Immune function of probiotic bacterial DNA and their advanced application to Food Immunology

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The toll-like receptor (TLR) family plays an important role in host defense through recognizing bacterial pathogen-associated molecular patterns. The induction of the host defense system begins with the recognition of pathogens. To date, 13 types of TLR, which function as a kind of pathogen sensor, have been identified. Our recent research on the physiological function of food products has investigated the immunoregulatory effects of probiotic lactic acid bacteria (LAB) via TLR. On the basis of our study, efforts have been made to develop a molecular immunoassay system for probiotic LAB and find novel immunostimulatory DNA sequences from probiotics and high potential immunobiotic LAB strains via TLR9 signaling. These findings may provide important clues at the molecular level on TLR signal transduction pathways and recognition mechanisms for the ligands. In addition to identifying immunoregulatory factor immunogenics from LAB, a better understanding of intestinal immune regulation through cytokine networks holds out promise for basic food immunology research and the development of immunobiotic foods/feeds to prevent specific diseases.

Keywords: probiotics, immunobiotics, immunostimulatory DNA, Functional Foods/Feeds

Introduction

The induction of the host defense system begins with the recognition of pathogens. To date, 13 types of toll-like receptors (TLR), which function as a kind of pathogen sensor, have been identified. The TLR family is important in all biological defense mechanisms and recognition of bacterial pathogenassociated molecular patterns that induce cytokine production and regulate the immune response in hosts. In particular, TLR9 recognize various cytosine-guanine sequences (CpG DNA) in bacterial genomes, respectively, trigger nuclear factor-kappaB (NF-κB) *via* the adapter molecule MyD88, induce production of inflammatory cytokines (e.g. tumor necrosis factor a, interleukin (IL)-6, IL-12, interferon (IFN)-α, and IFN-γ), and induces expression of cell surface co-stimulatory molecules. The development of vaccines containing the functional cell wall components and DNA from bacteria shows promise in the prevention and treatment of infectious diseases, inflammatory diseases, cancer and allergies. “Probiotics” refers to a category of microorganisms that provide health benefits in the host by improving the intestinal microflora. Research on the physiological function of probiotics has generally focused on controlling so-called harmful bacteria and increasing beneficial bacteria in the intestinal microflora to improve gut health. However, in addition to their current research of intestinal regulatory effects various authors have also aimed at elucidating the effects of probiotics on intestinal mucosal immunity. In 2003 Clancy proposed the concept of “immunobiotics” with reference to microorganisms that stimulate activation of mucosal

immunity. This has prompted an interest in research and development of novel immunobiotic foods using the specific immunobiotics. Our research on the bioregulatory function of food products, particularly in maintaining biological homeostasis via intestinal immunity, has investigated the immunoregulatory effects of probiotic lactic acid bacteria (LAB) and led to the identification of activation sequences including specific DNA motifs in the genomic DNA (Shimosato *et al*. 2005, 2006, 2009). Furthermore, in line with other recent publications, our previous study showed that structural, chemical and conformational differences in cell surface constituents occur even in genetically related LAB strains containing functional cell wall components, affecting the different immunoregulatory effects among genetically related LAB strains. Studies in swine, which are often used as a model for organ transplantation in humans, have examined intestinal immunoregulation by the probiotic LAB in food products. Efforts have also been made to develop an immunoassay system for probiotic LAB DNA and cell wall components to evaluate immunoregulation by the LAB, with the aim of designing functional food products. In this review we present our current research on swine intestinal immunity as a human model and trends in the potential applications of immunobiotic LAB with immunoregulation mediated via TLR.

