Immunostimulatory probiotic *Lactobacillus rhamnosus* HN001 and *Bifidobacterium lactis* HN019 do not induce pathological inflammation in mouse model of experimental autoimmune thyroiditis

J.S. Zhou*, H.S. Gill

Milk and Health Research Center, Institute of Food Nutrition and Human Health, Massey University, Palmerston North, New Zealand

Received 19 March 2004; received in revised form 25 August 2004; accepted 30 November 2004

**Abstract**

The possibility that intestinal microflora contribute to the pathogenesis of autoimmune diseases has raised issues regarding the safety of probiotic organisms, especially those with immunostimulating properties, in individuals with such immune dysfunctions. In this study, the effect of consumption of probiotic lactic acid bacteria (LAB) strains *Lactobacillus rhamnosus* HN001(HN001) and *Bifidobacterium lactis* HN019 (HN019) on the induction and progression of experimental autoimmune thyroiditis (EAT) was investigated in CBA/CaH (H-2k) mice. HN001 or HN019 in skim milk were fed to mice daily (1–1.5×10⁸ cfu/mouse/day) for 5 to 9 weeks. A mild form of EAT was induced by subcutaneous injection of mouse thyroglobulin (MTg) with either Freund’s adjuvant (complete and incomplete, CFA and IFA) or lipopolysaccharide (LPS). The proliferative responses of spleen lymphocyte to MTg stimulation in vitro and the presence (and degree) of mononuclear cell infiltration in thyroid gland tissues were examined to assess the development and severity of EAT. The levels of serum anti-MTg antibodies (IgG1 and IgG2a) and spleen weight index were determined to detect the presence of autoimmune responses of mice receiving MTg. Results showed that 8 weeks after immunization, 16.67–50% of the mice developed mild EAT with lymphocyte infiltration in the thyroid glands. Probiotic feeding did not induce full-blown EAT. There were no differences in spleen weight index or the proliferative spleenocytes in response to PMA between mice that received MTg alone and mice that received MTg and probiotic LAB strains.

© 2005 Elsevier B.V. All rights reserved.

**Keywords:** Probiotic; *Lactobacillus; Bifidobacterium;* Experimental autoimmune thyroiditis; Autoimmunity

* Corresponding author. Current address: Division of Rheumatology, Immunology and Allergy, Brigham and Women’s Hospital, Harvard Medical School, 1 Jimmy Fund Way, Smith Building Rm 636, Boston, MA 02115, United States. Tel.: +1 617 525 1285; fax: +1 617 525 1310. E-mail address: jzhou@rics.bwh.harvard.edu (J.S. Zhou).

1 Current address: Primary Institutes Research Victoria, Department of Primary Industries-Werribee Centre, 600 Sneydes Road, Werribee, VIC 3030, Australia.

0168-1605/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
1. Introduction

Autoimmune diseases are thought to have multiple etiologies. The penetration of gut bacterial antigens into lymphoid tissues is one of the suggested initial factors leading to a loss of tolerance towards self-components in genetically predisposed individuals (Hamilton et al., 1998; Famularo et al., 1997). There is growing evidence that the composition of the endogenous intestinal microflora may have an important role in the expression of systemic autoimmunity in both humans and animal models. Decreased susceptibility to autoimmunity in specific pathogen-free (SPF) or germ-free (GF) animals compared with conventionally housed animals has been reported in models of autoimmune thyroiditis (Penhale and Young, 1988), hemolytic anaemia (Murakami et al., 1994), arthritis (Kool et al., 1992; Thompson and Elson, 1993), systemic lupus erythematosus (SLE; Apperloo-Renkema et al., 1994; Terada et al., 1991), and inflammatory bowel disease, including Crohn’s disease and ulcerative colitis (Rutgeerts et al., 1991; Jewell and Patel, 1985).

