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ORIGINAL INVESTIGATION

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Dietary intake of *Lactobacillus rhamnosus* HN001 enhances production of both Th1 and Th2 cytokines in antigen-primed mice

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Abstract Probiotic lactobacilli have been proposed as a potential oral bacteriotherapeutic means of modulating immune phenotype expression in vivo, via their ability to promote cytokine production. This study investigated the ability of a known interferon (IFN)γ-promoting probiotic (*Lactobacillus rhamnosus* HN001) to modulate cytokine production in mice expressing an on-going Th2-type immune response. BALB/c mice were primed to ovalbumin in alum adjuvant to invoke antigen-specific Th2 cytokine-secreting cell populations. Mice that were fed *Lb. rhamnosus* HN001 during antigen sensitization produced higher levels of lymphocyte-derived IFNγ, but also interleukin (IL)-4 and IL-5, in comparison to control animals. Although HN001 was additionally shown to induce pro-IFNγ monokine (IL-12, IL-18) secretion in macrophages in vitro, its ability to invoke mixed lymphocyte cytokine production during an on-going Th2-type immune response in vivo suggests that this probiotic is a general immunostimulatory agent, in contrast to the pro-Th1/anti-Th2 immunoregulation reported for some strains of IFNγ-promoting lactobacilli.

Keywords Probiotic · Lactobacilli · Interferon · Immune modulation · Cytokines

Introduction

Recent reports have indicated that Gram-positive bacilli signal the mammalian immune system via cognate leukocyte surface receptors, in particular Toll-like receptor 2 [24]. In doing so, a variety of inflammatory-type monokines are induced via the stimulation of NF-κB and STAT activation pathways [14, 16]. Of particular interest among immunologists investigating early stages in immune activation is the ability of some Gram-positive bacilli to induce high levels of the monokines IL-12 and IL-18 [10, 15], since these molecules act synergistically to enhance IFNγ production and can thus favor a burgeoning Th1 response during MHC-restricted antigen recognition [22].

Safe and effective means of delivering pro-Th1-activating signals are desirable from a therapeutic/prophylactic viewpoint for activating anti-tumor immunoresponses, microbicidal defense mechanisms, and possibly for regulating atopic reactions [4, 13]. In this context, gut-colonizing Gram-positive lactobacilli have received a considerable amount of research attention as a non-pathogenic means of delivering pro-Th1 immune signals. Certain strains of lactobacilli have been shown to be potent inducers of pro-IFNγ monokines (IL-12 and IL-18) following contact with human or murine leukocytes [15, 23], and can promote elevated IFNγ production both in vitro and in vivo (i.e., following oral delivery as a probiotic [1, 5, 6, 19]). *Lactobacillus casei* (Shirota strain), for example, has been shown to induce IL-12 and to increase IFNγ production in both naïve and antigen-primed mice [10, 13], and in the latter case this action was sufficient to lower the production of Th2-type cytokines (IL-4, IL-5) when the bacillus was included in antigen-stimulated splenocyte cultures [23].

Recent research from our laboratory has identified a food-derived strain of *Lactobacillus* (*Lb. rhamnosus* HN001) that is safe for oral consumption and that can transiently colonize the human intestinal tract following oral delivery [20, 25]. In dietary supplementation studies, humans and mice fed HN001 exhibited increased NK cell tumoricidal activity [5, 6, 7], which in the latter was accompanied by an increased ex vivo capacity of mitogen-stimulated splenic lymphocytes to secrete IFNγ, suggestive of pro-Th1 immune stimulation in naïve hosts. However, oral delivery of HN001 was also shown to increase intestinal tract and serum antibody responses

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to orally or parenterally administered T-dependent vaccine antigens in mice [5, 6], possibly indicating that the form of immunostimulation exerted by this *Lactobacillus* could also impact on Th2 immunoresponses. In this study, we have sought to understand the mode of immunomodulation exerted by HN001 by examining patterns of cytokine production during an on-going antigen-specific immune response. For this study, we primed mice systemically to ovalbumin in alum adjuvant to invoke an IL-4/IL-5-secreting T lymphocyte response, and we investigated the ability of oral feeding with HN001 to affect patterns of ex vivo cytokine production.

Materials and methods

Animals

Six-week-old conventional female BALB/c mice were housed in cages at a controlled temperature (22 ± 2°C) with a 12-h light/dark cycle, and maintained on a standard mouse chow diet (Diet 86, Sharps, Lower Hutt, New Zealand) with free access to water at all times throughout the study. For an acclimatization period of 7 days prior to commencement of feeding experiments, mice were fed 50 µl of reconstituted skim milk powder (SMP) by dropper pipette daily, in addition to the above food and water.

