ORIGINAL ARTICLE

Maltooligosaccharides from JEG-3 Trophoblast-Like Cells Exhibit Immunoregulatory Properties

Aiping Zhu[1], Roberto Romero[2,3], Ji-Biao Huang[1], Andrea Clark[1], Howard R. Petty[1]

[1]Departments of Ophthalmology and Visual Sciences and Microbiology and Immunology, The University of Michigan Medical School, Ann Arbor, MI, USA;
[2]Perinatology Research Branch, Division of Intramural Research, Eunice Kennedy Schriver National Institute of Child Health and Human Development, NIH, Bethesda, MD and Detroit, MI, USA;
[3]Center for Molecular Medicine and Genetics, Wayne State University, and Hutzel Women's Hospital at the Detroit Medical Center, Detroit, MI, USA

Keywords
Immune regulation, leukocytes, saccharides

Correspondence
Howard R. Petty, Department of Ophthalmology and Visual Sciences, 1000 Wall Street, The University of Michigan Medical School, Ann Arbor, MI 48105, USA.
E-mail: hpetty@umich.edu

Submitted February 18, 2010;
accepted March 4, 2010.

Citation
Zhu A, Romero R, Huang J-B, Clark A, Petty HR. Maltooligosaccharides from JEG-3 trophoblast-like cells exhibit immunoregulatory properties. Am J Reprod Immunol 2011; 65: 54–64

doi:10.1111/j.1600-0897.2010.00851.x

Problem
To better understand the immunoregulatory properties of trophoblasts, we have searched for small immunologically active carbohydrates derived from intact trophoblast-like cells.

Method of study
Using solid phase extraction coupled with HPLC and mass spectrometry methods, we have characterized a low molecular weight carbohydrate-rich fraction associated with JEG-3 cells. We have also tested the bioactivities of selected authentic oligosaccharides found in the oligosaccharide fraction.

Results
The most abundant components of the low molecular weight carbohydrate-rich fraction were maltotriose and maltotetraose, with detectable amounts of maltopentaose. When authentic maltooligosaccharides were tested using lymphocytes, IL-2 inhibition was observed. This activity was dependent upon the number of saccharide subunits, stereochemistry, and concentration. To further test maltooligosaccharide properties, maltopentose was attached to glass cover slips. Although spontaneous neutrophil motility was observed on unmodified and control surfaces, it was inhibited on maltooligosaccharide-derivatized surfaces.

Conclusion
Maltooligosaccharides are associated with the trophoblast's surface where they may exhibit immunoregulatory activities.

Introduction

Carbohydrates are broadly important molecules in cells and tissues that provide structural assemblies, metabolic energy, and store information as disparate as blood group, intercellular recognition signals, and trafficking commands within their sequences.[1] Cell surface saccharides are well-known regulators of cell-to-cell interactions and downstream signal transduction events in developmental and host defense pathways.[2,3] For example, one mechanism regulating leukocyte trafficking is mediated by selectins, which are lectins expressed by leukocytes, endothelial cells, platelets, and other cell types.[4] Selectins recognize the terminal tetrasaccharide sequence: NeuAcα2 → 3Galβ1 → 4[Fucα1 → 3]GlcNAcβ1 (sLe^X). Selectins mediate initial leukocyte–endothelial cell contact leading to tight integrin-mediated contact

and subsequent extravasation.[4] Other cell surface receptors such as complement receptor type 3 and dectin-1 bind to polysaccharides and oligosaccharides known as β-1,3-D-glucans, which are found in yeast cell walls.[5] Hence, oligosaccharides play central roles in adaptive and innate immune responses with relevance to infectious diseases, autoimmunity, and cancer.[6,7]

An important physiological niche exhibiting modified leukocyte function is the maternal–fetal interface. For example, trophoblasts have been shown to protect the conceptus from destruction by activated macrophages.[8] Moreover, macrophages activated by multiple mechanisms display cytotoxicity against tumor cell targets, but not trophoblasts.[9] Included among the various molecules proposed to influence leukocyte activation in pregnancy are carbohydrates. Previous studies by Arkwright et al.[10] demonstrated that neutral oligosaccharides of syncytiotrophoblast membranes suppress allogeneic reactivity using the mixed lymphocyte reaction assay. Muchmore et al.[11] have suggested that the disaccharide α-D-Man-(1 → 6)-α-D-Man, derived from pregnancy urine, is capable of inhibiting early events during lymphocyte proliferative responses. We have found that maltooligosaccharides can be released from JEG-3 trophoblast-like cells, and that these saccharides reduce IL-2 production by stimulated Jurkat lymphocytes at concentrations expected for interfacial cell–cell interactions. Furthermore, when attached to substrates, maltooligosaccharides reduce the spontaneous motility of neutrophils. We speculate that maltooligosaccharides expressed at the trophoblast's surface have immunosuppressive characteristics.

Materials and methods

Materials

Maltotriose, maltotetraose, maltopentaose, cellotriose, isomaltotriose, and panose, were obtained from Sigma-Aldrich (St. Louis, MO, USA). Alexa-488-conjugated phytohemagglutinin (PHA) was obtained from Molecular Probes (Eugene, OR, USA).

Neutrophils

Peripheral blood was collected from healthy human donors in compliance with the guidelines of the University of Michigan Institutional Review Board for Human Subject Research. Neutrophils were isolated using Ficoll-Histopaque (Sigma) density gradient centrifugation, resuspended and washed in PBS by centrifugation.