Immunoassay system for probiotics

We have shown that cell wall components and DNA motifs from immunobiotic LAB can induce the immunoactivation of GALT. In addition, TLR9 is strongly expressed in the GALT. These findings demonstrate that TLR9 is able to recognize DNA from pathogenic bacteria, commensal bacteria and dietary LAB, thereby contributing to immunoregulation in the GALT. Establishing molecular immunoassay systems for these functional components will enable researchers to evaluate both the harmful effects of pathogenic bacteria and the beneficial effects of dietary LAB. This will also be an important tool in the development of physiologically functional food products such as Food for Specified Health Uses (FOSHU). Functional studies must ultimately be conducted on human subjects, but basic research using animal cells and experimental animals is also essential. Therefore, to develop a molecular immunoassay system for functional cell wall components and DNA motifs from probiotic LAB, especially immunobiotics, we constructed a transfectant of TLR9 with mammalian cells (Shimosato *et al*. 2005). The immunoassay system for intact LAB DNA that we developed is a three-step process for screening the TLR9 mediated immunostimulation of various DNA motifs by evaluating uptake, transcription activity of the intracellular signaling molecule NF-κB and cytokine induction (Figure 1). The cytokine assay combines both quantitative polymerase chain reaction and enzymelinked immunosorbent assay to provide accurate evaluation of functional activity. This enables screening for factors with potent immunoactivity from various cell wall components and DNA sequences in immunobiotic LAB. Elucidation of the TLR9 mediated immune response mechanism to immunobiotic DNA is essential for the future development of vaccine adjuvant using normal microflora and dietary LAB. This system could help in the development of new physiologically functional foods contributing to immunoregulation such as the maintenance of Th1/Th2 balance.

