accompanied by a marginal decrease in T-cell proliferation (by 8%) at the optimal ConA concentration. The higher PJS dose had little effect on mitogen-activated lymphocyte proliferation.

Time profile relationship. To assess the effect of the bacterial treatment period on immune responses, mice were treated with LGG or PJS (perorally, 10^{12} bacteria/kg/day) for 3, 7, or 14 days. T-cell proliferation at the optimal ConA concentrations was decreased by the 3- and 7-day LGG treatments (Table 2). However, this marginal decrease, and the enhanced B-cell proliferation observed after 7 days of LGG treatment, both appeared to be transient changes, since the 14-day treatment had no significant effects on B-lymphocyte proliferation or T-cell mitogenesis at the optimal ConA concentration. Instead, the 14-day LGG treatment appeared to enhance the basal lymphoproliferation and T-cell mitogenesis at the supraoptimal ConA concentration. Similarly, the significant decrease in basal splenocyte proliferation observed with the 3- and 7-day PJS treatments also appeared to be transient, since the 14-day treatment was comparable to control levels. However, the latter treatment significantly enhanced the proliferative response of T cells to optimal and supraoptimal ConA concentrations.

MLR. The effect of bacterial treatment on the proliferative response of isolated T lymphocytes to surface antigens on allogeneic cells was assessed by using the MLR assay. There was a trend towards increased MLR activity after 7 days of

TABLE 2. Dose-response and time profile relationships for the effects of *L. rhamnosus* GG (LGG) and *P. freudenreichii* subsp. *shermanii* JS (PJS) treatment on the proliferation activity of unstimulated (basal), ConA-stimulated, LPS-stimulated, and allogeneic-cell-stimulated (MLR) splenic lymphocytes

Bacterial strain	Dose (viable bacteria/kg [body wt]/day)/ treatment duration (days)	Relative proliferation indices (SEM for 3–8 mice)[a]							
		Basal	ConA at:			LPS at:			MLR
			2.5 μg/ml	5 μg/ml	10 μg/ml	12.5 μg/ml	25 μg/ml	50 μg/ml	
L. rhamosus GG (LGG)	10^9/7	113 (22)	118 (0)	169 (5)†	85 (8)	111 (16)	182 (18)*	157 (30)	152 (40)
	10^{12}/3	97 (23)	86 (2)	81 (7)	104 (13)	94 (9)	111 (7)	110 (8)	99 (11)
	10^{12}/7	95 (29)	98 (8)	92 (1)*	127 (37)	116 (7)†	132 (17)*	139 (17)*	119 (5)
	10^{12}/14	168 (35)	105 (9)	102 (4)	219 (42)†	111 (13)	115 (13)	109 (12)	109 (21)
P. freudenreichii subsp. *shermanii* JS (PJS)	10^9/7	58 (8)*	118 (10)	184 (31)†	75 (23)	89 (17)	157 (20)†	171 (28)†	133 (40)
	10^{12}/3	62 (9)*	115 (7)	112 (9)	99 (6)	87 (3)	85 (5)	64 (6)†	ND
	10^{12}/7	68 (19)*	94 (7)	90 (5)	112 (31)	100 (24)	98 (22)	83 (32)	126 (14)
	10^{12}/14	90 (14)	94 (5)	124 (2)*	180 (23)*	87 (18)	94 (19)	94 (24)	ND

[a] Significant differences: *, significantly different ($P < 0.05$) from saline-treated control group as calculated by two-tailed *t* test; †, significantly different ($0.1 > P > 0.05$) from the saline-treated control group as calculated by two-tailed *t* test. ND, not determined.

treatment with the low dose of LGG and both doses of PJS; however, these effects did not reach statistical significance (Table 2).

DISCUSSION

Lymphoproliferation is commonly examined when analyzing the efficacy of an immunosuppressive or immunoenhancing therapy, when testing chemicals for their immunotoxic potential, and when monitoring congenital immunological defects. The doses of probiotic bacteria used in this study were chosen on the basis of the usual human consumption levels. The lower experimental dose of 10^9 viable bacteria/kg (body weight)/day corresponds to the lactic and propionic acid bacterial intake of an adult human with an average daily consumption of yogurt (400 g) and cheese (20 g) (5). Similar intakes can also be accomplished by consuming commercial Gefilus capsules (Valio, Ltd.) or other dietary probiotic supplements. These capsules each contain a minimum of 5×10^9 CFU of lyophilized LGG, and the daily intake that is recommended by the manufacturer is two capsules per day. The higher experimental dose of 10^{12} viable bacteria/kg (body weight)/day was chosen specifically since it represented the maximal concentration of bacteria that could be reproducibly gavaged and was therefore a practical maximum dose to investigate whether repeated dosing was tolerated by the animals. This high dose would presumably be the upper dose limit in any future safety evaluations and is equal to a total daily dose of 7×10^{13} viable bacteria for a 70-kg human, which is more than 1,000-fold that received from a normal Western diet.

