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Comparison of oligosaccharides derived from salivary mucin of Japanese secretor and non-secretor individuals of blood group type-A

Takeshi Ohmori[1], Hidenao Toyoda[2], Toshihiko Toida[2], Toshio Imanari[2] and Hajime Sato[1]

[1] *National Research Institute of Police Science, 6-3-1, Kashiwa-no-ha, Kashiwa, Chiba, 277-0882, Japan,* [2] *Graduate School of Pharmaceutical Science, Chiba University, 1-33, Yayoi-cho, Inage-ku, Chiba, 263-8522, Japan*

Comparison of oligosaccharide components derived from salivary mucin was performed between secretor and non-secretor individuals. Salivary mucin was collected from four secretors and three non-secretors having blood group type-A. Compositional analysis showed that the contents of galactose and N-acetylglucosamine in the non-secretor were higher than those in the secretor. The O-linked oligosaccharides obtained by treatment with alkaline borohydride were separated by gel filtration using Sephadex G-50. The results indicated that the size of the type-A active oligosaccharides from the secretor was similar to or smaller than that of the non-secretor. Ion-exchange chromatography showed that the secretors had strong type-A activities in both the neutral and acidic fractions but the non-secretors showed type-A activity mainly in the neutral fraction. These results suggest that compositional differences in blood group substances exist between secretors and non-secretors.

Keywords: **ABO blood group, saliva, monoclonal antibody**

Introduction

In criminal investigations, saliva and salivary stains are important as evidential materials because ABO blood group substances are secreted into the saliva [1–4]. Thus, research on the ABO blood group substances of saliva continues to be important issue in forensic science.

It is well known that individuals can be divided into two groups, secretors and non-secretors, depending on the presence or absence of ABH antigens in their saliva, respectively [1–4]. Genetic differences between secretors and non-secretors have recently been reported [1,2,4–9] and ethnic specificities among non-secretor individuals in various populations have also been reported [10–17]. Japanese non-secretor individuals secrete a small amount of ABH antigen with their saliva, indicating that ABO blood group can be determined, even though an evidential sample is obtained from a non-secretor.

Previous reports indicated that the reactivity of monoclonal antibodies with type-A saliva is different among the commercially available individual monoclonal antibodies. The reactivity of each antibody does not always reflect the amount of type-A substances in the saliva [18–21]. Most antibodies react strongly with a secretor but to a lesser extent with a non-secretor [18–23]. However, antibodies which do not react with a non-secretor have also been reported [18–21].

In this paper we therefore investigated not only the differences in oligosaccharide compositions of the blood group substance between secretors and non-secretors but the chemical properties of the type-A substance in saliva obtained from them.

Materials and methods

Materials

Human whole saliva was collected from seven healthy donors of blood type-A, four secretors and three non-secretors. The secretor donors consisted of three Le(a − b+) phenotypes and one Le(a − b−) phenotype. The Lewis blood types of the donors were determined by a hemagglutination test.

Anti-A monoclonal antibodies and anti-Lewis blood type antibodies were obtained from Biotest (Dreieich, Germany) and Ortho diagnostics (Tokyo), respectively. The specificity of the antibodies used were verified by ELISA using synthetic antigens, bound to polyacrylamide (Syntesome, Munich, Germany). The anti-A antibody used is specific to a type-A determinant (GalNAcα1-3[Fuc α1-2]Galβ1-) but is not reactive to GalNAcα1-, GalNAcα1-3Galβ1-, H type-1, H type-2, type-B(Galα1-3[Fuc α1-2]Galβ1-), Le^a or Le^b determinants. The anti-Le^a antibody used is specific to the Le^a determinant but

is not reactive to Le^b, H type-1 or H type-2 determinants. The anti-Le^b antibody used is specific to the Le^b determinant but is not reactive to Le^a, H type-1 or H type-2 determinants. Sepharose CL-6B, Sephadex G-10, Sephadex G-50(fine) and QAE-Sephadex A25 were purchased from Pharmacia Biotech (Uppsala, Sweden).