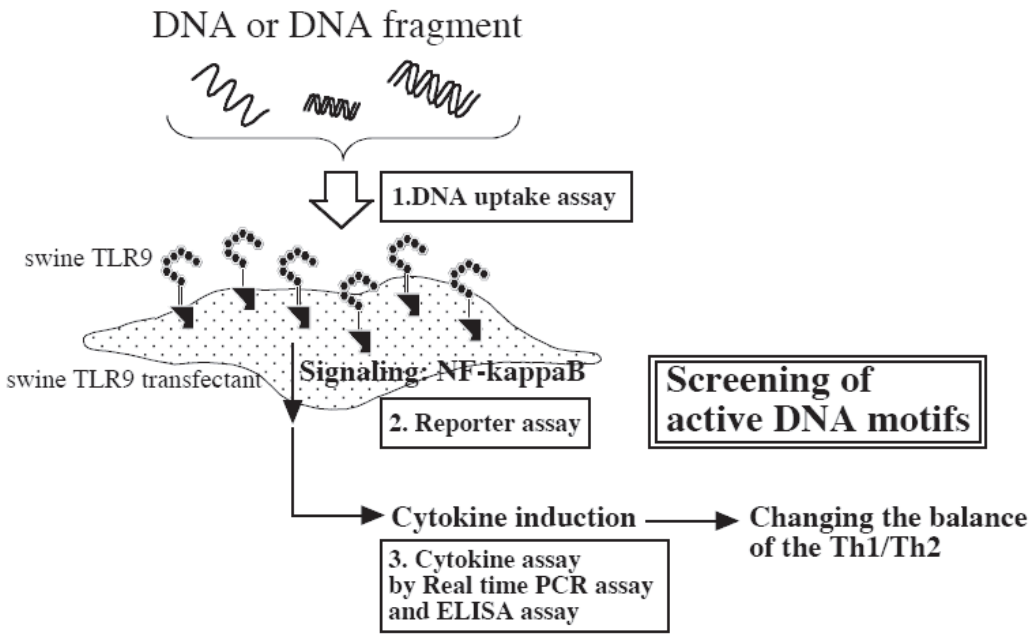

Figure 1. Possible functional assay for immunobiotic DNA using TLR9 transfectant

Discovery of immunostimulatory DNA from probiotics

We have proposed immunogenics that include extracellular and intracellular bacterial components with immunoregulatory abilities such as extracellular phosphopolysaccharides (EPS), peptideglycan, lipoteichoic acid and DNA. By using the constructed molecular immunoassay system via TLR mentioned above, we tried to find certain immunogenics in immunobiotics. Up to date, several immunostimulatory DNA sequences have been found in genomic sequences of immunobiotic LAB as summarized in Table 1. As the ligands for TLR9, bacterial DNA and synthetic oligodeoxynucleotide (ODN) containing unmethylated CpG-ODN have been known as specific sequence contexts for the TLR9 ligand. We found that not only CpG ODN but also ODN containing ATTTTTA core sequence (AT-ODN), which contained no CpG sequences, could augment Th-1 type immune responses via TLR9 (Shimosato *et al*. 2005). Moreover, the core sequence of 5′-ATTTTTA-3′ in AT-ODN from *L. gasseri* JCM 1131^{T} genome was involved in inducing cytokines such as IL-12, IFN-α and IFN-γ via TLR9 (Shimosato *et al*. 2006). To find the strong immunostimulatory AT-ODN containing the core sequence, we searched and identified 280 kinds of AT-ODN in the genomic sequence of probiotic *L. gasseri* OLL2716 used in producing a new type of probiotic yoghurt, LG21. Mitogenicity and NF-kB gene reporting assays showed that 13 of the 280 AT-ODN were strongly immunostimulatory in the TLR9 transfectant. Of these, with respect to the induction of Th1-type cytokines, AT-ODN (LGAT-243) had the greatest activity and was more potent than the porcine prototype, ODN D25. We further found that a six-base secondary loop structure containing a self-stabilized 5′-C . . . G-3′ stem sequence is required for the potent immunostimulatory activity (Figure 2). Finally, we found that novel strong immunostimulatory non-CpG AT-ODN exists in the genome of probiotic LAB. These results show for the first time that a non-CpG, AT-ODN with a specific loop and stem structure, belongs to the ligands of TLR9 (Shimosato *et al*. 2006). Understanding how the TLR signaling pathway mediates the functional activity of immunogenics such as immunostimulatory ODN should help in the development of oral vaccines and physiologically functional foods that specifically target immune responses.

Table 1. Immunostimulatory ODNs from lactic acid bacteria and bifidobacteria

Lactobacillus bulgaricus NIAIB6	OLLB7: CGGCACGCTCACGATTCTTG
Lactobacillus rhamnosus GG	ID35: ACTTTCGTTTTCTGCGTCAA
Bifidobacterium longum BB536	BL7: GCGTCGGTTTCGGTGCTCAC
Lactobacillus gasseri JCM1131^T	AT5ACL: TATAATTTTTACCAACTAGC
Lactobacillus gasseri OLL2716	LGAT243: TTAACAATTTTTACCCAAGA
Streptococcus thermophilus ATCC19258	MsST: CAGGACGTTGTATCACTGAA