The ability of probiotic bacteria to modulate immune responses has been demonstrated in a large number of studies (Gill et al., 2000; Matsuzaki and Chin, 2000). For example, consumption of probiotic lactic acid bacteria (LAB) has been associated with the enhancement of both innate and adaptive immune responses. Some LAB strains have also been shown to provide significant adjuvanticity in animal models (Gill et al., 2000; Pouwels et al., 1996; Claassen et al., 1995). Therefore, the use of a Lactobacillus-based live vector vaccine for oral delivery has been proposed (Pouwels et al., 1996). The beneficial effects of the consumption of immune-enhancing LAB strains to immunocompetent hosts are obvious, but the consequence of over-activation of the immune system by these organisms in hosts with immune dysfunctions, such as individuals genetically predisposed to autoimmunity, has raised some concerns (Guarner and Schaafsma, 1998; Wagner and Balish, 1998). For example, it has been demonstrated experimentally that Lactobacillus casei cell wall components (given intraperitoneally) are able to induce cardioangitis (an autoimmunity-associated heart disease) in mice (Okitsu-Negishi et al., 1996).

It has been reported in several studies that some LAB strains can translocate from the gut into tissues (liver, spleen, and kidney) or the bloodstream in immunodeficient hosts (Ha et al., 1999; Wagner et al., 1997; Link et al., 1995; Sussman et al., 1986). With respect to the association between bacterial antigens and autoimmune responses and the adjuvant activity of LAB strains, the involvement of LAB in the pathogenesis of some models of autoimmunity in experimental animals and possibly in humans has been suggested (Famularo et al., 1997). Thus, from a safety point of view, the potential of probiotic bacteria (especially the immunostimulatory strains), to induce destructive inflammation or autoimmunity needs to be investigated (Wagner and Balish, 1998).

Murine experimental autoimmune thyroiditis (EAT) mimics the main cellular and pathological manifestations of Hashimoto’s thyroiditis (a human organ-specific autoimmune disease) and has been frequently used as a model to study human autoimmunity (Alimi et al., 1998; Via and Shearer, 1988; Simon et al., 1985). EAT can be induced with thyroglobulin (Tg), a known thyroid autoantigen common to both mouse and humans (Kong, 1996). The major purpose of the present study was to investigate the effect of oral consumption of the probiotic strains Lactobacillus rhamnosus HN001 (HN001) and Bifidobacterium lactis HN019 (HN019), which enhance immune function (Gill et al., 2000), on the development and progression of EAT in inbred mice with the H-2k haplotype, such as CBA/J mice, are the most susceptible animal models for the induction of EAT (Bhatia et al., 1996; Kong, 1996; Nicoletti et al., 1994; Esquivel et al., 1977). CBA/CaH mice, which have a genetic phenotype similar to CBA/J mice including the H-2k haplotype were used in this study because we found they have a modest response in terms of the incidence and degree of EAT, thus permitting an analysis of whether LAB induce full-blown autoimmune disease under conditions that would otherwise induce only mild inflammation.

2. Materials and methods

2.1. Animals

Female mice (4–6 weeks old) were purchased from the Animal Resources Centre, Perth, Western Aus-
tralia, and acclimatized to the housing conditions for 2 weeks before the commencement of treatment. Fresh water and standard mouse chow (Unifeeds, Palmerston North, New Zealand) were supplied ad libitum. During the experimental period, the animals’ health status was observed daily: the body temperatures were taken every other day with an electronic anal thermometer, and body weights were measured once a week. At the end of experiment, mice were humanely euthanized by isoflurane overdose inhalation to collect the blood and thyroids. The experimental protocols, including the use of animals, were approved by Massey University Animal Ethics Committee (Palmerston North, New Zealand).