Preparation of type-A active mucin

Whole saliva was collected, dialyzed against distilled water and freeze-dried. The freeze-dried saliva was suspended in 50 mM Tris-HCl (pH 7.6) containing 6 M guanidine-HCl and centrifuged. The supernatant was subjected to gel filtration on a Sepharose CL-6B column (3.0×95 cm) equilibrated with 10 mM Tris-HCl (pH 7.6) containing 4 M guanidine-HCl. Chromatography was performed with a FPLC system (Pharmacia Biotech). Type-A active fractions were collected, dialyzed against distilled water, and freeze-dried.

The type-A active mucin obtained was subjected to capillary electrophoresis on a CE-800 system (JASCO, Tokyo, Japan). The separation was performed with an uncoated fused-silica capillary (75 μm i.d. $\times$ 55 cm effective length) and 50 mM sodium borate buffer (pH 8.9) containing 0.1% triethylamine and 0.1% Tween 20 as the eluent. The glycoprotein samples were introduced by pressure injection for 30 seconds. The operating voltage was 10 kV and the separation was monitored at 210 nm.

Analysis of monosaccharide composition of salivary mucin

The neutral and amino sugars contained in the salivary mucin that was prepared by Sepharose CL-6B chromatography were analyzed by high performance liquid chromatography (HPLC) with a post-column derivatization system as described previously [24]. The neutral sugar composition was determined by using anion-exchange HPLC (TSKgel sugar AXl 4.6 mm i.d. $\times$ 150 mm, Tosoh, Tokyo) after hydrolysis of the salivary mucin in 2.5 M trifruoloacetic acid for 6 h at 80°C. The amino sugar composition was determined using cation-exchange HPLC (TSKgel SCX 4.6 mm i.d. $\times$ 150 mm, Tosoh, Tokyo) after hydrolysis of the salivary mucin in 6 M HCl for 2 h at 80°C. The monitoring was performed at 280 nm.

Preparation of O-linked oligosaccharides

Samples of salivary mucin components (82 mg from Le(a $-$ b+) secretors, 90 mg from Le(a + b$-$) non-secretors and 98 mg from Le(a $-$ b$-$) secretors) were suspended in 15 ml of water, an equal volume of 0.2 M NaOH-2 M $NaBH_4$ solution was added to the mucin suspension followed by stirring at 45°C for 18 h. The reaction mixture was neutralized with acetic acid and then evaporated. Methanol was added to the residue and the solution was evaporated again. After dissolving the residue in distilled water, the solution was desalted by treatment with Sephadex G-10. The desalted oligosaccharide-alditols were freeze-dried.

Separation of oligosaccharides by gel filtration

The O-linked oligosaccharide-alditol obtained by treatment with NaOH-$NaBH_4$ was separated by gel filtration on Sephadex G-50 (1.5 cm i.d. $\times$ 75 cm) equilibrated with 20 mM NH_4HCO_3. The flow rate was 0.2 ml/min. The monitoring was performed at 210 nm and the type-A activity of each fraction was measured by enzyme-linked immunosorbent assay (ELISA). Type-A active fractions were collected.

Separation of oligosaccharides by ion-exchange chromatography

The type-A active fractions obtained by Sephadex G-50 gel filtration were further chromatographed on a QAE-Sephadex A-25 column (1.2 cm i.d. $\times$ 9 cm) equilibrated with 10 mM sodium bicarbonate. The column was washed with 12 ml of 10 mM sodium bicarbonate buffer (pH 9.6). The elution was performed by a linear concentration gradient from 0 M to 0.2 M NaCl. The flow rate was 0.2 ml/min. The monitoring was performed at 210 nm and the type-A activity of each fraction was measured by ELISA.

