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Effect of Human Milk Oligosaccharides on Messenger Ribonucleic Acid Expression of Toll-like Receptor 2 and 4, and of MD2 in the Intestinal Cell Line HT-29

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Abstract: It is believed that human milk oligosaccharides (HMO) act as receptor analogues that inhibit the attachment of pathogenic microorganisms to the infant colon, but the direct involvement of HMO in anti infection has not as yet been demonstrated. The present study was conducted to clarify whether HMOs as well as commercially available galacto oligosaccharides (GO), from which lactose has been removed, affect the expression of toll-like receptor (TLR) 2 and 4, and of MD2 mRNA in the HT-29, human colonic cell line. HT-29 cells were treated with neutral human milk oligosaccharides (nHMOs), which had been separated from the acidic oligosaccharide fraction and also from lactose, as well as with GOs. HT-29 cells were treated with specific oligosaccharides of major components of nHMO or GO. The mRNA expression of TLR2, 4 and MD2 were measured using Real-time RT-PCR normalized to glyceraldehydes 3-phosphate dehydrogenase (GAPDH) mRNA expression. The addition of nHMO affected the expression of TLR2. Treatment with 1.0 mg/mL of nHMO as well as with 0.5 mg and 1.0 mg/mL GO enhanced the mRNA expression of TLR4. In experiments with specific oligosaccharides, treatments with 3′-sialyllactose (3′-SL), 6′-sialyllactose (6′-SL) or 6′-galactosyllactose (6′-GL) increased the expression of TLR-2, while the administration of lacto-*N*-fucopentaose I (LNFP I), 3′-, 6′-SL or 6′-GL enhanced that of TLR4. These results suggest that HMOs as well as GOs had a direct effect on colonic epithelial cells, with induction of the mRNA expression of TLR.

Key words: human milk oligosaccharides, galacto-oligosaccharides, toll-like receptor, intestinal cell, HT-29 cells

The human milk oligosaccharide (HMO) fraction is the third largest solid component in milk/colostrum, after lactose and lipids. The maximum concentration of HMO is observed in colostrum (more than 20 g/L), while after about two weeks of lactation, this falls to about 12–14 g/L in milk.[1)] It is thought that human milk has a high content of neutral oligosaccharides, especially fucosyloligosaccharides.[2,3)]

Several studies on HMO have suggested that most of these substances remain undigested in the small intestine,[4–7)] and thus reach the colon where they stimulate the growth of colonic microorganisms beneficial to human health, specifically bifidobacteria and lactobacilli.[8–11)] The colonic microflora of the breast-fed infant contains more than 90% bifidobacteria and lactobacilli,[12)] whereas formula-fed infants are more often colonized by *Escherichia coli, Clostridium difficile* or Bacteroides.[13)]

In addition, other functions have been attributed to HMO, including serving as a component of innate immunity by preventing the attachment of potential pathogens to the intestinal tract.[14)] For example, some mainly fucosylated HMOs have been found to act as receptor homologs, inhibiting the binding of pathogens, such as enteropathogenic *E. coli, Campylobacter jejuni, Vibrio cholerae* and *Salmonella fyris*, to their host receptors.[15–18)]

A direct effect of HMOs or commercially available prebiotic oligosaccharides on the intestinal innate immune system has not, however, as yet been demonstrated. It has recently been reported that the feeding of prebiotic oligosaccharides (arabino-galactan, short-chain fructo-oligosaccharide, iso-malto-dextrins, *etc*.) together with bifidobacteria (*Bifidobacterium bifidum* and *B. longum* subsp. *infantis*) has effects that may alter the course of rotavirus disease in BALB/c mice.[19)] This suggests that these prebiotic saccharides not only promote the growth of colonic bifidobacteria, but also act as a direct regulator of the intestinal innate immune system.

