Oligosaccharides of Human Milk

II. ISOLATION AND CHARACTERIZATION OF A NEW PENTASACCHARIDE, LACTO-N-FUCOPENTAOSE III

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SUMMARY

A new pentasaccharide, "lacto-N-fucopentaose III" has been isolated from human milk and characterized as O-β-D-Gal(1 → 4)-O-[α-L-Fuc(1 → 3)]-O-β-D-GlcNAc(1 → 3)-O-β-D-Gal(1 → 4)-D-Glc. The preparation of O-β-D-GlcNAc(1 → 3)-O-β-D-Gal(1 → 2)-D-erythritol from lacto-N-tetraose is described.

The presence or absence of certain oligosaccharides in individual samples of human milk reflects the ability of the donor to synthesize heterosaccharide chains that occur in the complex molecules of cell surfaces and mucous secretions (1-4). In a previous publication, methods were described for the isolation and determination of some of these sugars (5). With these previous methods, a new pentasaccharide has been isolated, the structure of which is established by the data of the present paper as O-β-D-Gal(1 → 4)-O-[α-L-Fuc(1 → 3)]-O-β-D-GlcNAc(1 → 3)-O-β-D-Gal(1 → 4)-D-Glc (see Fig. 7). The sugar is a positional isomer of two previously described pentasaccharides, lacto-N-fucopentaose I (6) and lacto-N-fucopentaose II (7), and will be referred to in this publication as lacto-N-fucopentaose III.

EXPERIMENTAL PROCEDURE

Oligosaccharides—Lacto-N-neotetraose was prepared from LS-tetrasaccharide C by partial acid hydrolysis according to the procedure of Kuhn and Gauhe (8).

O-β-D-GlcNAc(1 → 3)-O-β-D-Gal(1 → 2)-D-erythritol was prepared from lacto-N-tetraose by periodate oxidation (9, 10) as follows: crystalline lacto-N-tetraose, 85 mg, was dissolved in a mixture of 30 ml of 0.05 N NaI04 and 1 ml of 5 M sodium acetate buffer, pH 5.4. After 24 hours at 5°C in the dark, 3 ml of 0.1 M ethylene glycol were added, and the reaction mixture was incubated for an additional 24 hours. The solution was cooled to 0°C, its pH was adjusted to 7.3 with 1 N NaOH, and 76 mg of NaBH4 in 10 ml H2O were added with stirring. The mixture was allowed to stand overnight at room temperature, an additional 10 mg of NaBH4 were added and incubation was continued for 4 hours to complete the reduction. Excess borohydride was decomposed by adjusting the pH of the solution to 5.0 with glacial acetic acid. The reaction mixture was then passed through a column (2 × 10 cm) of Amberlite AG 50(H+) and evaporated to dryness; borate was removed by the repeated addition and removal under vacuum of methanol (five times). The residue was chromatographed as a 100-cm band on Whatman No. 3MM paper for 24 hours with Solvent I. Periodate-benzidine reagent (11) revealed only one product. The material, which had a mobility approximately the same as lactose, was eluted from the chromatogram with H2O, and the resulting solution was deionized by passage through a mixed bed resin column (1 × 0.5 cm) containing Amberlite AG 50(H+) and 5.0 with glacial acetic acid. The reaction mixture was then continued for 4 hours to complete the reduction. Excess borohydride was decomposed by adjusting the pH of the solution to 5.0 with glacial acetic acid. The reaction mixture was then passed through a column (2 × 10 cm) of Amberlite AG 50(H+) and evaporated to dryness; borate was removed by the repeated addition and removal under vacuum of methanol (five times). The residue was chromatographed as a 100-cm band on Whatman No. 3MM paper for 24 hours with Solvent I. Periodate-benzidine reagent (11) revealed only one product. The material, which had a mobility approximately the same as lactose, was eluted from the chromatogram with H2O, and the resulting solution was deionized by passage through a mixed bed resin column (1 × 0.5 cm) containing Amberlite AG 50(H+) and AG 3(OH-). After evaporation to dryness, the residue was dissolved in 0.5 ml of H2O, and 3 ml of ethanol were added slowly with stirring. The product crystallized in needles and was collected by filtration and recrystallized by the same procedure (yield, 40 mg). Acid hydrolysis of a sample (1 N HCl at 105°C for 1 hour) resulted in the liberation of glucoseamine, galactose, and erythritol as revealed by paper chromatography with Solvent VI. For analysis, a sample was dried at 110°C under vacuum over P2O5 for 15 hours (melting point 275-277°C (decomp.); [α]D = +7.35).