Lymphocytes

Jurkat cells (ATCC, Manassas, VA, USA) were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% FCS and 1% antibiotics.

JEG-3 Trophoblasts

JEG-3 cells, derived from human choriocarcinoma, were obtained from the ATCC. JEG-3 cytotrophoblast-like cells were grown in RPMI 1640 containing 10% FCS and 1% penicillin G/streptomycin/amphotericin B (Invitrogen). The cell line was occasionally replaced with the original cell line as a quality control measure.

Cell Treatment

As many as 10–20 tissue culture flasks were used in each treatment. JEG-3 cells on tissue culture plates were washed with PBS (without Ca^{2+} and Mg^{2+}; Invitrogen) then incubated with PBS (without Ca^{2+} and Mg^{2+}) on a shaker at 37°C for 24 hr to non-lytically promote the release of glycocalyx materials without cell debris. Alternatively, in some experiments cells were incubated with recombinant PNF-ase F (BioLabs, Boston, MA, USA) (10 min at 37°C) to promote the release of these materials. After the supernatant was collected from multiple flasks, the combined supernatants were ultrafiltrated with CentriPlus filter (YM-10; Millipore, Billerica, MA, USA), which has a molecular weight cutoff of 10 K, to isolate a low molecular weight fraction. As negative controls, other cell lines (e.g., Jurkat cells) were subjected to the same experimental conditions. The samples were stored at −80°C until used.

Concentration and Desalting of Samples

A non-porous graphitized carbon column (4 mL, Carbograph SPE; Alltech, Deerfield, IL, USA) was pre-activated with 2 mL of 25% MeOH and 2 mL of water. Thirty milliliters of sample was passed through the column at a flow rate of 1 mL/min. The column was washed with water to remove salts and then eluted with 2 mL of 25% MeOH in 10 μM NH_4OAc to obtain the oligosaccharide fraction.[12,13] The eluate was concentrated to 0.5 mL in a Speed-Vac evaporator (Thermo Fisher Sci., Waltham, MA, USA).

Electrospray Mass Spectrometry

Electrospray ionization mass spectrometry (ESI-MS) spectra were recorded on a ThermoElectron Finnigan LTQ linear ion trap mass spectrometer equipped with an electrospray ionization source. Full scan mass range m/z was 100–2000. For positive-ion mode MS experiments, the ESI voltage was set at 3.2 kV, the capillary voltage at 25.5 V, and the tube lens voltage at 131 V. For negative mode MS experiments, the ESI voltage was set at −2.1 kV, the capillary voltage at −25 V, and tube lens voltage at −79 V. The capillary temperature, sheath gas, and auxiliary gas were 250°C, 20 units, and 5 units, respectively, for both modes.

High-Performance Anion-Exchange Chromatography (HPAEC) Analysis

The chromatography system was a Dionex (Sunnyvale, CA, USA) BioLC, which included a GP50 gradient pump and an ED50A electrochemical detector. The standard carbohydrate waveform was used for ED50A. Oligosaccharides were separated on a CarboPac PA100 column (9 × 250 mm; Dionex) with 0.1 M NaOH as solvent A and 0.1 M NaOH/0.5 M NaOAc as solvent B. The following linear gradient was used: 0–12.5% B for 15 min, and then increased to 25% B for 25 min.

IL-2 Production

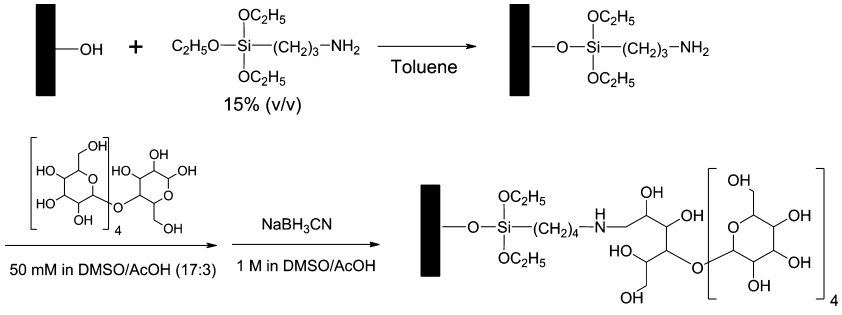
Aliquots of 10^6 cells/mL/well of Jurkat cells (clone E6-1) were treated with various concentrations of maltooligosaccharides (DP3, DP4, DP5, DP6; DP is the degree of polymerization), isomaltotriose, cellotriose, and panose (Sigma) in the presence or absence of the stimulant PHA (10 μg/mL) for 24 hr in a 5% CO_2 incubator at 37°C. Culture supernatants were collected for IL-2 measurement using a kit from R&D Systems (Minneapolis, MN, USA). Briefly, supernatants were incubated in the anti-IL-2 antibody-coated plates at room temperature for 2 hr; after washing, plates were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for another 2 hr before color generation. Optical density was measured by a Spectra Max 190 (Molecular Devices, Sunnyvale, CA, USA).

PHA Receptor Staining

Jurkat cells were pre-treated with 20 mM DP4 or DP5 at room temperature for 30 min followed by the addition of 10 μg/mL Alexa-488-PHA (Molecular Probes) for an additional 30 min. Microscopy was performed as described later.