All microorganisms contain distinct structures or microbe-associated molecular patterns that are recognized via pattern recognition receptors (PRRs). It is well known that an important class of PRRs is TLRs. The TLRs, TLR

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1−10, were discovered as homologues of Drosophila Toll proteins, playing an important role in differentiation, cellular/molecular mediation of bacterial-epithelial crosstalk and defense.[20]

Among the TLRs, TLR2 and 4 recognize different bacterial cell wall components. Recent data indicate that TLR 2 is mainly involved in responses to cell wall components of gram-positive bacteria, while TLR4 has a role in the recognition of gram-negative bacterial compounds.[21,22] TLR2 is required for the recognition of Gram-positive constituents, including bacterial lipopeptide, lipoteichoic acid, peptidoglycan and soluble tuberculosis factor.[21−24] TLR4, on the other hand, has been shown to be required for the recognition of lipopolysaccharide (LPS), which is mainly a gram-negative bacterial wall component. Mutations or absence of TLR4, in addition, abolish the response to LPS.[25,26] Further studies have shown that the MD2 protein is also required for effective LPS signal transduction, forming a complex with the extracellular domain of TLR4.[27,28]

The present studies were done to clarify whether HMOs affect the expression of TLR2, 4 and MD2 mRNA in HT-29, an intestinal cell line. This approach may help to elucidate a novel mechanism for the anti- pathogenic effect of HMO. Their expression in HT-29 as affected by HMO was compared with the effects of a commercially available GO mixture of prebiotic saccharides.

Table 1. The oligosaccharides used in this study.

	Oligosaccharides
Neutral human milk oligosaccharide (nHMO)	2′-Fucosyllactose*
	3-Fucosyllactose
	Difucosyllactose
	Lacto-*N*-tetraose (LNT)*
	Lacto-*N*-neotetraose (LNnT)
	Lacto-*N*-fucopentaose I (LNFP I)*
	Lacto-*N*-fucopentaose II (LNFP II)
	Lacto-*N*-fucopentaose III (LNFP III)
Galacto-oligosaccharide (GO)	3′-Galactosyllactose
	4′-Galactosyllactose
	6′-Galactosyllactose

*Major oligosaccharides.

Specific oligosaccharide	Structure
2′-Fucosyllactose (2′-FL)	Fucα1-2Galβ1-4Glc
3-Fucosyllactose (3-FL)	Calβ1-4[Fucα1-3]Glc
Lacto-*N*-tetraose (LNT)	Galβ1-3GlcNAcβ1-3Galβ1-4Glc
Lacto-*N*-fucopentaose I (LNFP I)	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc
3′-Sialyllactose (3′-SL)	Neu5Acα2-3Galβ1-4Glc
6′-Sialyllactose (6′-SL)	Neu5Acα2-6Galβ1-4Glc
Sialyllacto-*N*-tetraose c (LSTc)	Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc
4′-Galactosyllactose (4′-GL)	Galβ1-4Galβ1-4Glc
6′-Galactosyllactose (6′-GL)	Galβ1-6Galβ1-4Glc

Glc, glucose; Gal, galactose; Fuc, fucose; GlcNAc, *N*-acetylglucosamine; Neu5Ac, *N*-acetyl neuraminic acid. *Major oligosaccharide.

MATERIALS AND METHODS

Oligomate 55 (lot YPS1214N), GO, was kindly supplied from Yakult Pharmaceutical Industry Co., Ltd. (Tokyo, Japan). 2′-fucosyllactose (2′-FL), 3-fucosyllactose (3-FL), Lacto-*N*-tetraose (LNT), Lacto-*N*-fucopentaose I (LNFP I), Sialyllacto-*N*-tetraose (LSTc), 3′-sialyllactose (3′-SL) and 6′-sialyllactose (6′-SL) were purchased from Dextra Laboratories (England). 4′-Galactosyllactose (4′-GL) and 6′-galactosyllactose (6′-GL) were obtained from Dr. Tadashi Nakamura (Obihiro University); their purities were checked by high performance liquid chromatography (HPLC) after pyridylamination prior to the successive study[29] and were found to be 83.8 and 71.8% pure, respectively.

Preparation of oligosaccharide fraction from human milk. The components of nHMO and GO are shown in Table 1. Human colostrum samples were collected in Keiai Hospital from twenty-four healthy mothers during the first 3 days of lactation, as previously described.[29] We obtained informed consent from all the donors to use their samples, using the protocol approved by Obihiro University of Agriculture and Veterinary Medicine.