ELISA

Fifty μl of each fraction that had previously separated by chromatography was added to each well of a multi well plate (SUMILON MS-7196F, Sumitomo Bakelite, Tokyo) and incubated for 1 h at 37°C. After the solution was discarded, 200 μl of 1% BSA solution was added to each well and the plate was left at 4°C overnight. After discarding the BSA solution, each well was washed with 10 mM PBS containing Tween 20 and 50 μl of anti-A, anti-Le^a or anti-Le^b monoclonal antibody solution was added. The plate was incubated at 37°C for 1 h. After washing the plate, 50 μl of anti-mouse IgM antibody (American Qualex, San Clemente, USA) solution containing 1% of goat serum was added. The plate was incubated at 37°C for 1 h. After washing, 50 μl of 0.1 mg/ml o-phenylenediamine/0.1 M Tris-HCl (pH 7.6) solution containing 0.006% H_2O_2 was added to each well and the plate was held at room temperature for 30 min in the dark. The reaction was stopped by adding 100 μl of 2N H_2SO_4 and the absorbance at 490 nm was measured by a microplate reader (Powerwave200, Bio-Tek instrument).

Results

Separation of salivary mucin by gel filtration

The type-A active salivary glycoproteins were separated by gel filtration on a Sepharose CL-6B column with 4 M guanidine HCl. Elution profiles are indicated in Figure 1. Type-A activity, measured by ELISA using an anti-A monoclonal antibody, was

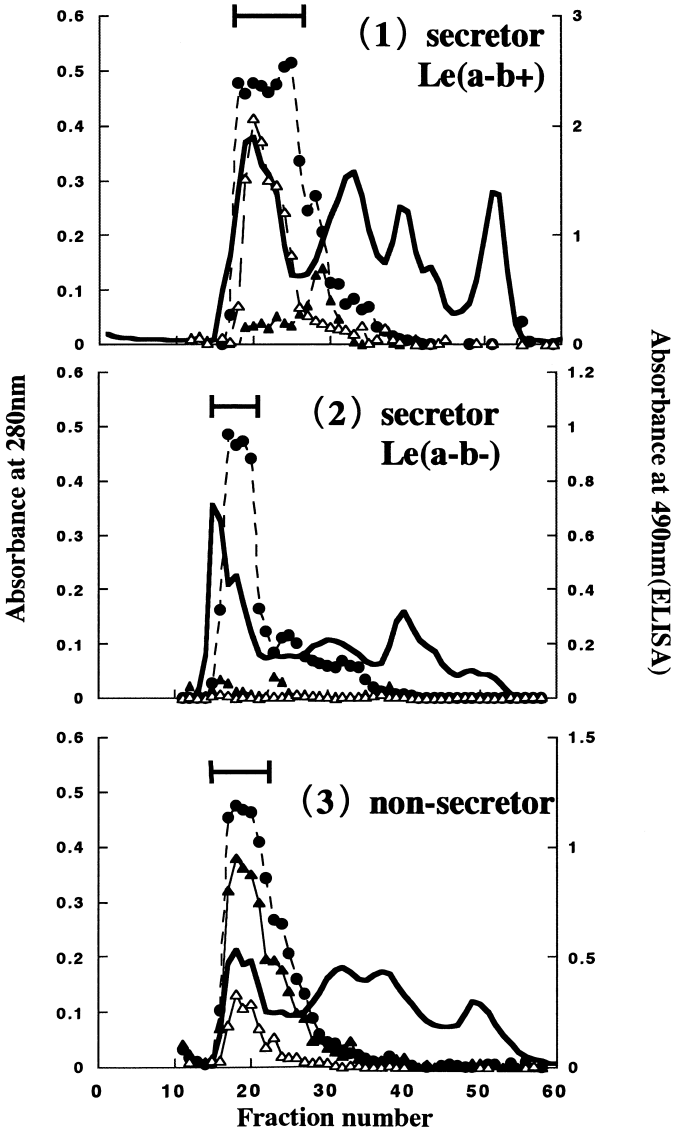

Figure 1. Gel filtration of freeze-dried saliva on a Sepharose CL-6B column (3.0 × 95 cm), equilibrated with 10 mM Tris-HCl (pH 7.6) containing 4 M guanidine-HCl. The protein content of each fraction was analyzed by an absorbance measurement at 280 nm (solid line). The activities of type-A (●), Le^a (▲) and Le^b (△) were measured by ELISA. The bar in each figure indicates fractions pooled as a tuype-A active fraction.

eluted in the void volume of Sepharose CL-6B column. Lewis blood type activities were also observed in the void volume.