C16H24O11N

Calculated: C 44.40, H 6.78, N 2.88
Found: C 44.23, H 7.07, N 2.62

5496
Laeto-N-fucotetraose I and lacto-N-fucohexaose II were prepared by reducing lacto-N-fucotetraose I and lacto-N-fucohexaose II with sodium borohydride according to the procedure of Kuhn, Helmut, and Gauhe (7). Laeto-N-fucopentaose III was prepared from lacto-N-fucopentaose III by the same procedure. For analysis, the product was dried at 110° under vacuum over P2O5 for 15 hours (αD = -28.0).

\[ \text{CnH2nNOz2H2O} \]

Calculated: C 43.09, H 6.90
Found: C 43.40, H 7.15

O-β-d-Gal-(1→3)-β-GlcNAc was prepared before lacto-N-fucopentaitol by partial acid hydrolysis in 0.1 M HCl at 100° for 40 min and isolated by paper chromatography with Solvent I. The chromatographic mobility of the disaccharide is shown in Fig. 3. O-β-d-Gal-(1→4)-GlcNAc was kindly provided by Dr. A. Gauhe. 3-O-Methyl-α-methyl-N-acetylgalactosaminide was prepared according to the method of Neuberger (12).

**Paper Chromatography**—Descending paper chromatography was performed with the following solvents: I, upper layer of ethyl acetate-pyridine-H2O (2:1:2); II, ethyl acetate-pyridine-acetic acid-H2O (5:5:1:3); III, 2-propanol-H2O (4:1); IV, 1-butanol-pyridine-H2O (6:4:3); V, lower layer of phenol-formic acid-2-propanol-H2O (80:10:5:100); VI, upper layer of pyridine-ethyl acetate-acetic acid-H2O (1:0:3:6:1:15); VII, phenol-H2O-concentrated acid-2-propanol-H2O (80:10:5:100); VIII, upper layer of ethyl acetate-acetic acid-H2O (3:1:3).

Oligosaccharides were located with AgNO3 reagent (13), or with periodate-benzidine reagent (14). Morgan-Elson reagent (15) was used to detect oligosaccharides that contain sialic acid (18). The occurrence of lacto-N-fucopentaose III in isolated samples of “lacto-N-fucopentaose II” could be assayed by using the fact that lacto-N-neotetraose obtained from lacto-N-fucopentaose III by mild acid hydrolysis does not react in the Morgan-Elson test for amino sugars (18) while lacto-N-tetraose obtained from lacto-N-fucopentaose II does. Samples of pentasaccharide were hydrolyzed in 0.01 M HCl at 100° for 30 min, and the tetrasaccharide product was isolated by paper chromatography with Solvent I. The isolated tetrasaccharides were then assayed for total sugar with the phenol-sulfuric acid reagent (22) and for amino sugar with the Morgan-Elson reagent (18).

X-ray diffraction powder patterns were recorded on a Sutter precision camera with flat film.

**Hemagglutination Studies**—Studies on the inhibition of hemagglutination were carried out as follows: 15 μl of the different antisera (obtained from commercial sources) were incubated with 55 μg of lacto-N-fucopentaose III for 30 min at 22°. A 2% suspension of the appropriate red cells, 15 μl, was added, and incubation was continued for an additional 30 min. The suspensions were then centrifuged and read for agglutination both microscopically and macroscopically and compared with agglutination obtained in the absence of lacto N-fucopentaose III.

**RESULTS AND DISCUSSION**

Isolation of Lacto-N-fucopentaose III—Lacto-N-fucopentaose III is quite similar to lacto-N-fucopentaose II in its chromatographic properties, and no separation of the two isomers was achieved with any of the solvents tested. However, individuals with the blood type Le(a-b-) lack the enzyme GDP-α-fucose-N-acetyl-β-d-glucosaminylsaccharide 4→α-fucosyltransferase (2), and their milk is missing lacto-N-fucopentaose II, lacto-N-difucohexaose I, and lacto-N-difucohexaose II. Consequently, the new pentasaccharide is best isolated from milk obtained from these individuals, who comprise about 5% of the population (23).