HMOs were isolated from colostrum as desribed by Gnoth *et al*.[7] and Kunz *et al*.,[30] with a few modifications. The colostrum was centrifuged at 3000 × *g* at 4°C for 30 min; the upper lipid layer was removed, the aqueous phase was decanted and then one volume was treated with two volumes of precooled 95% ethanol to precipitate the protein. The solution was mixed gently overnight, centrifuged at 3000 × *g* at 4°C for 30 min and the supernatant was freeze-dried. To remove sialyloligosaccharides, lactose and monosaccharides, 100 mg of the freeze-dried supernatant was dissolved in 1 mL H_2O, filtered through a 0.45-μm filter (Advantec Toyo Kaisha, Ltd., Tokyo, Japan) and applied to a BioGel P-2 fine column (particle size 45−90 μm, 250 cm × 4.6 cm i.d., Bio-Rad Laboratories, Hercules, USA). The elution solvent was water (flow rate 0.25 mL/min) and 5 mL fractions were collected. Saccharides were detected by refractive index measurements using Smart Chrom (KYA Technology, Tokyo, Japan). Finally, the whole nHMO fraction was isolated by filtration through a BioGel P-2 extrafine column (particle size < 45 μm, 250 cm × 4.6 cm i.d., Bio-Rad Laboratories) with water as the eluent, to remove lactose. The composition of all nHMO fractions was examined using HPLC after derivatization with 1-phenyl-3metyl-5pyrazolon and 2-aminopyridine in order to confirm.[29]

GO was separated from lactose, and the presence of each component was monitored as described previously for Oligomate 55 (Yakult Pharmaceutical Industry Co., Ltd.). To this end, Oligomate 55 was diluted with water, followed by freeze-drying, and 100 mg was applied to a Bio Gel P-2 extrafine column, to remove all traces of lactose. The components in the eluted fraction were monitored by reverse-phase HPLC with PA-labeling to confirm that the lactose in Oligomate 55 was removed successfully, and that the fraction contained 4′-, 6′- and 3′-GL, their proportions being approximately 60, 30, 10%, respectively.

Cell culture. HT-29, a human colonic carcinoma cell line (ATCC HTB-38, Lot number 3441400), was purchased from American Type Culture Collection (Manassas, USA). The cells were cultured as described by Furrie *et al*.,[31] with a few modifications. Cultures were maintained at 37°C in 5% CO_2 in 75 cm^2 tissue culture flasks

Table 2. Primer pairs of TLR2, TLR4, MD2 and GAPDH in the present studies.

Gene	Association number		Sequence (5′-3′)	Product size
TLR-2	U88878	Forward	GGGTTGAAGCACTGGACAAT	205
		Reverse	TGTTGTTGGACAGGTCAAGG	
TLR-4	U88880	Forward	CGTTTTATCACGGAGGTGGT	211
		Reverse	CCCCATCTTCAATTGTCTGG	
MD-2	AB018544	Forward	ATTGGGTCTGCAACTCATCC	236
		Reverse	GTCATCAGATCCTCGGCAAA	
GAPDH	M33197	Forward	GAGTCAACGGATTTGGTCGT	201
		Reverse	TGGGATTTCCATTGATGACA	

in Dulbecco's modified Eagle's medium, low glucose type (DMEM, Invitrogen Corporation, Carlsbad, USA), supplemented with 10% fetal bovine serum (FBS, HyClone, South Logan, USA), and 1% penicillin/streptomycin (Invitrogen Corporation). The cells were passaged at preconfluent densities using 0.05% trypsin (Invitrogen Corporation) twice before use.

Treatment of HT-29 cell lines with nHMO, GO, and specific milk oligosaccharides. HT-29 cells were plated in six-well dishes at an initial density of 1.0×10^6/wells and cultured at 37°C in 5% CO_2 in DMEM with 10% FBS. Once confluent, the cells were washed two times with Dulbecco's phosphate-buffered saline (−) (PBS) at 37°C, and left for 24 h in DMEM without FBS. The cells were stimulated with 0.5 mg or 1 mg/mL of nHMO and GO in DMEM without FBS for 24 h. In other experiments, the cells were treated with 40 μM (40 nmol/well) of each specific milk oligosaccharide. After removal of the media, the cells were washed two times with PBS.