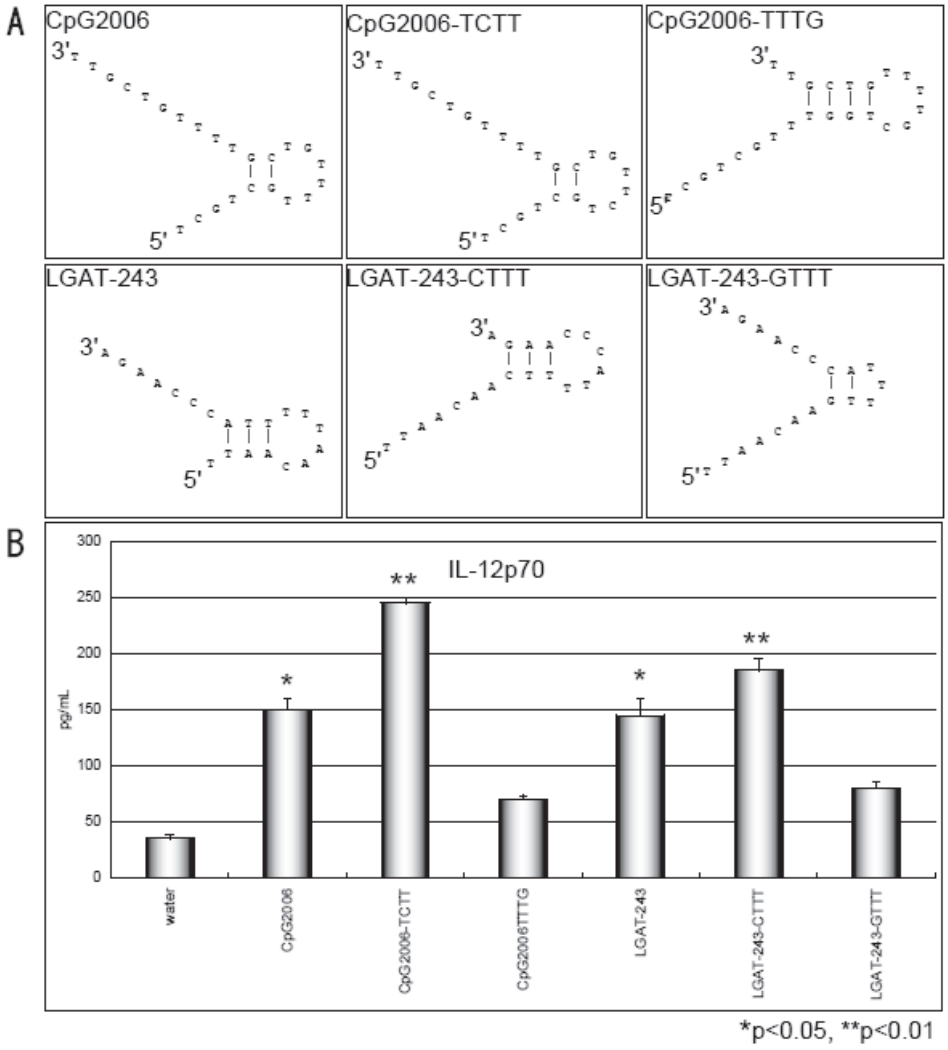

Figure 2. (A)Secondary structural analysis of immunostimulatory CpG ODNs (variants of CpG2006) and non-CpG ODNs (variants of LGAT-243). Secondary structures as determined by GENETYX. (B)The mitogenic activity of CpG2006 and LGAT-243 variants with a substitution in the loop of a T by an A, G, or C. IL-12p70 production in human PBMCs stimulated with various ODNs. Error bars represent standard deviations. All experiments were repeated three times using hPBMC from different donors. ***p<0.01* and **p<0.05 vs.* identically treated cells cultured in the absence of ODNs.

Future trends in immunobiotic LAB DNA

LABs are technologically and commercially important and have also various beneficial effects on the improvement of gut health through the control of intestinal ecosystem. In many studies whole cells, including live and heat-killed cells, cell wall and cytoplasmic fractions of LAB have been shown to have various biological functions. The surface cell wall properties of LAB are especially important in fermentation technology, but they are also thought to play an important role in the immunoregulation of the host. In addition to the cell wall components, DNA in the cytoplasmic fractions has been shown to be a major immunoregulatory substance. An initial report in 1988 by Tokunaga on the anti-tumor effects of *Mycobacterium bovis* BCG DNA, and a later report in 1995 by Krieg on stimulation of B cells by CpG DNA motifs, has prompted keen interest in immunoactivation by microbial DNA sequences. Further understanding of the effects of CpGDNAon the immune system came in 2000 with the identification of TLR9 as the certain receptor molecule for CpG DNA one of the non-CpG ODN, exerts potent immunostimulation in humans as well as in swine rather than mice. This mandates the use of animal models other than mice to evaluate the immune effects of ODN for use in humans. Immunologically functional food factors are thought to regulate intestinal immunity through the contact and stimulation of immunocompetent cells in the gastrointestinal tract and regulate cytokine networks through induction of cytokine production. In this new world of food immunology, however, much remains unknown about the underlying mechanisms of intestinal mucosal immunity. Accordingly, many details remain unclear about the effects of food components including immunogenics on intestinal immune responses. The findings in our research activities may provide important clues at the molecular level on TLR signal transduction pathways and recognition mechanisms. They also provide the impetus to further delineate the activation mechanism of the innate immune response. In addition, identification of immunobiotic LAB strains with certain immunogenics substances such as cell wall components and DNA, and better understanding of intestinal immunoregulation by cytokine networks hold promise in basic research and development of immunobiotic foods to prevent allergies, infectious and inflammatory diseases. This also can benefit humankind by offering immunobiotic foods as a safer alternative to conventional drug therapy.

Acknowledgements

Financial support for this study was provided by the Special Coordination Funds for Promoting Science and Technology of the Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government.

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