Synthesis of Maltooligosaccharide Surfaces

As maltooligosaccharides are highly soluble in water and adsorb poorly to glass, we covalently linked DP5 to glass surfaces. The synthetic organic preparation was carried out as outlined in the Scheme 1. Briefly, glass cover slips (25 × 25 mm, Corning Glass Works, Corning, NY, USA) were cleaned for 30 min in 0.1 M NaOH followed by 30 min in concentrated H_2SO_4 then rinsed exhaustively with deionized water. After drying under a nitrogen flow, silanization was performed for 2 hr in 10% v/v 3-aminopropyltrimethoxysilane solution in toluene. The cover slips were rinsed sequentially with toluene, ethanol, and deionized water, respectively. After drying, the cover slips were treated with 10-mM DP5 or cellotriose (β-D-Glc-(1 → 4)-β-D-Glc-(1 → 4)-D-Glc) in DMSO/AcOH (17:3) for 2 hr and followed by 1 M $NaBH_3CN$ in DMSO for 3 hr. After the synthetic procedure was complete, the modified cover slips were washed with DMSO and deionized water in that order.



Scheme 1 Immobilization of DP5 on the surface of cover slips.

Neutrophil Spontaneous Motility

The spontaneous motility of neutrophils was assessed using time-lapse optical microscopy. Control or oligosaccharide-modified cover slips were employed. Cells were allowed to acclimate to the surface at 37°C for one hour. A low magnification field was then chosen to permit visualization of 50–60 cells. A time series of images was taken using a QImaging Retiga 1300 camera (QImaging, Surrey, BC, Canada) and a 40x/0.6 NA Nikon Plan Fluor objective (Nikon Inst., Inc., Melville, NY, USA). The series of photographs were taken at a rate of 1/min using MetaMorph software. Cell movement was scored over 30 min, with movement being defined as a cell moving at least 10 μm, or the approximate average diameter of a neutrophil. Cells were tagged using the manual count function in MetaMorph, and results were tabulated and plotted using Microsoft Excel.

Microscopy

Cells were observed using a Nikon Eclipse TE2000 Quantum inverted fluorescence microscope (Nikon Instruments, Inc., Melville, NY, USA) with mercury illumination interfaced to a computer using Metamorph (Molecular Devices, Danville, PA, USA) software. Images were taken with an Andor Technologies iXon model DV8 16-bit electron multiplying CCD camera cooled to −90°C (Andor Technologies, South Windsor, CT, USA). A filter module comprised of a D470/40x exciter, 505DCLP dichroic, and an E515LPv2 emitter was used for imaging Alexa-488-conjugated PHA. Time-lapse imaging was performed in transmitted bright field imaging using the Metamorph software without the electron-multiplying channel activated.

Results

Structural Studies

The low molecular weight carbohydrate-rich fraction obtained by using the protocol described earlier was analyzed by MS. In the positive-ion mode (Fig. 1a), there are two major ions at m/z 527.4 and 689.5, whereas in the negative-ion MS mode, more major ions were observed (503.1, 539.3, 563.1, 665.2, 701.2, 725.1, 677.3, 839.3, and 611.3) (Fig. 1b). When the positive-ion mode species at m/z 527.4 and 689.5 were further investigated using MS^2, they showed typical oligosacchride characteristics in their spectra (see Fig. 1c,d). In the negative-ion mode, we assign m/z 503.1 and 665.2 as a trisaccharide (M_1) and tetrasaccharide (M_2), respectively. Furthermore, the species at m/z 539.3 and 563.1 are $[M_1+Cl]^-$ and $[M_1+CH_3CHOO]^-$ and the species at 701.2 and 725.1 are $[M_2+Cl]^-$ and $[M_2+CH_3CHOO]^-$, respectively. In the positive mode, the species at m/z 527.2 and 689.2 are sodium adducts of M_1 and M_2. The component at m/z 611.3 is glutathiol (confirmed by multi-MS and HPLC). The species at m/z 677.3 and 839.3 also exhibit oligosaccharide characteristics; they are likely adducts of M_1 and M_2 (677.3 = $[M_1+173]^-$, 839.3 = $[M_2+173]^-$). As the m/z 527.4 peak of Fig. 1a appears as a breakdown product in the MS^2 spectrum of the m/z 685.5 component in Fig. 1d, as well as several smaller fragments, M_1 is a fragment of M_2. Therefore, we focused on the structural analysis of M_1.

Further experiments were performed to better understand the nature of M_1. The fragmentation pattern in positive-ion mode MS may not be structure-specific. For example, oligosaccharides may be protonated at several positions, resulting in a potential lack of linkage specificity. Consequently, most carbohydrate linkage determinations have been performed using negative-ion mode MS. However, in our experiments using Na^+ adducts, the positive-ion MS mode gave us more structural information. Moreover, the sensitivity was improved dramatically in comparison with the negative-ion mode. In these experiments, three commercially available trisaccharides containing glucose with differences in the anomeric carbon and linkage positions were selected as standard samples for comparison with M_1. These saccharides were maltotriose (α-D-Glc-(1 → 4)-α-D-Glc-(1 → 4)-D-Glc), cellotriose (β-D-Glc-(1 → 4)-β-D-Glc-(1 → 4)-D-Glc), panose (α-D-Glc-(1 → 6)-α-D-Glc-(1 → 4)-D-Glc), and isomaltotriose (α-D-Glc-(1 → 6)-α-D-Glc-(1 → 6)-D-Glc). Like M1, these trisaccharides only exhibit sodium adducts, because the sodiated ions are more stable than their protonated counterparts in positive-ion mode MS (Fig. 2). Fig. 2 shows that trisaccharides with different linkages (1 → 4 and 1 → 6) and anomeric configurations (α and β) show different fragmentation profiles. The molecular weight and MS^2 spectrum of maltotriose are identical to that of M_1, indicating the identity of these two molecules. These findings are summarized in Fig. 3, which shows the molecular fragments corresponding to the peaks given in Figs 1 and 2 as well as the measured abundances of these