Real-time RT-PCR analysis of TLR2, 4 and MD2 expression in HT-29. The total RNA was extracted from HT-29 cells using ISOGEN according to the manufacturer's instructions. cDNA was synthesized using the Exscript RT reagent kit (Takara Bio Inc., Tokyo, Japan) from 2 μg of total RNA diluted with 40 μL of EAZY Dilution (Takara Bio Inc.). Those were quantified at 260 nm and checked for quality by 260/280 nm ratio.

Quantitative real-time PCR for TLR2, 4, MD2 and GAPDH was performed using 5 μL of the cDNA as template in a final volume of 50 μL by ABsolute QPCR SYBR Green Mix (NIPPON Genetics Co., Ltd., Tokyo, Japan) with a 7300 Real-Time PCR system (Applied Biosystems, Foster City, USA). Primer pairs of TLR2, 4, MD 2 and GAPDH used in PCR were obtained from Sigma-Aldrich Corporation (St. Louis, USA), as shown in Table 2. After an initial denaturation at 95°C for 10 min, PCR reactions for all amplifications were conducted using 40 cycles at 95°C for 30 s, 59°C for 30 s and then 72°C for 30 s, and the measured values were normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression. The PCR reactions were performed in triplicate, and each PCR product size was checked using 2% agar gel electrophoresis.

Statistical analysis. All results are expressed as a mean plus standard deviation. Statistical analysis was done using JMP 6.03 software (SAS Institute Inc., Cary, USA). Data ($n = 6$) on the treatment of HT-29 with nHMO or GO were subjected to one-way analysis of variance for comparison of means, and significant differences were calculated using the Tukey-Kramer test. Significant differences between the results using specific oligosaccharides and those with controls (medium only) were determined using Dunnett's test.

RESULTS AND DISCUSSIONS

To our knowledge, this is the first study showing a direct effect of nHMO and GO on the innate immune systems of colonic cells. Although there have been many studies suggesting the prevention of attachment of pathogens to the colonic mucosa,[32] there have been no experiments investigating the influence of HMO or of specific milk oligosaccharides on the gene expression of TLR2, 4 and MD2 on intestinal cells such as HT-29 cells. As the unresponsiveness of Caco-2, the major colonic cell line, has been shown to LPS stimulated TLR4 expression,[33] we used only the HT-29 cell line in these experiments. According to the result of this study, the gene expressions of TLR2, 4 and MD2 in HT-29 were affected by 0.5 and 1.0 mg/mL of nHMO or GO administration. Further study is required to establish the most suitable concentration of HMO that has effects on the intestinal cell line.

The effects of nHMO or GO administration on gene expression of TLR2, 4 and MD2 in HT-29.

The mRNA expression of TLR2, 4 and MD2 in HT-29 after the treatments with nHMO or GO are shown in Fig. 1. The addition of 1 mg/mL of nHMO remarkably increased the mRNA expression of TLR2 compared with the control and that of GO (Fig. 1 (A)). It is known that the concentration of nHMO in colostrum or mature milk is much higher than that of acidic oligosaccahrides.[1] It has been reported that various microbial cell wall constituents act as signals via TLR2. Representative examples of these constituents are lipoteichoic acid and peptidoglycan, which are cell wall constituents of some bifidobacteria and lactobacilli.[34] Our results suggest that nHMO may affect the bifidus flora of the infant colon through the enhancement of TLR2 expression.

Treatment with 0.5 mg or 1.0 mg/mL of GO, and with 1.0 mg/mL of nHMO enhanced the gene expression of TLR4 (Fig. 1 (B)). Administration of nHMO at 1.0 mg/mL enhanced the gene expression of TLR4 in HT-29 cells more than that at 0.5 mg/mL. Although two dose treatments with GO maintained the stimulation at high expression, there were no differences between the treatment at