Microorganisms

Lb. rhamnosus strain HN001 was obtained as a lyophilized seed stock from the New Zealand Dairy Research Institute Culture Collection (DR20™; NZDRI, Palmerston North, New Zealand). Stock cultures were transferred to de Man Rogosa Sharpe (MRS) broth and subcultured in the same medium. Cells in log-phase growth were harvested and washed, re-suspended in MRS broth and incubated at 37°C overnight. Bacteria were re-harvested, centrifuged and the culture supernatant discarded. Bacterial cell suspensions for feeding to mice were subsequently prepared by washing and re-suspending the bacterial pellet to the desired concentration in 10% re-constituted SMP, based on the number of viable colony forming units (CFU) grown on MRS agar plates.

Dietary regimes and antigen sensitization

Thirty-six mice were utilized in this study. Test group mice ($n = 15$) were administered 10^9 viable CFUs of *Lb. rhamnosus* HN001 in 25 µl reconstituted SMP, by feeding via a dropper pipette, daily for a period of 28 days; control group mice ($n = 15$) received un-supplemented skim milk for the same period. On days 14 and 21 of the feeding regime mice were antigen-sensitized by intraperitoneal injection of 50 µg ovalbumin (OVA, Sigma Chemicals, Perth, Australia) absorbed onto alum adjuvant, and on days 26 and 27 the response was boosted by administering 20 µg OVA in saline intranasally. To confirm antigen-specific sensitization, two groups of three mice per group were fed skim milk with or without probiotics (as above) but not primed to OVA. All mice were killed on day 28.

Preparation of splenic and peritoneal leukocytes

Single-cell leukocyte suspensions were prepared from the spleens of each mouse individually (erythrocytes were removed by lysis using ACK buffer) and peritoneal lavages were undertaken. Viable splenic lymphocytes and peritoneal macrophages were enumerated by flow cytometry, and cell suspensions were adjusted to the desired concentrations in RPMI 1640 medium, supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 5 nM 2-mercaptoethanol, 50 µg/ml gentamicin (all reagents Sigma, Perth, Australia) and 10% fetal calf serum (Bioclone Laboratories, USA). Splenic lymphocytes were seeded at 3×10^6 cells/well of a 24-well tissue culture plate and stimulated for 48 h with 50 µg/ml OVA, or left un-stimulated. Macrophages were seeded at 3×10^5 cells/well of a 96-well tissue culture plate and stimulated for 18 h with 4×10^6 heat-killed *Lb. rhamnosus* HN001 bacilli (HK-HN001), or left un-stimulated. Culture supernatants were collected and cleared of cells by centrifugation.

Cytokine assays

Levels of cytokines were determined by commercial ELISA detection kits [R&D Systems, Minneapolis, USA for IFNγ, IL-4, IL-12 (p70) and IL-18; PharMingen Inc., San Diego, USA for IL-5]) as described elsewhere [3]. Results were calculated against standard curves generated using known amounts of recombinant cytokine, according to the manufacturer's instructions. Data were compared between probiotic-fed/ and control-fed/OVA-sensitized groups by two-sample Student's *t*-tests; a P value < 0.05 was taken to indicate significance.

Results and discussion

Antigen sensitization invoked Th2-type immune reactivity among the OVA-primed mice, as signified by specific antigen-stimulated production of IL-4 and IL-5 by splenic lymphocytes in vitro (Fig. 1) and an excessive Th2 cytokine:IFNγ ratio (Table 1). In vitro production of IFNγ and IL-4 by OVA-stimulated splenocytes was significantly higher among probiotic-fed mice compared to control-fed animals (Fig. 1), while production of IL-5 was also higher (but not significantly so) in the probiotic-fed mice ($P = 0.091$). Calculated on a mouse-by-mouse basis, the mean IL-4:IFNγ and IL-5:IFNγ ratios were marginally (although non-significantly) reduced in probiotic-fed mice compared to controls (Table 1). HK-HN001 bacilli induced secretion of significant levels of IL-12 and IL-18 following in vitro co-culture with peritoneal macrophages (Fig. 2). Levels of HN001-induced monokine secretion were similar in cells derived from probiotic-fed/ or control-fed/OVA-sensitized mice.