A chromatographic analysis by methods described previously (5) of the oligosaccharides in milk from a Le(a-b-) donor is shown diagrammatically in Fig. 1. This analysis should be carried out before a large scale isolation of lacto-N-fucopentaose III is attempted as the new oligosaccharide was missing in one out of four samples of milk from Le(a-b-) donors that were analyzed (see “Occurrence of Lacto-N-fucopentaose III”).
Approximately 0.5 pmole of that contained sialic acid. The effluent was concentrated lacto-N-fucopentaose III (e.g. Fractions 22 to 32 in Fig. 1) were lacto-N-fucopentaose II as a standard. The fractions containing N-fucopentaose III by paper chromatography with authentic and protein, was removed by centrifugation and washed twice night at 0 °. The white precipitate that formed, mainly lactose sugars were visualized with AaN reagent (13).

From each fraction was spotted 1 cm apart on Whatman No. 1 characterized heptasaccharide. 2'-fucosyllactose; LD, lactodifucohexaose; LNF, lacto-N-tetraose; LNF-I, lacto-N-fucopentaose I; LNF-III, lacto-N-fucopentaose III; N-I, a new, partially characterized heptasaccharide.

similar chromatograms of the oligosaccharides in milk from individuals with the blood type Le(a+) or Le(b+), lacto-N-fucopentaose III was isolated as follows. A liter of LNT, lacto-N-tetraose; LNF, lacto-N-fucopentaose II; lacto-N-fucopentaose III resulted in the same products as did acid hydrolysis of lacto-N-fucopentaose II. As shown in Fig. 2, paper chromatography revealed the liberation of glucose, galactose, glucosamine, and fucose. Reduction of the two oligosaccharides before hydrolysis resulted in disappearance of glucose with the concomitant appearance of material in the orbito area of the chromatogram, indicating that glucose is at the reducing end of both sugars. By colorimetric analysis and analysis by gas chromatography lacto-N-fucopentaose III contains 1 glucosamine, 1 fucose, and 2 galactoses for each glucose, as shown in Table I.

Like lacto-N-fucopentaose II, acid hydrolysis of lacto-N-fucopentaose III liberated free fucose and resulted in the formation of a tetrasaccharide with the same chromatographic mobility and monosaccharide constituents as lacto-N-tetraose, as shown in Fig. 3 and Table I. However, unlike lacto-N-tetraose (the tetrasaccharide derived from lacto-N-fucopentaose II), the tetrasaccharide derived from lacto-N-fucopentaose III did not react with Morgan-Elson reagent (18). In addition, as shown in Fig. 3, the partial hydrolysis pattern of the tetrasaccharide was quite different from that of lacto-N-tetraose but was identical in every detail with that of lacto-N-neotetraose. The tetrasaccharide was prepared on a large scale for x-ray diffraction studies as follows. Lacto-N-fucopentaose III, 47 mg, was hydrolyzed for 40 min in 10 ml of 0.01 N HCl at 100 °. The hydrolysate was evaporated to dryness, and the residue was taken up in water, applied as an 80-cm band on Whatman No. 3MM paper, and chromatographed for 36 hours with Solvent I. The tetrasaccharide was eluted from the paper with water, and the eluate was passed through a small column of mixed bed resin containing Amberlite AG 50(H+) and AG 3(OH-). After evaporation to dryness, the resulting colorless syrup was dissolved in 300 µl of water, and 2 ml of 95% ethanol was added slowly with stirring. The solution became cloudy, and the tetrasaccharide, which started to crystallize as needles, was harvested by filtration after standing overnight (yield 18 mg). The crystalline tetrasaccharide was identified as lacto-N-neotetraose by a comparison of its x-ray diffraction pattern with that of authentic lacto-N-neotetraose, as shown in Fig. 4.