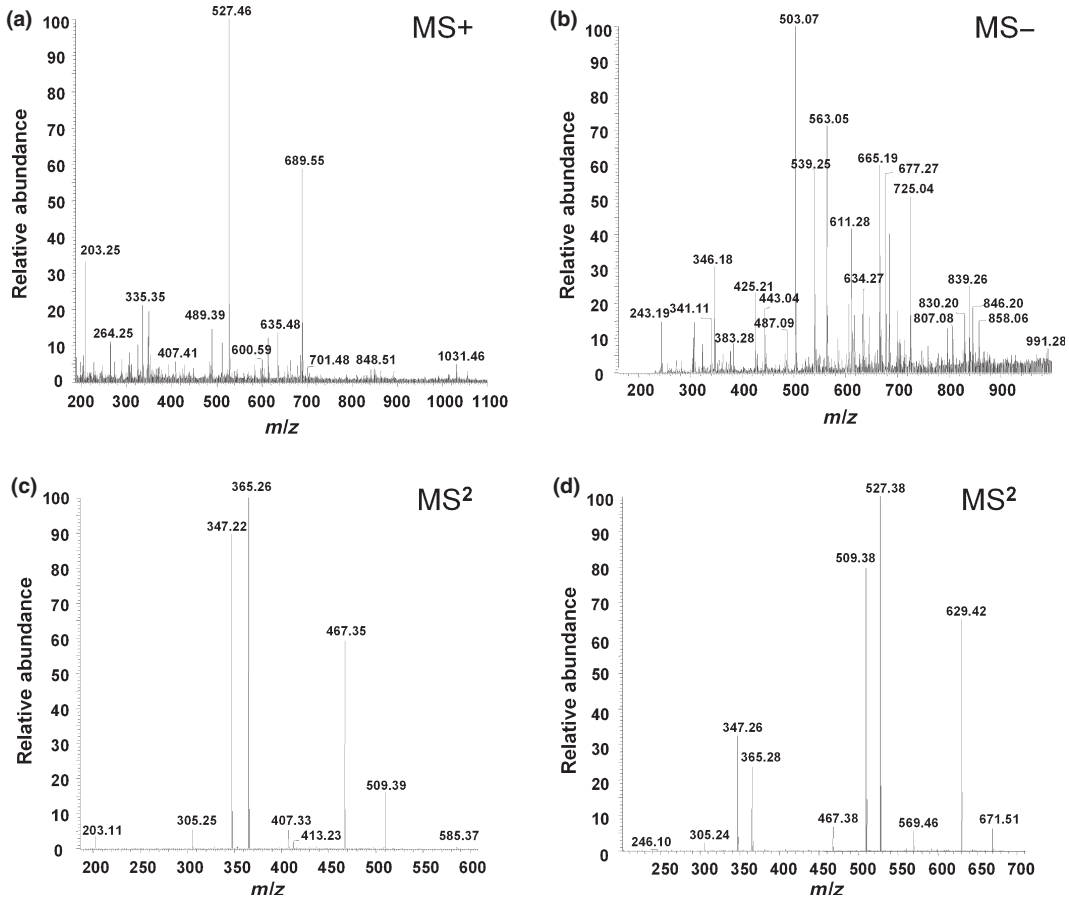


Fig. 1 Mass spectrometry of trophoblast oligosaccharides. Mass spectroscopy (MS) spectra of SPE fraction in the positive-ion mode (panel a) and negative-ion mode (panel b) are shown. In addition, MS^2 spectra of m/z 527.4 (panel c) and m/z 685.5 (panel d) in the positive-ion mode are given. The similarities in these fragmentation patterns in panels c and d suggest structural similarities in these two compounds.

various fragments for the listed panel of glucose-containing isomeric trisaccharides. These data further support the identification of M_1 as maltotriose.

To provide an independent line of evidence to test the correspondence of M1 with maltotriose, we performed HPAEC analyses for M_1, M_2, maltotriose, and maltotetraose under identical conditions. The chromatographs are shown in Fig. 4. Fig. 4a shows the separation of solid phase extraction (SPE) fraction. Two major peaks with retention times of 21.97 and 27.00 min are observed. These two peaks correspond to the most abundant molecules in the extract, namely M_1 and M_2. The retention times and the shapes of peaks match well with those of authentic maltotriose and maltotetraose (Fig. 4b,c). Hence, the two major compounds released from JEG-3 cells were maltotriose and maltotetraose, which were determined by electrospray mass spectroscopy (ESI-MS) and HPAEC. Although maltooligosaccharides were found to be associated with JEG-3 cells, it is possible that they originated in the culture medium then adsorbed to the cells. When the cell growth medium was examined by HPAEC, maltooligosaccharies could not be detected (data not shown), thus indicating that the maltooligosaccharides originate from the cells, not the culture medium. As a negative control experiment, we found that maltooligosaccharides could not be isolated from Jurkat cells (data not shown).