Certain strains of probiotic lactobacilli have been shown to induce Type I IFN (IFNα) [2, 11] and pro-IFN monokines (IL-12 and IL-18) [15, 21], and this has been suggested to be the primary mechanism driving enhancement of IFNγ production (and hence elevated pro-Th1 cell-mediated effector responses) in probiotic-fed animals and humans [4, 6, 11]. Previously it has been reported that lactobacilli exhibiting this effect can promote increased IFNγ production concomitant with either no effect [10] or a decrease [13] in Th2 cytokine production, suggesting that IFNγ-inducing probiotics regulate immune responses in a pro-Th1/anti-Th2 manner. Here we have demonstrated that the effect may not be as clearly delineated as that, in that orally delivered *Lb. rhamnosus* HN001 certainly promotes IFNγ production [5, 6] and pro-cell-mediated effector responses [7], but in antigen-primed mice seems to

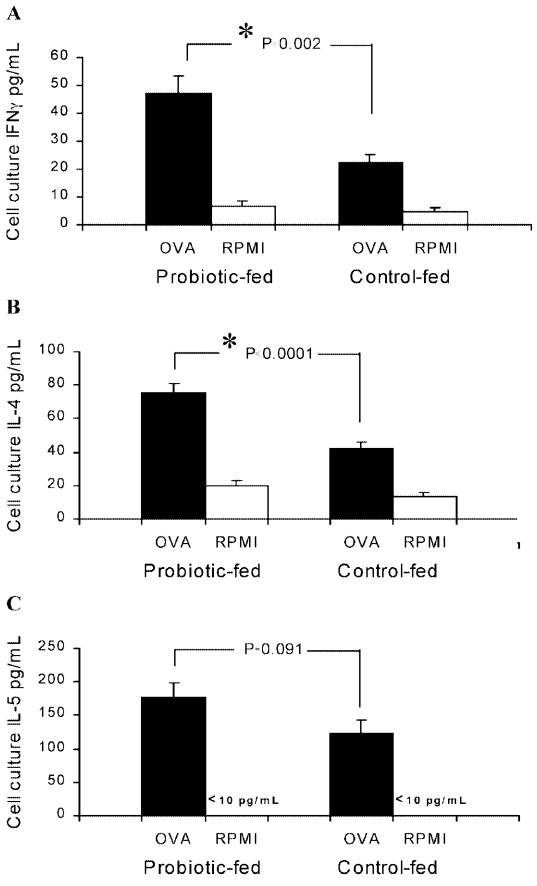

Fig. 1A–C. Effect of probiotic feeding on the ex vivo antigen-driven production of T cell cytokines by splenic lymphocytes derived from OVA-sensitized mice. Splenocytes were derived from OVA-primed mice that had or had not been fed a probiotic (HN001) during antigen sensitization. Splenocytes were cultured in vitro for 48 h with 50 μg/ml OVA or RPMI, and the secretion of IFNγ (**A**), IL-4 (**B**) and IL-5 (**C**) was determined by ELISA. Splenocytes of probiotic-fed mice produced higher levels of all T cell cytokines in response to antigen stimulation than did control mice (*asterisks* and *P* values refer to levels of significance). OVA-stimulated cytokine levels in non OVA-sensitized mice ($n = 3$) were < 10 pg/ml for each cytokine (data not shown)

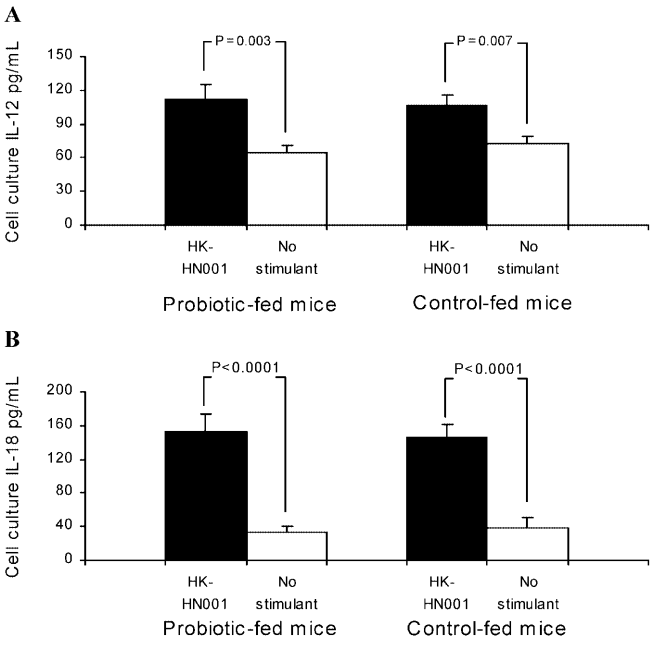

Fig. 2A, B. Ability of heat-killed *Lactobacillus rhamnosus* HN001 to stimulate pro-interferon monokine production in vitro. Peritoneal macrophages, derived from OVA-primed mice that had or had not been fed a probiotic during antigen sensitization, were co-cultured in vitro with heat-killed *Lb. rhamnosus* HN001 bacilli (HK-HN001) for 18 h. Levels of IL-12 (**A**) and IL-18 (**B**) were determined in the culture supernatants by ELISA. *P* values refer to cytokine levels in macrophages exposed to HK-HN001 that were significantly higher than levels observed in non-stimulated cells