2.2. Preparation of mouse thyroglobulin (MTg)

Thyroglobulin (Tg) is the main protein synthesized in thyroid follicle cells and accounts for up to 75% of the total protein in the thyroid (Kong, 1996; Verschueren et al., 1991). Tg from different species (cow, rodent, pig, human) is similar with regard to its physical, biochemical, and molecular properties. However, to ensure induction of optimal autoimmune responses, MTg rather than Tg from other species was used in this study. To prepare MTg, frozen thyroids from 300 inbred mice (BALB/c and CBA/CaH mice, mixed sex, age 6 15 weeks, from the retired naive recycled mouse stock of Massey University Small Animal Production Unit) were thawed at 4 °C and transferred into a chilled 15-ml heavy-duty centrifuge tube. The thyroids were homogenized with an Ultra-Turrax T25 homogeniser (Janke and Kunkel, IKA® Labortechnik, Germany) at maximum speed (scale 6 or 35,000 rpm) for 1 min with intervals of 10 s on ice. The homogenate was ultracentrifuged in a Beckman Ultracentrifuge (NV Ti 90 rotor, 100,000×g or 35,000 rpm) at 4 °C for 1 h. The clarified supernatants (free of membranous debris) were collected and separated on a Sephacyr l-300 gel column (Sephacryl S-300 HR Columns, cat: 17-0599-01, Pharmacia Biotech, Piscataway, NJ, USA) on a fast protein liquid chromatography (FPLC) system (Pharmacia Biotech). The protein fractions and concentrations were monitored at 280 nm. The first major protein peak containing MTg, which appeared at about 120 to 160 min (just before the hemoglobin peak; Fig. 1), was collected in 2-ml fractions in glass tubes using a computer-controlled automatic fractionation system (Liquid Chromatography Controller LCC-500 plus, Pharmacia Biotech). Fractions within the range of the MTg peak were pooled, and the protein concentration was determined with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The MTg in the pooled fractions was concentrated to >800 μg/ml with a protein concentration unit (Centriplus™ Concentrators, Amicon, Beverly, MA, USA). The purity of the thyroglobulin was determined by SDS-PAGE analysis and compared with a commercially-purified bovine thyroglobulin preparation (Thyroglobulin from bovine thyroid, T-1001, Sigma Chemical, St. Louis, MO, USA; Fig. 2). The purified thyroglobulin suspension (in PBS) was dispensed into 2-ml tubes and stored at −70 °C until use.

Fig. 1. Separation of MTg from thyroid extracts. The thyroid extracts prepared by the method described in Section 2.2 were purified using a Sephacryl-300 gel column with a FPLC system. The column was run with PBS (pH 7.4) at a flow rate of 0.3 ml/min. The fractions from 95 to 180 min were collected. Protein concentration was monitored using UV absorbance (280 nm). 10 fractions with the highest protein concentration within the first major peak (MTg) were pooled and used for further analysis. MTg: mouse thyroglobulin; HB: hemoglobin.
2.3. Feeding of probiotics

HN001, HN019, and a commercial probiotic strain *L. rhamnosus* GG (*L. GG*) were prepared using the method described previously (Zhou et al., 2000). The cell populations were adjusted to 2–5 \times 10^9 cfu/ml. Thirty microliters of bacterial suspension was fed to each mouse on a daily basis using a sterile pipette. Feeding of probiotics commenced 1 week prior to the beginning of MTg injections and was sustained until the end of the experiment.

2.4. Induction of EAT

2.4.1. MTg+CFA and IFA

MTg (800 \mu g/ml in PBS) was emulsified (1:1) with complete Freund’s adjuvant (CFA, F5881, Sigma Chemical) or incomplete Freund’s adjuvant (IFA, F5506, Sigma Chemical) using 2-ml Luer-lock syringes. One hundred microliters of MTg emulsion (400 \mu g/ml in CFA) was injected subcutaneously at multiple sites on the inside of the hind legs of each mouse on day 0. After 7 days, a booster injection of MTg (400 \mu g/ml in IFA) was given to each mouse.

2.4.2. MTg+LPS

One hundred microliters of MTg (400 \mu g/ml in PBS, pH 7.4) was injected subcutaneously into mice as described above. Three hours following each MTg injection, 100 \mu l of lipopolysaccharide (LPS, 200 \mu g/ml, TCA-precipitated from *E. coli* 055:B5, L6529, Sigma Chemicals, St. Louis, MO, USA) was injected into each mouse (in the contralateral leg to that of MTg injections). A third dose of MTg (100 \mu l, 400 \mu g/ml in PBS) and LPS (20 \mu g in 100 \mu l PBS) was injected into each mouse 5 weeks after the first MTg injection. Animals were humanely euthanized at 4, 7, or 8 weeks after the first MTg injection to collect blood, spleen, and thyroid glands. Lymphocytic infiltration in the thyroid and spleen cell proliferative responses to MTg were assessed as indicators of autoimmune responses.

2.5. Autoantibody detection

The amounts of anti-MTg IgG1 and IgG2a antibodies in serum were determined by ELISA to confirm the establishment of an autoimmune reaction (Bhatia et al., 1996). The wells of a 96-well microtitre plate (Nunc-Immuno™ plate, Nalge Nunc International, Denmark) were coated with 200 \mu l coating buffer (Gill et al., 2000) containing MTg (2 \mu g/ml) by incubation at RT overnight. Antibody levels of individual samples were presented as the OD values (absorbance at 405 nm).