The methanolysate of the fully methylated lacto-N-fucopentaol III was identical to that obtained from fully methylated lacto-N-fucopentaol II; both contained 2,3,4-tri-O-methyl-
Fro. 2. Paper chromatogram of products derived from lacto-N-fucopentaose II and lacto-N-fucopentaose III. Hydrolysis was carried out in 1 N HCl at 100° for 60 min. The chromatogram was developed with Solvent I for 15 hours and the sugars were visualized with AgNO₃ reagent (13). 1, hydrolysate of lacto-N-fucopentaose II; 2, hydrolysate of lacto-N-fucopentaose III; 3, hydrolysate of lacto-N-fucopentaitol II; 4, hydrolysate of lacto-N-fucopentaitol III; 5, lacto-N-fucopentaitol II; and 6, lacto-N-fucopentaitol III.

Fig. 3. Paper chromatography of the partial acid hydrolysis products of lacto-N-fucopentaose II, lacto-N-fucopentaose III, lacto-N-tetraose, and lacto-N-neotetraose. The chromatogram was developed with Solvent I for 20 hours and the sugars were visualized with AgNO₃ reagent (13). The spots circled are sugars that also react with Morgan-Elson reagent (14). 1, lacto-N-fucopentaose III; 2 and 3, hydrolysis products (0.01 N HCl at 100° for 30 min) of lacto-N-fucopentaose II and lacto-N-fucopentaose III, respectively; 4, 5, and 6, hydrolysis products (0.01 N HCl at 100° for 40 min) of the tetrasaccharides obtained from lacto-N-fucopentaose III, authentic lacto-N-neotetraose and authentic lacto-N-tetraose, respectively. LAC, lactose; LNT, lacto-N-tetraose.

Table I

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Analytical method</th>
<th>Monosaccharide molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Galactose</td>
</tr>
<tr>
<td>Lacto-N-fucopentaose III</td>
<td>Gas chromatography</td>
<td>1.00</td>
</tr>
<tr>
<td>Tetrasaccharide</td>
<td>Colorimetric</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Gas chromatography</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Colorimetric</td>
<td>1.00</td>
</tr>
</tbody>
</table>

methyl-fucoside, 6-O-methyl-methyl-N-acetylgalactosaminide, 2,4,6-tri-O-methyl-methylgalactoside, 2,3,4,6-tetra-O-methyl-methylgalactoside, and 1,2,3,5,6-penta-O-methyl-sorbitol, as shown in Fig. 5. These results show that the fucosyl residue of lacto-N-fucopentaose III is attached to the C-3 position of the N-acetylgalactosaminyl residue of lacto-N-neotetraose as sub-
stitution at other positions would not yield methylated derivatives identical with those obtained from lacto-N-fucopentaitol II.

The molar rotation of lacto-N-fucopentaitol III is -13,900 while the molar rotation of lacto-N-neotetraose is +19,089 (24). As the molar rotations of \( \alpha \) and \( \beta \)-methyl-\( \alpha \)-fucopyranoside are -35,100 and +2,900, respectively (25), it is clear from Hudson's rule (26) that the fucosyl residue is attached to lacto-N-neotetraose in an \( \alpha \) linkage.