Table 1. Effect of probiotic feeding on the mean Th2 cytokine:IFNγ ratios for OVA-sensitized mice. Values refer to the mean Th2 cytokine:IFNγ ratios (± SEM) for both dietary groups of OVA-sensitized mice, calculated on a case-by-case basis for each individual animal (data derived from pg/ml of each cytokine produced by OVA-stimulated splenic lymphocytes, as depicted in Fig. 1)

	IL-4:IFNγ ratio	IL-5:IFNγ ratio
Control-fed mice	2.53 ± 0.46	8.53 ± 2.33
Probiotic-fed mice	2.15 ± 0.38	5.20 ± 1.13

stimulate a mixed Th1/Th2 cytokine pattern. While the Th2 cytokine:IFNγ ratios observed following in vitro stimulation of splenocytes with specific antigen were marginally reduced in probiotic-fed mice, the overall results indicated that lymphocytes of these mice produced elevated levels of all T cell cytokines measured (IFNγ, IL-4 and IL-5) in comparison to cells of control-fed mice. It thus appears that, in the course of a comparatively short-term (28 days) antigen-specific sensitization regime such as the one employed here, feeding HN001 to mice as a probiotic can elevate general T cell-mediated cytokine output. Quite possibly, the Th2 component of this mechanism is sufficient to stimulate the previously observed elevation of antibody responses against experimental vaccine antigens in mice fed HN001 over a 28-day period [6]; however the role of other antibody-enhancing cytokines (e.g., IL-6, TGFβ) in this model needs to be determined.

In previous studies, systemic or oral delivery of IFNγ-promoting lactobacilli has been shown to increase in vivo (plasma) and/or ex vivo IL-12 production in mice [17, 18, 21, 23], while direct co-culture of lactobacilli with leukocytes in vitro has been shown to stimulate the production of IL-12 and IL-18 [9, 10, 15]. In common with these reports, *Lb. rhamnosus* HN001 was shown here to induce IL-12 and IL-18 following direct in vitro co-culture with macrophages, indicating that this strain is capable of directly stimulating the production of pro-IFNγ monokines. In vitro studies have clearly demonstrated the essential role for IL-12 in IFNγ-promotion by lactobacilli, in that IL-12-neutralizing monoclonal antibodies, added

to co-cultures of antigen-responsive murine splenocytes and *Lb. casei* Shirota, can effectively reduce IFNγ production and increase IL-4 and IL-5 output [10]. Thus, we believe that *Lb. rhamnosus* HN001 probably stimulates IFNγ production in vivo by a similar mechanism, i.e., by directly inducing IL-12/IL-18 in leukocytes populating the gut environment (since it is known that dietary components can induce pro-IFNγ monokine expression in the intestinal epithelium; [12]). However, in contrast to the reported effects of *Lb. casei* Shirota, *Lb. rhamnosus* HN001 feeding here did not appear to down-regulate Th2 cytokines in antigen-primed mice, suggesting a different mode of immune regulation. It has been suggested that different strains of immunomodulatory lactobacilli exert differing influences on the immune system primarily via their ability to induce IL-12 or the regulatory cytokine IL-10 [8], and since another *Lb. rhamnosus* strain (GG) has been shown to induce IL-10 in addition to IL-12 [14, 15], it is likely that the balance between these two cytokines may determine the character of probiotic-induced immunoregulation. Future research will be necessary to confirm what mechanisms are operative at the systemic level for HN001 feeding to simultaneously increase Th1 and Th2 cytokine production during antigen-specific reactions, and whether this mixed response will subsequently become more dichotomized over a longer time frame (e.g., during a memory/recall response).

Probiotic lactobacilli have been proposed as a non-pathogenic bacteriotherapeutic means of modulating immune phenotype expression [5, 13], and in particular those strains that are known to induce IFNγ may have value as biomodulatory agents to boost Th1 effector responses (e.g., in cancer immunotherapy) [18] and to down-regulate Th2 responses (e.g., in anti-allergy immunotherapy) [4]. Some strains of lactobacilli (such as *Lb. plantarum* L-137 or *Lb. casei* Shirota) have been shown to induce IL-12 and promote IFNγ production, and can strongly down-regulate production of Th2-type cytokines in mice [13, 17, 23]. However, the conclusion from the present study is that not all IL-12–inducing/IFNγ-promoting lactobacilli are likely to be exclusively pro-Th1 immunomodulatory agents, and thus careful consideration should be given to characterizing the immunoregulatory effects of different strains of probiotic lactobacilli for their relevance to human health.

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