The type-A active fractions, indicated by bars in Figure 1, were pooled and analyzed for purity by capillary electrophoresis. The type-A active fraction gave a single broad peak by capillary electrophoresis (Figure 2). This result suggests that the fractions separated by gel filtration consist of heterogeneously glycosylated proteins. Figure 2 also shows that the migration time of each fraction obtained from Le(a − b+)/secretors and non-secretors is similar, indicating that these components are inseparable by capillary electrophoresis.

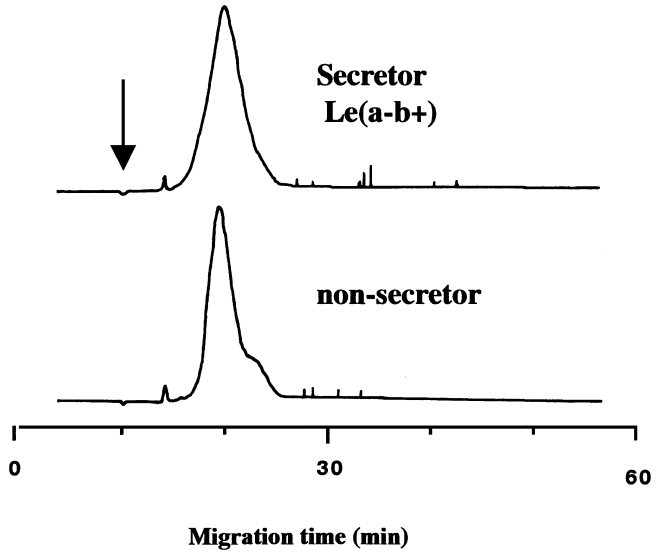

Figure 2. Electropherogram of the type-A active salivary mucin separated by gel filtration. Capillary electrophoresis condition: capillary, un-coated fused-silica capillary (75 μm i.d. × 55 cm effective length); buffer, 50 mM sodium borate (pH 8.9), 0.1% Triethylamine, 0.1% Tween; Operating voltage, 10 kV; monitoring, 210 nm. The arrow indicates the position of the neutral marker (Acetone).

Monosaccharide composition of salivary mucin

The monosaccharide compositions of salivary mucin obtained from secretors (Le(a − b+) and Le(a − b−) phenotype) and non-secretors (Le(a + b−) phenotype) are shown in Table 1. These compositions are characteristic of mucin-type glycoproteins. The molar ratio of galactose (Gal) to N-acetylglucosamine (GlcNAc) in the salivary mucin was 1:1. The contents of Gal and GlcNAc in salivary mucin obtained from non-secretors were considerably higher than those obtained from secretors. The contents of fucose (Fuc) and N-acetylgalactosamine (GalNAc) for the Le(a − b+) secretors were slightly higher than those for the non-secretor. Trace amounts of mannose were detected but xylose and glucose were not. The monosaccharide composition of salivary mucin obtained from Le(a − b−) secretors was almost similar to that obtained from Le(a − b+) secretors.

Separation of oligosaccharide by gel filtration

A size fractionation of oligosaccharides obtained from salivary mucin was performed by gel filtration on a Sephadex G-50 column (Figure 3). The type-A activity measured by ELISA using anti-A antibody is indicated by a broken line in Figure 3. The type-A active oligosaccharides obtained from the Le(a − b+)/secretor showed two major peaks at fraction numbers 40 and 46. The type-A active oligosaccharides obtained from the non-secretor had one peak in fraction number 40, corresponding to the larger molecular weight fraction

Table 1. Saccharide composition of salivary mucin from Japanese subjects

		Monosaccharide (nmol/μg mucin)						
Secretory status	*Lewis phenotype*	*Man*	*Fuc*	*Gal*	*Xyl*	*Glc*	*GlcNAc*	*GalNAc*
Secretor	Le(a − b+)	Trace	1.24	1.60	N.D.	N.D.	1.58	1.12
	Le(a − b−)	Trace	1.10	1.29	N.D.	N.D.	1.18	0.93
Non-secretor	Le(a + b−)	Trace	1.06	2.20	N.D.	N.D.	2.20	0.75

Man: mannose, Fuc: fucose, Gal: galactose, Xyl: xylose, Glc: glucose, GlcNAc: N-acetylglucosamine, GalNAc: N-acetylgalactosamine, N.D.: not detected.

of type-A active component of the secretors. The result obtained from the Le(a − b−)/secretor was similar to that for the Le(a − b+)/secretor.