Functional Studies

Previous studies from other laboratories[10] have suggested that trophoblast oligosaccharides exhibit immunoregulatory capacity. To test the potential immunoregulatory capacity of maltooligosaccharides,

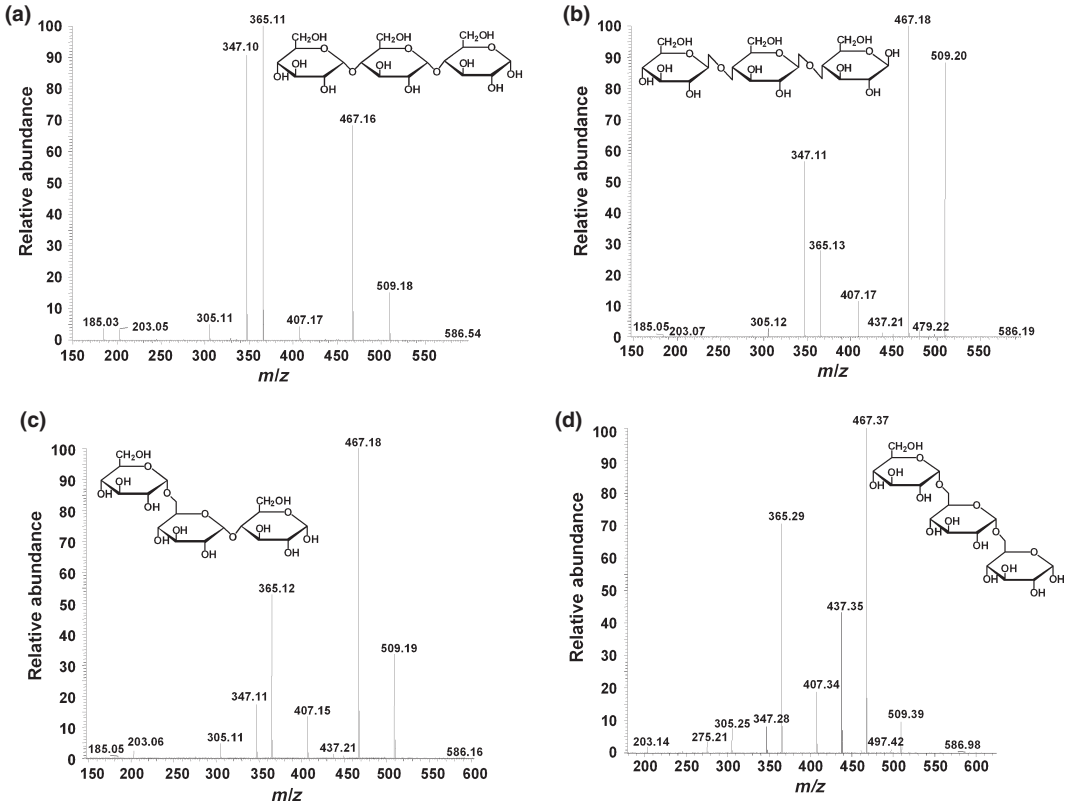


Fig. 2 Analysis of a family of structural isomers. MS^2 of maltotriose, cellotriose, panose, and isomaltotriose in the positive-ion mode are shown in panels a–d, respectively. Note that panel a in this figure is identical to panel c in Fig. 1. MS, mass spectroscopy.

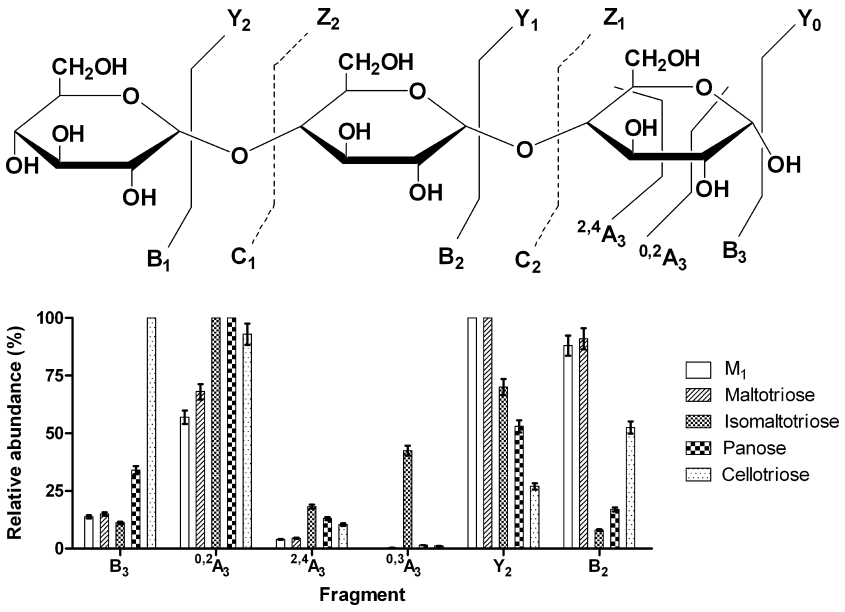


Fig. 3 Relative abundances of fragments for M_1 and standard trisaccharides using the fragmentation nomenclature of Domon and Costello.[29] This analysis shows clearly the correspondence between M_1 and maltotriose.