Confirmatory evidence for the linkage of fucose to the 4 position of the \( \alpha \)-acetylglucosaminyl residue of lacto-N-neotetraose was obtained by the following results, which eliminate as possibilities the other seven hydroxyls. (a) The C-2 positions on glucose and the 2 galactosyl residues can be eliminated as linkage possibilities by the alkaline lability of the fucosyl residue. Large amounts of free fucose were liberated from lacto-N-fucopentaitol III by heating in 0.05 M NaK\(_2\)CO\(_3\) at 100°. Fucose linked to the C-2 of glucose or the 2 galactosyl residues would be stable under the above conditions (27). (b) The C-3 position of the glucose and the C-3 position of the terminal galactosyl residue of lacto-N-fucopentaitol III are shown to be unsubstituted by periodate oxidation. Lacto-N-fucopentaitol III, 1.7 mg, was oxidized with 0.2 ml of 0.1 M Na\(_2\)O\(_x\) in 0.5 M sodium acetate buffer, pH 4.5, in the dark at 5° for 24 hours. Ethylene glycol, 0.2 ml of 0.1 M, was added to destroy excess oxidant, and the reaction mixture was passed through a mixed bed resin column (2 x 0.5 cm) containing Amberlite AG 50(H\(^+\)) and AC 3(OH\(^-\)). The column was washed with \( \text{H}_2\text{O} \), and the eluate and washing were combined and evaporated to dryness. Na\(_2\)B\(_4\)H\(_4\), 0.4 ml of 0.2 M, was added twice at 4-hour intervals. After the mixture was allowed to stand for 4 hours at room temperature, 0.01 ml of glacial acetic acid was added to destroy the excess Na\(_2\)B\(_4\)H\(_4\), and the reaction mixture was passed through a column (2 x 0.5 cm) containing Amberlite AG 50(H\(^+\)) and evaporated to dryness under reduced pressure. Borate was removed by the repeated addition and evaporation of methanol (five times), and the residue was dissolved in 0.4 ml of 1 N HCl and heated in a boiling water bath for 1 hour. Acid was removed by repeated addition and evaporation of water, and the hydrolysate was analyzed by paper chromatography with Solvent VI. Erythritol, galactose, and glucosamine were detected. If the glucose were substituted with fucose on its C-3, arabitol instead of erythritol would be formed by periodate oxidation, as is the case when authentic lacto-N-fucohexaose II was subjected to the same procedure. When the hydrolysis was performed under mild conditions (0.5 ml of 0.1 N H\(_2\)SO\(_4\) at room temperature for 24 hours), a sugar with the chromatographic mobility in Solvents I, II, III, IV, and VIII of \( \beta \)-\( \alpha \)-GlcNAc-\( \beta \)-d-\( (1 \rightarrow 3) \)-\( \beta \)-d-Gal-(1 \rightarrow 2)\)-d-erythritol was obtained in high yield. This result indicates that the terminal galactose residue was oxidized and that its C-3 position is therefore unsubstituted. (c) The C-4 position of the terminal

![Fig. 4. X-ray diffraction patterns of the tetrasaccharide obtained by partial acid hydrolysis of lacto-N-fucopentaitol III (left) and authentic lacto-N-neotetraose (right).](http://www.jbc.org/)

Fig. 4. Gas chromatograms of the methanolysate of permethylated milk oligosaccharides. A, standard 3-O-methyl-\( \alpha \)-methyl-\( \beta \)-acetylglucosaminide; B, lacto-N-fucopentaitol II; C, lacto-N-fucopentaitol III; D, lacto-N-tetraose. The identity of the numbered peaks are as follows: 1, 2, 3, 4-tri-O-methyl-\( \beta \)-methylfucoside; 2, 3, 4, 5, 6-penta-O-methyl-\( \alpha \)-methylgalactoside; 3, 1, 2, 3, 5, 6-penta-O-methyl-sorbitol; 4, 2, 4, 6-tri-O-methyl-\( \beta \)-methylgalactoside; 5, 2, 4, 6-tri-O-methyl-\( \alpha \)-methylgalactoside; 6, and 7, 4, 6-di-O-methyl-methyl-\( \beta \)-acetylglucosaminide; 8 and 9, 6-O-methylmethyl-\( \beta \)-acetylglucosaminide; and 10, 3-O-methyl-\( \alpha \)-methyl-N-acetyleucosaminide.
galactose and the C-6 position of glucose are free, based on the results of periodate consumption. Lacto-N-fucopentaisol III consumed 7 moles of periodate, as shown in Fig. 6. Under the same conditions, lacto-N-fucopentaisol I, lacto-N-fucopentaisol II, and sorbitol consumed 6, 7, and 5 moles of periodate, respectively. The moles of formaldehyde formed after 5 hours of oxidation per mole of substrate (21) were 1.93 from lacto-N-fucopentaisol III, 1.98 from lacto N-fucopentaisol I, and 1.91 from lacto-N-fucopentaisol II, with a sorbitol standard (2.00 moles).

The structure of lacto-N-fucopentaisol III that is consistent with all of the above data is given in Fig. 7.