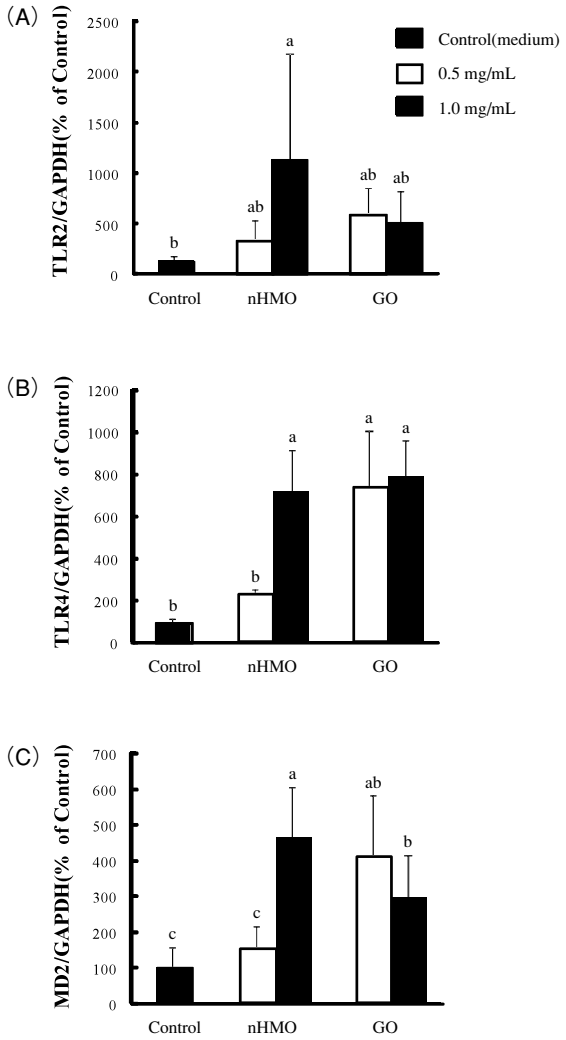

Fig. 1. The effect of the nHMO or GO administration on the mRNA expressions of TLR2, 4 and MD2 in HT-29 cell.

Treatment of 0.5 mg/mL of nHMO or GO is shown as open bars (n = 6) and that of 1.0 mg/mL is shown as solid (n = 6). Values are the means of percentage of control as 100%, and vertical lines represent the SD. Significant differences, which were determined by the Tukey-Kramer test, are shown by different superscript letters (p < 0.05). Abbreviations in this figure are explained in Table 2.

0.5 mg/mL and at 1.0 mg/mL. These results raise the possibility that GO was more effective in increasing TLR 4 expression at lower levels than nHMO. It has been shown that TLR4 plays a role in the recognition of the gram-negative bacterial substance, LPS.[22] In addition, some reports have suggested that TLR4 induces the activation of nuclear factor-κB and the expression of the pro-inflammatory cytokines in human intestinal cells.[35–37] The administration of nHMO and GO may evoke such immunoreactions in the intestine, by induction of the expression of TLR4.

The expression of MD2 after the addition of 0.5 mg/mL of GO was higher than that of 1.0 mg/mL GO (Fig. 1(C)). The administration of nHMO and GO significantly increased the expression of MD2 in HT-29 cells, similar to that of TLR4. However, a different result was observed with MD2 in that addition of 0.5 mg/mL of GO produced higher expression than 1.0 mg/mL. This indicated that treatment with GO at 0.5 mg/mL was adequate for the largest expression of MD2 under our experimental conditions. MD2 is a protein that is located outside TLR4 on the cell surface.[38] TLR4 without the expression of MD2 alters inactive pro-inflammatory cytokine gene expression in response to LPS;[27,39] this shows that MD2 has significant effects on innate immunity. Since these oligosaccharide fractions were shown to have a stimulating effect on MD2 expression as well as on TLR4 expression, they may have a role in preventing the invasion of the cells by gram-negative bacteria.

The effects of specific oligosaccharides that are major components of HMO and GO on gene expression of TLR2, 4 and MD2 in HT-29 cells.

According to our previous studies,[29] the major components of HMO in colostrum are 2′-FL, LNT and LNFP I. Their percentages are about 20, 15 and 20%, respectively. Thus, it was concluded that 0.5 mg/mL of the nHMO fraction contained LNFP I (Mw 853.7) at about 100 mM in this study. The concentration of nHMO at 10 μg/mL, which contained 2 μg/mL (2.34 μM) or less of LNFP I, and 1 μg/mL of acidic human milk oligosaccharide fraction (aHMO), used by Eiwegger *et al*.[40] in their experiment must have been within physiological conditions. Interestingly, they found that the aHMO, containing 3′-, 6′-SL, and LSTc, was more effective in cytokine production and activation of cord blood derived T cell *in vitro* than nHMO.