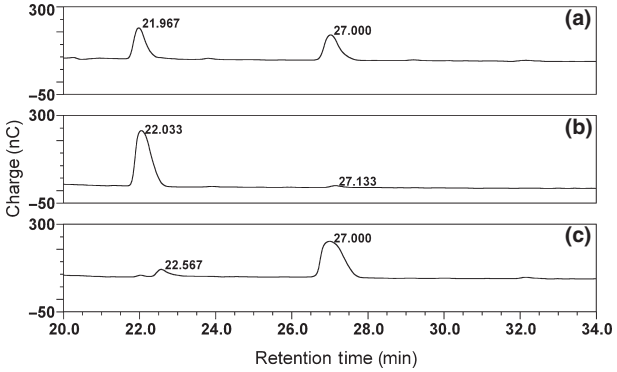


Fig. 4 HPAE chromatographs for the SPE fraction (panel a), as well as authentic maltotriose (panel b) and maltotetraose (panel c) are shown. The chromatogram peaks for the trophoblast carbohydrate sample at 22 and 27 min agree with those of maltotriose and maltotetraose.

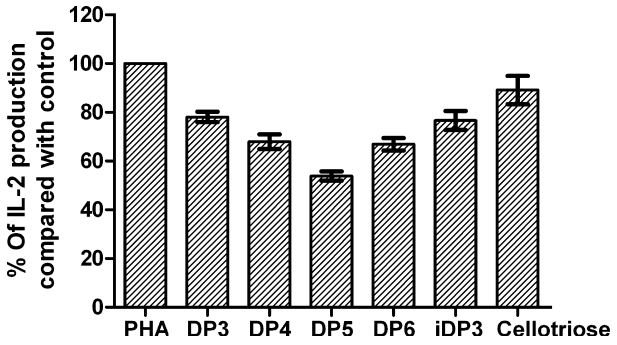


Fig. 5 Effect of various saccharides on IL-2 production by Jurkat cells. Cells were stimulated with 10 μg/mL phytohemagglutinin (PHA) in the absence or presence of DP3, DP4, DP5, DP6, iDP3 (isomaltotriose), and cellotriose (all at 20 mM). The saccharide most effective at blocking IL-2 production was DP5 (PHA versus DP3 and DP4, $P < 0.005$; PHA versus DP5 and DP6, $P < 0.001$; iDP3 versus DP4, DP5 and DP6, $P < 0.001$; iDP3 versus DP3, $P < 0.005$).

Jurkat T cells were tested for IL-2 production during treatment with various oligosaccharides. In the first series of experiments a saccharide concentration of 20 mM was used because it approximates the interfacial maltooligosaccharide concentration expected at a site of cell–cell contact. Cells were incubated with or without PHA for 24 hr at 37°C. As shown in Fig. 5, the isomers cellotriose (β-D-Glc-(1 → 4)-β-D-Glc-(1 → 4)-D-Glc) and isomaltotriose (α-D-Glc-(1 → 6)-α-D-Glc-(1 → 6)-D-Glc) did not have a statistically significant effect on IL-2 production. However, DP3, DP4, and DP5 increasingly reduced IL-2 production, reaching about 50% inhibition for DP5 at 20 mM. DP6 had somewhat less efficacy than DP5, which is likely because as the sequence nears DP7, the conformation becomes more circular in shape.[14] These findings strongly suggest that the reported effects are specific, as small changes in oligosaccharide stereochemistry resulted in significant changes in IL-2 production.

We also examined the dose–response properties of maltooligosaccharides. In this series of experiments we incubated T cells with various concentrations of DP4 and DP5, as described in the previous paragraph. As shown in Fig. 6a,b, respectively, DP4 and DP5 dose-dependently inhibit IL-2 production by Jurkat T cells. At higher concentrations, DP5 was somewhat more effective than DP4. At 40 mM, DP5 reduced IL-2 production to about 30% of control values. Hence, maltooligosaccharides significantly inhibit the production of IL-2 by T cells.

Although maltooligosaccharides do not resemble the complex oligosaccharides recognized by PHA, we nonetheless controlled for the possibility that DP4 and DP5 blocked the binding of PHA to Jurkat cell surfaces. Cells were pre-treated with 20-mM DP4 or DP5

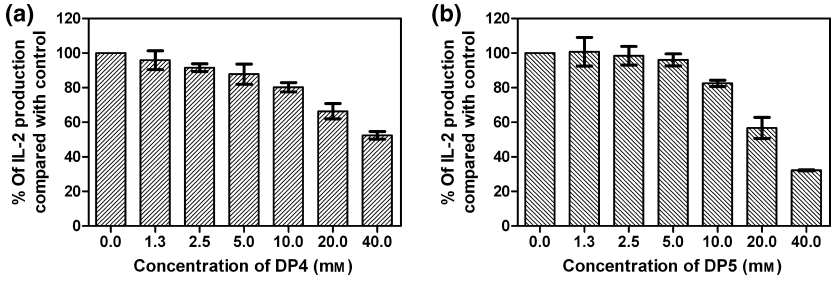


Fig. 6 Effect of DP4 (panel a) and DP5 (panel b) on IL-2 production by Jurkat cells. Cells were treated with various concentrations of DP4 or DP5 in the presence or absence of 10 μg/mL phytohemagglutinin (PHA) for 24 hr at 37°C. IL-2 production was assessed using an ELISA kit and plate reader. A dose-dependent reduction in IL-2 release was observed, although DP5 exhibited greater efficacy than DP4 (For DP4 and DP5, PHA versus 2.5, 5.0, 10, 20, and 40 mM, $P < 0.005$).