**Occurrence of Lacto-N-fucopentaisol III—Lacto-N-fucopentaisol III** was found in 3 out of 4 of samples of milk from donors with the blood type Le(a-b-). The milk lacking the oligosaccharide did not contain any oligosaccharide with the grouping O-α-Fuc-(1 → 3)-Glc, which includes 3-fucosyllactose and lacto-difucotetraose, and was unique in the more than 50 samples analyzed. The simultaneous absence of both these oligosaccharides and of lacto-N-fucopentaisol III suggests that the fucosyltransferase that forms the O-α-Fuc-(1 → 3)-Glc linkage in lacto-N-fucopentaisol III is also responsible for the formation of the O-α-Fuc (1 → 3)-Glc linkage in the other oligosaccharides. This dual specificity would be analogous to the lactose synthetase-N-acetyllactosamine synthetase relationship reported recently by Brew, Vanaman, and Hill (28).

Lacto-N-fucopentaisol III in milk from donors with the blood type Le(a-b-) or Le(b+), although obscured on chromatograms by lacto-N-fucopentaisol II, can be assayed as described in "Analytical Procedures". Samples of "lacto-N-fucopentaisol II" isolated from five individuals with the blood type of Le(a +) or Le(b+) were found to be approximately one-third lacto-N-fucopentaisol III. The levels of lacto-N-fucopentaisol III in the different samples ranged from 0.05 to 0.2 μmole per ml of milk.

**Serological Specificity of Lacto-N-fucopentaisol III—**Two kinds of carbohydrate chains, known as type I and type II, are part of the antigenic determinants of soluble blood group substances with A, B, H, Leα, and Leβ specificities (29, 30).

Lacto-N-fucopentaisol II has the following structure in common with the nonreducing end of a type I chain with Leα specificity.

\[
\beta-d-Gal-(1 \rightarrow 3)-\beta-d-GlcNAc-(1 \rightarrow 3)-\beta-d-Gal \ldots
\]

\[
\alpha-L-Fuc-(1 \rightarrow 4)
\]

and consequently it is a potent haptenic inhibitor of the agglutination of Leα red cells by Leα antisera (31). Lacto-N-fucopentaisol III resembles the corresponding structure in the type II chain,

\[
\beta-d-Gal-(1 \rightarrow 4)-\beta-d-GlcNAc-(1 \rightarrow 3)-\beta-d-Gal \ldots
\]

\[
\alpha-L-Fuc-(1 \rightarrow 3)
\]

which, unlike the type I structure, does not inhibit Leα-anti-Leα hemagglutination and has no known serological or antigenic specificity (20). For this reason, lacto-N-fucopentaisol III was tested for haptenic activity with antisera directed against various red cell types in hopes of finding some serological specificity.

However, 1.6 mg of pentasaccharide per ml did not inhibit hemagglutination by any of the antisera tested, including anti-A, anti-B, anti-D, anti-M, anti-N, anti-S, anti-s, anti-I, anti-P, anti-K, anti-Kp+, anti-Kp+, anti-K, anti-Fy+, anti-Fy+, anti-JK+, anti-JK+, anti-Le+, anti-Le, anti-e, anti-e, anti-C, and five different preparations of anti-I. Under the conditions of agglutination, 30 μg of lacto-N-fucopentaisol II per ml completely inhibited the agglutination of Leα cells by anti-Leα serum. As suggested by Lloyd and Kabat (29), it is possible that the fucose attached to N-acetylgalactosamine in the type II chain may form an "interaction product" with other substituents such as fucose or N-acetylgalactosamine attached to the terminal galactose in the type II chain shown above. Lacto-N-fucopentaisol III would not be expected to inhibit antisera directed against such compound antigens. Also, no conclusion can be drawn from the fact that the blood type of the donor, who is unable to synthesize lacto N-fucopentaisol III, does not appear to be unusual (see Footnote 3). Twenty per cent of the population (classified as "nonsecretors") are unable to synthesize oligosaccharides that contain the grouping O-α-L-Fuc-(1 → 2)-O-β-D-Gal (1 yet are able to synthesize the same grouping in the complex saccharides responsible for A, B, or O specificity found on their cell surfaces.

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Oligosaccharides of Human Milk: II. ISOLATION AND
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