2.6. Spleen lymphocyte proliferative responses to MTg

Spleen lymphocyte proliferative responses to MTg stimulation in vitro were determined using a commercial cell proliferation kit (Cell Proliferation ELISA kit, BrdU Colorimetric kits, 1647229, Boehringer Mannheim, Indianapolis, USA) as described previously (Cross and Gill, 1999). Results were expressed as the proliferation index [the ratio of the OD value (absorbance at 450 nm) of MTg+ wells to that of MTg wells].

2.7. Histology

Thyroid glands attached to the larynx and trachea were excised carefully and immediately immersed in
Bouin’s fluid (24% formalin, 71% saturated picric acid and 5% glacial acetic acid). The thyroids were transferred to 70% ethanol within 8 to 12 h and processed for hematoxylin and eosin (H and E) staining. Six sections (6 μm thick) from each thyroid (both left and right side of the thyroid) were cut and mounted on glass slides, so that 12 sections were made for each sample. The pathological changes were evaluated according to the extent of mononuclear cell infiltration in the thyroid tissues. A scoring system of 0–4 (Table 1) as described previously by Kong (1996) and Peterson and Braley-Mullen (1995) was used to evaluate the pathological index (PI). Animals with at least one section with mononuclear cell infiltration in thyroid tissues and a PI of equal to or greater than 0.5 were defined as EAT-positive.

3. Results

3.1. Clinical observations

All of the mice survived to the end of the experiments. There were no significant differences in the body weight (Fig. 3) or temperature of mice injected with saline alone, MTg (+CFA/IFA/LPS) alone, or fed with probiotics and injected with MTg.

3.2. Immune responses

MTg-immunized mice produced measurable titers of IgG1 and IgG2a antibodies against MTg (OD values of 2.3 ± 0.23 vs 0.0037 ± 0.0004 and 0.5497 ± 0.096 vs. 0.0094 ± 0.0011 respectively), confirming the onset of an autoimmune response. Animals treated with MTg+CFA/IFA or MTg+LPS had significantly higher spleen weight indices

![Graph](image-url)

Fig. 3. Changes in body weight of mice receiving MTg+CFA/IFA with or without probiotic feeding. Data represent the average body weight of mice in each group (n=12). Control: normal mice receiving saline only; MTg: mice treated with MTg only; MTg+HN019, HN001, or L. GG: mice treated with MTg and HN019, HN001, or L. GG respectively.
(4.114±0.103) than control mice (3.368±0.1011, P<0.05); probiotic feeding had no effect on this indicator.

Probiotic feeding also had no effect on lymphocyte proliferation induced by MTg+LPS, spleen cell proliferation indices of mice fed with probiotics receiving MTg injections (1.3049±0.094; 1.3415±0.052) were similar to those of mice that received MTg alone (1.3517±0.071, P>0.05).

3.3. Thyroid histological changes

Injections of MTg+CFA/IFA induced only mild thyroid histological changes in <25% of animals. The pathological index (PI) was around 0.5 to 1.0; i.e., mononuclear cell infiltration was not remarkable. As indicated in Table 2, the average PI in each group was less than 0.15 (MTg+CFA/IFA) or 0.5 (MTg+LPS). There was no significant difference in the incidence of EAT between treatment and control mice that received MTg+LPS (p=0.739 by Fisher’s Exact test). Approximately 50% of the animals developed EAT following MTg+LPS injections (Table 2).

### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of mice</th>
<th>EATa no. (%)</th>
<th>PIb mean±S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTg+ CFA/IFA</td>
<td>Control</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>MTg³</td>
<td>12</td>
<td>2 (18.18)</td>
</tr>
<tr>
<td></td>
<td>MTg+HN019</td>
<td>12</td>
<td>3 (25.00)</td>
</tr>
<tr>
<td></td>
<td>MTg+HN001</td>
<td>12</td>
<td>2 (16.67)</td>
</tr>
<tr>
<td></td>
<td>MTg+Lb. GG</td>
<td>11d</td>
<td>2 (18.18)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>59</td>
<td>8 (17.02)</td>
</tr>
<tr>
<td>MTg+LPS</td>
<td>Control</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>MTg³</td>
<td>12</td>
<td>6 (50)</td>
</tr>
<tr>
<td></td>
<td>MTg+HN019</td>
<td>12</td>
<td>5 (41.67)</td>
</tr>
<tr>
<td></td>
<td>MTg+HN001</td>
<td>12</td>
<td>6 (50)</td>
</tr>
<tr>
<td></td>
<td>MTg+Lb. GG</td>
<td>6</td>
<td>3 (50)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>54</td>
<td>20 (47.62)</td>
</tr>
</tbody>
</table>