Figure 3 also indicates that the quantity of type-A active oligosaccharides of the secretors was larger than that of the non-secretors.

Type-A active fractions were pooled as indicated by the bars in Figure 3.

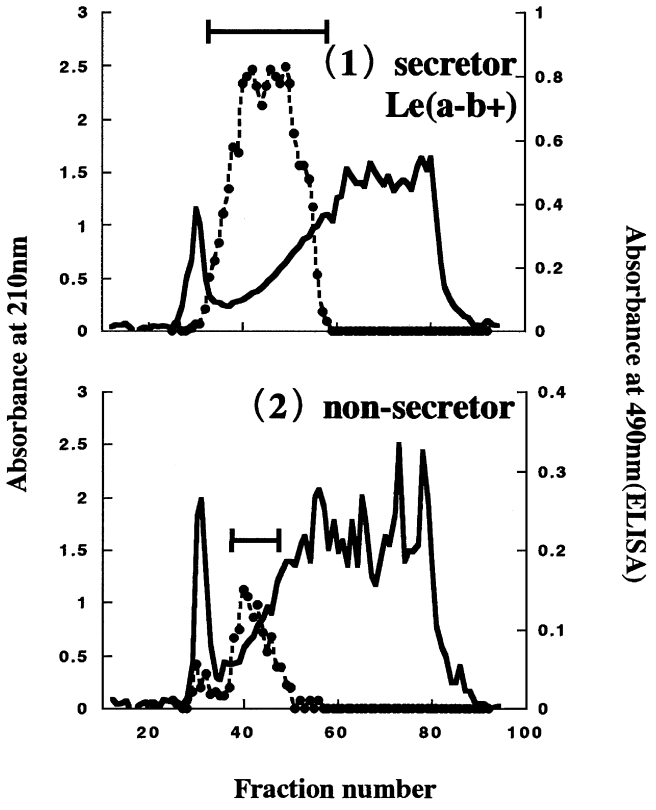

Figure 3. Gel filtration of oligosaccharides liberated from salivary mucin on a column (1.5 cm i.d. × 75 cm) of Sephadex G-50, equilibrated with 20 mM NH_4HCO_3. The oligosaccharide content of each fraction was analyzed by an absorbance measurement at 210 nm (solid line). The activities of type-A (●) were measured by ELISA. The bar in each figure indicates fractions pooled as a type-A active fraction.

Separation of oligosaccharide
by ion-exchange chromatography

The oligosaccharides separated by gel filtration were further separated by anion-exchange chromatography on QAE-Sephadex A-25 (Figure 4). The oligosaccharides obtained from salivary mucin were separated into neutral (flow-through fraction, fraction numbers 1–8) and acidic fractions (fraction numbers 15–25). In the case of secretors, large peaks corresponding to type-A activities were observed in both the neutral and acidic fractions, whereas the majority of the type-A activity of non-secretors was present in the neutral fraction. In the Le(a − b+) secretor, Le^b activity was detected in both the neutral and acidic fractions, whereas Le^a activity was not detected. The Le(a − b−) secretor had neither Le^a nor Le^b activity. In the non-secretors, the Le^a activity was detected in both the neutral and acidic fractions and the Le^b activity was detected only slightly in the neutral fraction.

Discussion

Since saliva and related stains are important as evidential materials in criminal caseworks, a more complete understanding of the blood group substance of saliva is also important in forensic science, even today. Nadano and Mukoyama [25] identified some oligosaccharide structures in human salivary mucin derived from a type-O secretor. Klein et al. [26] determined 37 oligosaccharide alditol structures in salivary mucin obtained from the pooled saliva of twenty type-O donors, but they did not provide information on the secretion status of donors. Thus, a further investigation of each blood group substance of a secretor and a non-secretor would be desirable because the reactivity of commercially available monoclonal antibodies do not always reflect the amount of blood type-A substances present in saliva obtained from secretors and non-secretors [18–21].