Treatments with 3′-, 6′-SL or 6′-GL increased the expression of TLR2 in HT-29 cells (Fig. 2 (A)). In the experiments on the administration of specific oligosaccharides that are components of HMO and GO, we observed that 3′-, 6′-SL as well as 6′-GL stimulated the expression of TLR2. Although nHMO was found to have a remarkable influence on TLR2 expression, the administration of specific components of nHMO, such as 2′-FL, LNT and LNFP I, had no effect. Administration of 40 μM of each specific oligosaccharide used in this experiment might not have been enough to affect the mRNA expression of TLR 2, while 40 μM treatment with specific acidic oligosaccharides used in this study should be enough to affect TLR2 mRNA expression, as mentioned above. Velupillai *et al*.[41] have reported that some minor oligosaccharides of human milk, such as lacto-*N*-fucopentaose III (LNFP III) and lacto-*N*-neotetraose (LNnT), had an effect on murine Interleukin-10 secretion. This suggests that these minor oligosaccharides in nHMO may affect the expression of TLR2 in HT-29 cells.

Treatment with LNFP I, 3′-, 6′-SL or 6′-GL enhanced the expression of TLR4 (Fig. 2 (B)). In this study, 3′- and 6′-SL stimulated the expression of both TLR2 and 4. These results might have indicated some specific aHMO influences on innate immune systems of the human colon even at low concentration, compared with nHMO. Schumacher *et al*.[42] have shown interference by aHMO with specific P-selectin ligand binding, but not by nHMO. Similar results were also obtained by Bode *et al*.[43] who showed that an aHMO fraction decreased the formation of a complex between platelets and neutrophils.

Both human milk and bovine colostrum contain 3′- and 6′-SL. Although neutral oligosaccharides predominate over acidic oligosaccharides in human milk, there have

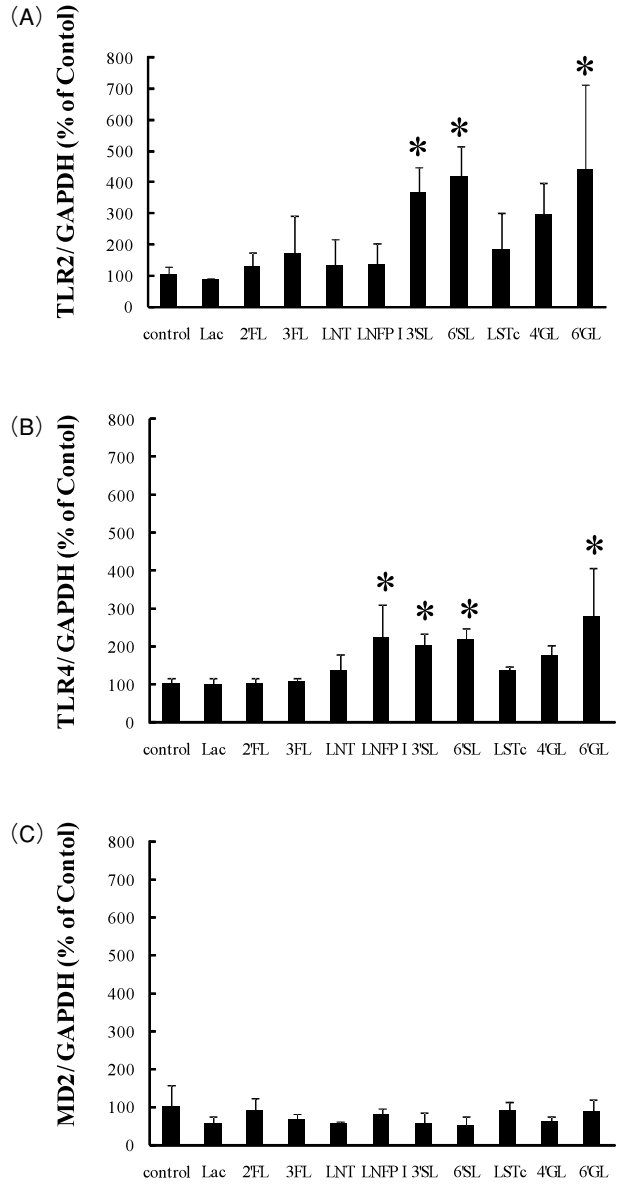

Fig. 2. The effect of the addition of 40 μM sole oligosaccharides on the mRNA expressions of TLR2, 4 and MD2 in HT29 cells.