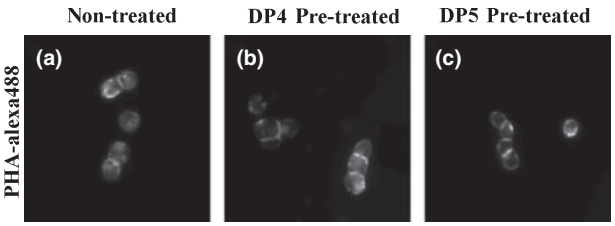


Fig. 7 Effect of maltooligosaccharides on phytohemagglutinin (PHA) binding to lymphocytes. Cells were incubated with buffer (panel a), 20 mM DP4 (panel b), or 20 mm DP5 (panel c) for 30 min followed by the addition of 10 μg/mL Alexa-488-PHA for 30 min at 37°C. Samples were then washed by centrifugation and observed by fluorescence microscopy. As no significant differences in binding intensity or location were observed, these maltooligosaccharides do not inhibit the binding of PHA to cells.

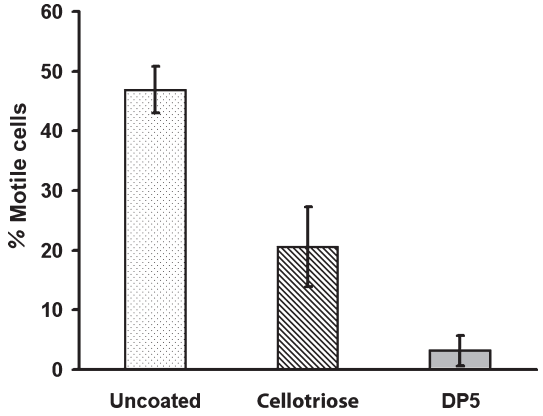


Fig. 8 Effect of surface-bound oligosaccharides on spontaneous neutrophil motility. Surfaces were prepared as outlined in Scheme 1. Neutrophils were added to unmodified glass cover slips (panel a) (n = 4), cellotriose-modified glass cover slips (panel b) (n = 8), or DP5-modified glass cover slips (panel c) (n = 9). Cells moving 10 μm or more during the incubation period were scored as positive. DP5-derivatized surfaces that displayed four glucose subunits (Scheme 1) dramatically inhibited spontaneous motility in comparison with both unmodified and cellotriose-modified glass surfaces (uncoated versus DP5, $P < 0.0001$; uncoated versus cellotriose, $P = 0.0005$; cellotriose versus DP5, $P = 0.0001$).

for 30 min followed by the addition of Alexa-488-PHA for 30 min. Fig. 7 shows fluorescence micrographs of Jurkat cells labeled with Alexa-488-PHA after incubation in the absence of oligosaccharides (Fig. 7a) or the presence of DP4 or DP5 (Fig. 7b,c, respectively). As these data show, there were no significant differences in the PHA labeling of Jurkat cells during these various conditions. Hence, the differences in IL-2 production cannot be accounted for by competition of the maltooligosaccharides with cell surface oligosaccharides for PHA binding.

To ascertain the breadth of its potential immunoregulatory capacity, we examined the ability of neutrophils to undergo spontaneous motility on maltooligosaccharide-modified glass cover slips. These covalently modified cover slips were prepared as described in the Materials and Methods. Neutrophils were added to these cover glass-bottom Petri dishes, then allowed to settle and attach to the surface for 1 hr at 37°C. To avoid confounding maltooligosaccharide effects with those of chemokinetic and chemotaxis effects, we examined spontaneous neutrophil motility. Motile cells were defined as those that moved at least one cell diameter during a timed observation period. Roughly one-half of the neutrophils demonstrated motility on unmodified glass surfaces (Fig. 8). However, this was significantly reduced ($P < 0.001$) on cellotriose-modified surfaces to about 20%. On DP5-modified surfaces, the motile cell percentage was reduced to about 4%, a dramatic reduction in cell movement. It is not surprising that some inhibition was obtained on cellotriose-modified surfaces because it is a glucose-based oligosaccharide whose sequence differs only in the anomeric carbon atom (β versus α) in comparison with maltooligosaccharides. Hence, maltooligosaccharides have immunoregulatory potential.

Discussion

In this study, we have found that maltooligosaccharides can be released from JEG-3 trophoblast-like cells and that these maltooligosaccharides may influence the behavior of leukocytes. Although the mechanisms responsible for maternal immunoregulation have not been completely described, emerging evidence suggests that they are multifactorial in nature and involve both adaptive and innate immune responses. For example, trophoblasts do not express immunostimulatory HLA class I antigens, but rather express HLA-G molecules, which promote tolerance.[15,16] As trophoblasts express B7-H2 and B7-H3 molecules, which interact with members of the CD28 family on lymphocytes, trophoblasts are prone to stimulate Th2-type responses.[17] Production of the immunoregulatory enzyme indoleamine-2,3-dioxygenase may also contribute to immunoregulation during pregnancy.[18] Although trophoblasts have been shown to protect the conceptus from destruction by activated macrophages[8] and evade destruction by activated tumoricidal macrophages,[9] the

regulation of the innate immune response in pregnancy is also incompletely understood. Maltooligosaccharides may be another local regulatory factor influencing the behavior of immune cells. As the JEG-3 line is derived from a choriocarcinoma, maltooligosaccharides may also be relevant in the context of leukocyte interactions with choriocarcinoma cells to deflect immune effector function. Thus, our findings may be relevant to one or more areas of reproductive immunology.