As can be seen in this report, although the differences in the blood group substance carrier proteins between secretors and non-secretors were not distinguished by capillary electrophoresis, the saccharide compositions in these glycoproteins were different between secretors and non-secretors. The most notable difference was that the contents of both Gal and GlcNAc in the non-secretors were about twice as high as those

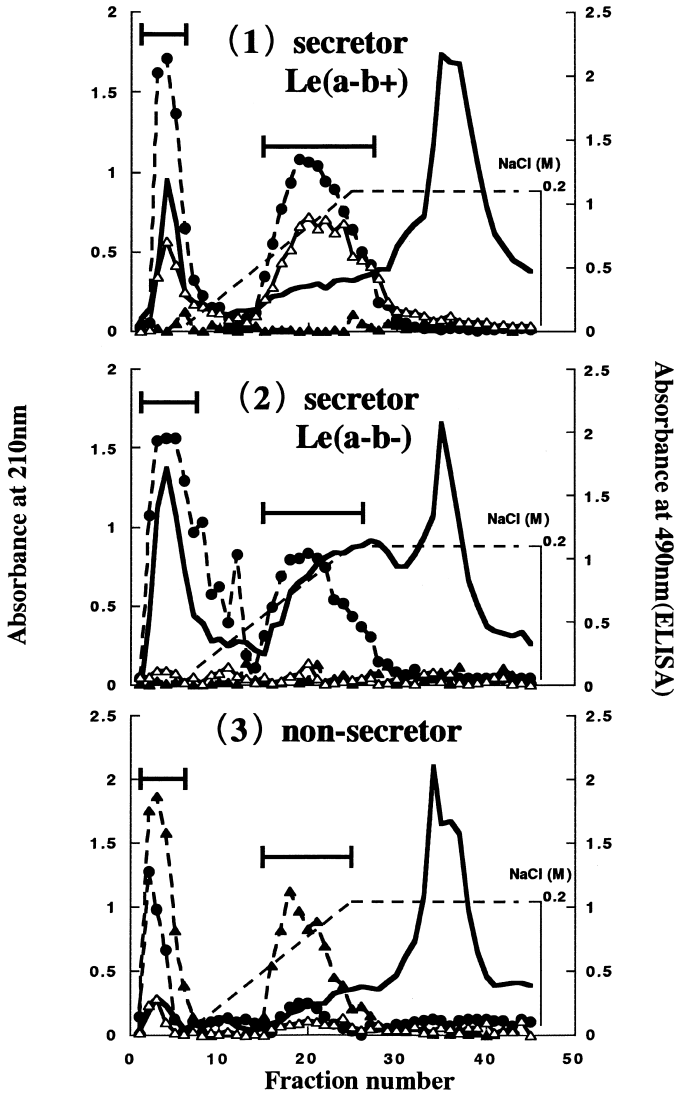

Figure 4. Ion-exchange chromatography of oligosaccharides on a QAE-Sephadex A-25 column (1.2 cm i.d. × 9 cm). The oligosaccharides separated by gel filtration on the column of Sephadex G-50, were applied to the column equilibrated with 10 mM sodium bicarbonate buffer (pH 9.6). The column was washed with 12 ml of 10 mM sodium bicarbonate buffer (pH 9.6). Elution was performed using a linear concentration gradient from 0 M to 0.2 M NaCl (dotted line). The oligosaccharide content of each fraction was analyzed by absorbance measurement at 210 nm (solid line). The activities of type-A (●), Le^a (▲) and Le^b (△) were measured by ELISA. The bar in each figure indicates the fractions pooled as a type-A active fraction.

in secretors and the molar ratio of Gal to GlcNAc was 1:1 (Table 1). These results suggest that the non-secretor produces more repeating units having an N-acetyllactosamine structure (Galβ1,4GlcNAc) than the secretor. The content of GalNAc in the secretor is greater than in the non-secretor, suggesting that secretors produce larger amounts of type-A structure than non-secretors (Table 1).