Values are the means of percentage of control as 100%, and vertical lines represent the SD. Significant differences compared with control in Dunnet's test are shown by $^{*}p < 0.05$. Abbreviations in this figure almost are explained in Table 2, and Lac means lactose.

been some reports about various effects by the latter.[40,44–46] In bovine milk, acidic oligosaccharides constitute most of the oligosaccharide fraction.[47] Bovine milk is generally used for preparing artificial formulas, and there is a possibility that bovine colostrum can be used as a source of 3′- and 6′-SL, which are present at concentrations of about 1.0 and 0.2 g/L, respectively.[48]

There was no effect on the expression of MD2 resulting from the addition of any of these oligosaccharides (Fig. 2 (C)). In the experiment with nHMO or GO treatment, however, MD2 mRNA expression was affected. The administration of specific oligosaccharides at 40 μM might be the threshhold level to increase the expression of MD2 mRNA. This suggests that mRNA expression of TLR4 and MD2 are promoted by different concentrations of some specific oligosaccharides. Lactose, the most dominant saccharide in milk, had no effect on these gene expressions in the present study.

Although nHMO had a significant effect on the stimulation of TLR4 expression, in the experiments with specific oligosaccharides only LNFP I at 40 μM had this effect. In future experiments, several concentrations of each oligosaccharide should be used to determine whether they affect expression of TLR4 in a dose-dependent manner. In addition, a further study is necessary to investigate in the signal pathway of HMO, GO and specific oligosaccharides for TLR expression.

The result of this study suggests that nHMO may contribute to infant colonic health induced by the increasing in the expression of TLR2, 4 and MD2.

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腸管細胞株 HT-29 における ヒトミルクオリゴ糖の Toll 様受容体 2，4 および MD2 遺伝子発現に及ぼす影響

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ヒトミルクオリゴ糖は新生児の腸管において，病原菌や毒素に結合することで感染を防ぐという間接的作用が明らかになっている．一方，ヒトミルクオリゴ糖 (HMO) の腸管への直接的作用はほとんど研究されていない．そこで本研究は，主要な HMO である中性ヒトミルクオリゴ糖画分 (nHMO)，対照としての市販のガラクトオリゴ糖画分 (GO)，さらにはそれぞれの画分を構成するオリゴ糖単独の腸管免疫調節への直接的作用を検討するため，これらの投与がヒト腸管細胞株 HT-29 における Toll 様受容体 2，4 (TLR2，4) および MD2 の遺伝子発現に及ぼす影響について検討した．ヒト腸管細胞株 HT-29 を 1×10^6/well で培養し，それぞれのオリゴ糖画分を 0.5 mg/mL，1.0 mg/mL で，単独のオリゴ糖では 40 nM で投与し，24 時間後に細胞を回収した．細胞における TLR2，4 および MD2 mRNA 発現を，逆転写 Real Time PCR を用いて比較検討した．グラム陽性菌の構成成分を認識する TLR 2 遺伝子発現において，nHMO 1 mg/mL 投与のみが有意な増加 ($p < 0.05$) を示した．また，グラム陰性菌の構成成分を認識する TLR 4 および MD 2 遺伝子発現においては，nHMO 1 mg/mL ならびに GO 0.5 および 1.0 mg/mL 投与により有意な増加 ($p < 0.05$) がみられた．単独のオリゴ糖投与では，TLR2 遺伝子発現においては，3′-シアリルラクトース (3′-SL)，6′-シアリルラクトース (6′-SL) そして 6′-ガラクトシルラクトース (6′-GL) が有意な増加を示した．TLR4 遺伝子発現においては，ラクト-*N*-フコペンタオース I (LNFP I)，3′-SL，6′-SL，6′-GL が有意な増加を示した．また，いずれのオリゴ糖投与においても，MD2 遺伝子発現に対する影響は認められなかった．nHMO の TLR 遺伝子発現への作用が明らかになり，このことはグラム陽性菌やグラム陰性菌に対する腸管免疫機能に関わる可能性も考えられる．