The immunomodulatory effects observed after the 7-day treatments with LGG and PJS did not exhibit the clear dose-response relationships that one associates with most therapeutic agents, and this should be an important consideration when selecting appropriate dose ranges for efficacy and safety testing. The factors and possible mechanisms which may be responsible for this lack of a dose-dependent relationship for the immunomodulatory effects of microorganisms have not been fully elucidated. In contrast, the regulatory mechanism implicated in the induction of oral tolerance to soluble proteins has previously been shown to be a clearly dose-dependent phenomenon (21).

Variations in the duration of the LGG and PJS treatments provided further insight into the time profile of the immunomodulatory effects of these bacteria. The inhibition of basal lymphoproliferation by PJS treatment, and the enhancement of B-cell mitogenesis by LGG treatment, both appeared to be transient phenomena which were not observed after bacterial treatment for 14 days. This may, at least in part, be explained by the time profile of the antibody-secreting cells. Their number is known to reach maximum levels in the peripheral blood 1 week after antigen exposure (6). The transiency may also be associated with induction of oral tolerance, since regulatory cells have been shown to appear in the spleen 4 to 7 days after orally administered antigens (21).

The inclusion of both suboptimal and supraoptimal mitogen concentrations in the lymphocyte proliferation assay enabled the investigation of the effects of bacterial treatments on the mitogen concentration-response curve. Certain bacterial treatments were able to modify the inhibitory effects of excessive LPS concentrations. In particular, 7-day treatment with either dose of LGG and the lower dose of PJS appeared to have an enhancing effect on B-cell proliferation and to improve the tolerance of the splenic lymphocytes towards the inhibitory effects of the supraoptimal concentration of LPS. LPS cytotoxicity is known to play a role in the pathogenesis of gram-negative bacterial sepsis and endotoxic shock by causing vascular endothelial cell injury (1, 9). Therefore, these results indicate that it also may be worthwhile to study whether treatments with these bacterial strains could enhance the tolerance of lymphocytes to endotoxins in vivo, since they may have potential for use in the prophylaxis and treatment of sepsis.

Certain bacterial treatments were also able to modify the inhibitory effects of excessive ConA concentrations. For example, the 14-day LGG and PJS treatments both appeared to enhance the proliferative response of splenic lymphocytes at the supraoptimal concentrations of ConA, which may indicate improved tolerance to the cytotoxic effects of this mitogen. In previous studies, increases in the relative concentration of ConA to the immune cell number has been shown to result in a "switchover" from mitogenicity to cytotoxicity (17). It has been hypothesized that T lymphocytes respond to the binding of this mitogen by producing a burst of reactive oxygen species, such as superoxide or hydrogen peroxide, which, if sufficiently elevated and prolonged, will result in cytotoxicity (17). Further evidence suggests that the cytotoxicity induced by ConA results in ultrastructural changes consistent with apoptotic processes (9, 15) and has consequently been suggested as a useful model for the study of lymphocyte apoptosis in AIDS (17). These changes in the ability of T cells to proliferate in the presence of high ConA concentrations may be due to variations in the proportion of T-cell subpopulations, especially in the ratio of

the $CD4^+$ Th1 and Th2 subclasses. There is recent evidence that Th1 cells may be more susceptible to apoptosis, which further supports this mechanism (4, 10). In this study, the ratio of the different T-cell subpopulations was not evaluated and neither was lymphocyte cell death. Thus, the time profile relationship between probiotic treatments and the proportional activity of different lymphocyte subpopulations should be characterized in future studies. It is also important to examine whether such treatments can influence the susceptibility of lymphocytes to antigen- or mitogen-induced apoptosis. Furthermore, it may be useful to study the effects of probiotic bacteria on the susceptibility of host lymphocytes to known cyto-, endo-, and immunotoxins.

Future studies that assess the immunological safety and properties of probiotic bacteria will need to employ a broad spectrum of immunoassays. In this study, a preliminary investigation of MLR was also made and, although treatment with a lower LGG dose for 7 days appeared to enhance MLR, this finding was not statistically significant. However, further studies may be needed to adequately assess the safety of probiotic bacteria in transplant patients who are also on immunosuppressive therapy to prevent rejection of the transplant.

The immunomodulatory effects which PJS demonstrated in this study are a particularly significant contribution, since there is very little previous data on the immunomodulatory properties of the cluster of propionic acid bacteria. As mentioned previously, some promising results have been reported in clinical trials with parenteral administration of their cutaneous counterparts (7, 8, 19). Therefore, an important area for further study is the investigation of whether some of the cutaneous propionibacteria strains currently used for immunomodulation in clinical trials could effectively be replaced by PJS.

In conclusion, LGG and PJS have specific dose- and duration-dependent immunomodulatory effects on the proliferative activity of B and T lymphocytes. These treatments may also alter lymphocyte sensitivity to the cytotoxic effects of lectin mitogens. Additional studies are needed to further evaluate the optimal and safe doses and treatment period regimens before any probiotic strain can be used appropriately for their immunomodulatory effects in therapeutic trials.

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