Le(a − b+)/secretors produce a large amount of type-A antigen and Le^b antigen (difucosyl antigen), and non-secretors produce a small amount of type-A antigen and Le^a antigen (monofucosyl antigen). Therefore, the content of Fuc is higher in the Le(a − b+)/secretors than in non-secretors. Furthermore, Fuc detected in Le(a − b−)/secretors, who do not produce Lewis antigens, is regarded as originating from fucosyl residues of the type-A antigen.

A difference in the size of the oligosaccharide chain was also observed between secretors and non-secretors. The non-secretors had type-A active oligosaccharides which were long in size, while secretors produced molecules of a similar or small size compared to those from the saliva from non-secretors. It is conceivable that this result is compatible with the compositional analysis because a large amount of N-acetyllactosamine structure leads to the presence of longer oligosaccharides. Henry et al. reported that an individual who is a weak secretor shows a tendency to produce more elongated Lewis-active glycolipids in the small intestine and plasma than secretors [27,28] and suggested that a product of the Se^w gene might lead to only the partial formation of the H antigen and would allow an increased elongation of precursor chains to occur and an increased production of Le^a [4]. Our results observed from the human salivary mucin might be explained by this suggestion, as proposed by Henry et al. [4].

The ion-exchange chromatography results (Figure 4) showed that the type-A active oligosaccharides of non-secretors were largely neutral oligosaccharides and those of secretors included both neutral and acidic oligosaccharides. Figure 4 also shows that Le^a and Le^b antigens are detected in both the neutral and acidic fractions. It was previously thought that the type-A and Le^b antigens found in the acidic fraction were not sialylated determinants [29].

The findings herein suggest that several compositional differences in oligosaccharides of salivary mucin exist between secretors and non-secretors in addition to a quantitative difference in blood group substances.

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References

1 Watkins WM, Molecular basis of antigenic specificity in the ABO, H and Lewis blood-group systems. In *Glycoproteins*, edited by Mountreuil J, Vliegenthart JFG, Schachter H (Elsevier Science, Amsterdam, 1995), pp. 313–90.

2 Oriol R, ABO, Hh, Lewis and secretion, In *Blood Cell Biochemistry, Vol. 6*, edited by Cartron JP, Rouger P (Plenum Press, New York, 1995), pp. 37–73.

3 Clausen H, Hakomori S, ABH and related histo-blood group antigens: Immunochemical differences in carrier isotypes and their distribution, *Vox Sang* **56**, 1–20 (1989).