Previous studies have suggested that pregnancy-associated carbohydrates, including those at the trophoblast's surface, affect the behavior of immune cells.[10,11,19,20] Although the structures of some of these compounds are known, the remainder of these potential immunoregulatory molecules is unknown. In this study, we first identified a major low molecular weight cell surface saccharides of JEG-3 cytotrophoblast-like cells then evaluated their bioactivity. To accomplish the first goal, we used high-HPAE with pulsed amperometric detection (HPAE-PAD), which is one of the most powerful techniques for underivated carbohydrate analysis, in conjunction with HPLC-ES-MS and MS^2. Using these tools, we determined the structures of two components of this fraction to be maltotriose and maltotetraose. Interestingly, the accumulation of maltotriose and maltotetraose in a cell surface fraction of syncytiotrophoblasts has been noted,[21] although they did not determine if maltooligosaccharides were externally expressed and their biological role was not addressed. Our structural analysis of JEG-3 cytotrophoblast-like oligosaccharides demonstrates that maltooligosaccharides are a significant carbohydrate structure of these cells. These maltooligosaccharides must be synthesized by JEG-3 cells and are presumably non-covalently associated with the cell surface in a fashion similar to hyaluronan and other glycocalyx components.

As previous studies have indicated that trophoblast membranes[22,23] and, specifically, their membrane-associated saccharides,[10] affect lymphocyte function, we assessed cytokine production by PHA-activated Jurkat lymphocytes. We found that maltooligosaccharides reduce cytokine release in a dose-dependent fashion, with an optimal chain length of DP5. This optimal chain length is likely because of the formation of secondary structures, such as rings, at longer chain lengths.[14] However, the use of millimolar concentrations of maltooligosaccharides in these experiments might seem too high. Although this may appear to be high, it is roughly what one would expect for a region of intercellular contact. Using HPAE chromatography of SPE isolates and authentic DP3, we estimate that 4×10^{-9} mol of DP3 can be isolated from 10^7 cells. Given the assumptions that the cell surface area equals 800 μm^2 and that maltooligosaccharides are uniformly distributed on their surfaces, we estimate $\sim 3 \times 10^5$ molecules of DP3 per μm^2. With approximately equal abundances of DP3 and DP4, a maltooligosaccharide value of $\sim 6 \times 10^5$ molecules of per μm^2 is suggested, which is roughly a quarter of the density of phospholipids in a membrane ($2.5 \times 10^6/\mu m^2$). We estimate that maltooligosaccharides may be distributed in a zone with a thickness of 0.04 μm. This does not seem unreasonable because the trophoectoderm and apical uterine epithelial membranes at the time of implantation approach as close as 0.0075–0.015 μm.[24] It is also consistent with the fact that the leukocyte glycocalyx is ~0.012 nm in thickness.[25] Using a thickness of 0.04 μm, we conservatively estimate a local concentration of ~24 mM. Hence, for the biological conditions of intercellular contact, the concentrations used in this study do not appear to be unreasonable.

Early studies have also suggested that trophoblasts affect the behavior of adherent mononuclear phagocytes.[8,9] To test this possibility in our maltooligosaccharide system, we synthesized DP5-modified glass cover slips (Scheme 1). In addition, the surface-associated DP5 is a better model of the trophoblast surface (both are essentially two-dimensional), in contrast to the bulk addition of DP5 in the lymphocyte experiments. As phagocytic cells are adherent, these surfaces allowed us to test the hypothetical bioactivity of DP5 in a chemically robust system while retaining a physiologically appropriate surface context for the assessment of bioactivity. Our experimental findings showed that spontaneous neutrophil motility was reduced on DP5-modified surfaces. This is consistent with other work suggesting that small oligosaccharides, including maltooligosaccharides, affect phagocyte functions such as oxidant production.[14] Although speculative, it is possible that maltooligosaccharides may act by diminishing glucose transport, a previously reported attribute of trophoblast membrane fragments,[26] Thus, the biological activities of both neutrophils and lymphocytes are affected by maltooligosaccharides.

Although our findings have suggested a previously unrecognized biological regulatory pathway based

upon maltooligosaccharides, there may be practical applications of these findings. For example, biomaterials are often compromised by immunological activation at their surfaces.[27] By using maltooligosaccharides as a coating component, in analogy with trophoblasts, it may be possible to improve the biocompatibility of implants. This proposal is not unreasonable because carbohydrates, such as hyaluronic acid, have already proven to be useful in mitigating host responses to implant materials. Indeed, maltooligosaccharide modification may be a generally useful tool in reducing immunologic/inflammatory responses to implants, polymeric injectables, and other substances.

In summary, we have demonstrated that maltooligosaccharides are present at the surface of JEG-3 trophoblasts. Further studies on the biochemical and physiological properties of maltooligosaccharides are underway. From a biological perspective, maltooligosaccharides would be expected to be an excellent immunological evasion tactic. As a component of glycogen, maltooligosaccharides are an ancient self-molecule. More importantly, as glycogen is a component of normal human serum (5 mg/100 mL[28]), mothers producing an anti-maltooligosaccharide antibody would succumb to serum sickness. Moreover, we have also shown that these molecules are capable of influencing the behavior of leukocytes. We suggest that maltooligosaccharides contribute to the carbohydrate surface coat of JEG-3 cells. To examine maltooligosaccharide expression in human placental tissue, we are developing novel fluorescent tools. We speculate that maltooligosaccharides may contribute to the mitigation of adaptive and innate immunologic responses at the maternal–fetal interface.

Acknowledgments

We thank Hongpeng Liu for technical assistance. This work was supported, in part, by the Division of Intramural Research of the Eunice Kennedy Schriver National Instutute of Child Health and Human Development, NIH, DHHS, contract number N01-HD-2-3342 and subcontract WSU04055.

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