a No. of mice with at least one thyroid section with histological changes characteristic of EAT (PI≥0.5). Overall EAT incidence: P>0.05 (LAB groups vs MTg); P<0.05 (control vs. MTg and LAB groups).

b Pathological index=total score/no. of sections examined; the average PI of mice in control group (did not receive MTg or LAB) was used as baseline. P>0.05 by ANOVA test (SAS-GLM).

d Mice treated with MTg only (no LAB supplementation).

d One sample damaged during processing was excluded.

4. Discussion

The experiments conducted here indicate that long-term, daily ingestion of living probiotic organisms B. lactis HN019 and L. rhamnosus HN001 had no adverse effect on the induction or progression of MTg-induced experimental autoimmune thyroiditis in CBA/CaH mice. In previous studies of experimental autoimmune thyroiditis (EAT), different animal models, adjuvants, and immunization protocols have been used. Complete and incomplete Freund’s adjuvants (CFA and IFA) are commonly used to facilitate the induction of EAT (Kong, 1996). Bacterial lipopolysaccharide (LPS) is another widely used adjuvant that assists in the induction of EAT in susceptible mice (Kong, 1996; Simon et al., 1985). The results from the experiment using CFA and IFA as adjuvant showed mild lymphocyte infiltration in thyroids from a few animals (Table 2). This could have been due to the mouse strain CBA/CaH not being profoundly susceptible to EAT or the antigen dose/adjuvant/MTg cocktail used. Administration of LPS leads to the abrogation of self-tolerance to MTg even in poor-responder mice, and the use of LPS as an adjuvant results in more uniform disease induction in experimental models of autoimmunity (Kong, 1996; Esquivel et al., 1977). Therefore, LPS instead of CFA and IFA was also used as the adjuvant. Detectable mononuclear cell infiltration in the thyroids of these mice was observed by 7 or 8 weeks after the first MTg injection, when LPS rather than CFA and IFA was used as the adjuvant, and three MTg injections were given. In general, the histological changes induced by MTg in the present study were less severe than those reported in other mouse strains (Damotte et al., 1997; Kong, 1996; Nicoletti et al., 1994). However, in some previous studies, an EAT incidence similar to that observed in the present study has also been reported (Bhatia et al., 1996; Rose et al., 1971). Nevertheless, compared with animals that were not fed with LAB, animals that received LAB feedings and MTg treatment did not exhibit any exacerbated histological manifestations in the thyroid.

To examine the effect of probiotic feeding on the development and progression (or severity) of EAT, probiotic feeding was initiated 1 week before the MTg immunizations and sustained until the end of this
experiment, i.e., these test probiotic strains and their products (antigens) reached the gastrointestinal tract of mice prior to the administration of MTg. If the antigens from these probiotic strains participated in the pathogenesis of EAT through activation of the immune system, there should be some effect on either EAT incidence or severity associated with the feeding of the specific LAB strains. Normally, spleen or lymph node cell proliferative responses to autoantigen (e.g., MTg) in vitro and the degree of mononuclear cell infiltration in thyroid tissues are used to determine the effect of treatment on the development and progression of EAT. This result is in accordance with previous studies on the effects of non-pathogenic LAB strains on autoimmunity (Claassen et al., 1995).

Overall, the results of this study suggest that immunostimulatory probiotic L. rhamnosus HN001 and B. lactis HN019 do not induce or enhance autoimmune responses in animals which have the genetic potential to develop autoimmunity.

Acknowledgements

Special thanks to Dr H. R. Katz from Harvard Medical School for his valuable editorial comments and to A. Broomfield, D. Johnson, K. Kennedy, L. Fray, and S. Blackburn for their skilled technical assistance.

References