4 Henry S, Oriol R, Samuelsson B, Lewis histo-blood group system and associated secretory phenoptypes, *Vox Sang* **69**, 166–82 (1995).
5 Le Pendu J, A hypothesis on the dual significance of ABH, Lewis and related antigens, *Immunogenetics* **16**, 53–61 (1989).
6 Oriol R, Le Pendu J, Mollicone R, Genetics of ABO, H, Lewis, X and related antigens, *Vox Sang* **51**, 161–71 (1986).
7 Oriol R, Genetic control of fucosylation of ABH precursor chains; Evidence for new epistatic interaction in different cells and tissues, *J Immunogenet* **17**, 235–45 (1990).
8 Kudo T, Iwasaki H, Nishihara S, Shinya N, Ando T, Narimatsu I, Narimatsu H, Molecular genetic analysis of the human Lewis histo blood group system, *J Biol Chem* **271**, 9830–37 (1996).
9 Kelly RJ, Rouquier S, Giorgi D, Lennon GG, Lowe JB, Sequence and expression of a candidate for the human *Secretor* blood group α(1,2)fucosyltransferase gene(*FUT2*), *J Biol Chem* **270**, 4640–49 (1995).
10 Koda Y, Soejima M, Liu Y, Kimura H, Molecular basis for secretor type α(1,2)fucosyltransferase gene deficiency in a Japanese population: A fusion gene generated by unequal crossover responsible for the enzyme deficiency, *Am J Hum Genet* **59**, 343–50 (1996).
11 Yu LC, Broadberry RE, Yang YH, Chen YH, Lin M, Heterogeniety of the human secretor α(1,2)fucosyltransferase gene amoung Lewis(a + b−) non-secretors, *Biochem Biophys Res Comm* **222**, 390–4 (1996).
12 Yu LC, Lee HL, Chu CC, Broadberry RE, Lin M, A newly identified nonsecretor allele of the human histo-blood group α(1,2)fucosyltransferase gene (FUT2), *Vox Sang* **76**, 115–9 (1999).
13 Henry S, Mollicone R, Lowe JB, Samuelsson B, Larson G, A second nonsecretor allele of the blood group α(1,2)fucosyltransferase gene (FUT2), *Vox Sang* **70**, 21–5 (1996).
14 Henry S, Mollicone R, Fernandez P, Samuelsson B, Oriol R, Larson G, Homozygous expression of a missense mutation at nucleotide 385 in the FUT2 gene associated with the Le(a + b+) partial-secretor phenotype in an Indonesian family, *Biochem Biophys Res Comm* **219**, 675–8 (1996).
15 Yu LC, Yang YH, Broadberry RE, Chen YH, Chan YS, Lin M, Correlation of a missense mutation in the human secretor α1,2-fucosyltransferase gene with the Lewis(a + b+) phenotype: A potential molecular basis for the weak *Secretor* allele (Se^w), *Biochem J* **312**, 329–32 (1995).
16 Koda Y, Soejima M, Kimura H, The polymorphisms of fucosyltransferases, *Legal Medicine* **3**, 2–14 (2001).
17 Tanegashima A, Nishi K, Fukunaga T, Rand S, Brinkmann B, Ethnic differences in the expression of blood group antigens in the salivary gland secretory cells from German and Japanese non-secretor individuals, *Glycoconjugate J* **13**, 537–45 (1996).
18 Ohmori T, Mizuno N, Sekiguchi K, Senju H, Sakai I, Application of ABO blood-grouping monoclonal antibodies to forensic samples, *Jpn J Sci Tech Iden* **1**, 43–8 (1996).
19 Ohmori T, Kirihara S, Sato H, Evaluation of commercially available monoclonal antibodies for ABH blood grouping of forensic samples, *Reports of the National Research Institute of Police Science* **52**, 116–23 (1999).
20 Ohmori T, Sakai I, Isolation of type-A blood group active glycoproteins from salivas and their reaction with monoclonal antibodies, *Jpn J Legal Med* **53**, 322–9 (1999).
21 Takizawa N, Ohba Y, Mukoyama R, Komuro T, Mukoyama H, Takei T, Determination of ABO blood groups from saliva and salivary stains by an indirect enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies, *Jpn J Legal Med* **43**, 294–302 (1989).
22 Wang B, Wang O, Koda Y, Akiyama K, Kimura H, Measurement of ABH blood group substances in human saliva by immunoassay using artificial antigens as standard substances, *Jpn J Legal Med* **50**, 46–9 (1996).
23 Nagai K, Yokota M, Sakota S, Imura M, Maeda H, A comparative study on applicability of commercially available monoclonal ABO blood grouping reagents to identification of forensic materials, *Acta Crim Japon* **61**, 30–69 (1995).
24 Toyoda H, Demachi Y, Komoriya S, Furuya N, Toida T, Imanari T, Characterization and determination of human urinary keratan sulfate, *Chem Pharm Bull* **46**, 97–101 (1998).
25 Nadano D, Mukoyama H, Isolation of H and Le^b active human salivary mucin and structures of their sugar chains with the H and Le^y(Y) determinants, *Jpn J Legal Med* **44**, 259–66 (1990).
26 Klein A, Carnoy C, Wieruszeski JM, Strecker G, Strang AM, van Halbeek P, Roussel P, Lamblin G, The broad diversity of neutral sialylated oligosaccharides derived from human salivary mucins, *Biochemistry* **31**, 6152–65 (1992).
27 Henry SM, Samuelsson BE, Oriol R, Immunochemical and immunohistological expression of Lewis histo-blood group antigens in small intestine including individuals of the Le(a + b+) and Le(a − b−) nonsecretor phenotypes, *Glycoconjugate J* **11**, 600–7 (1994).
28 Henry SM, Oriol R, Samuelsson BE, Detection and characterization of Lewis antigens in plasma of Lewis-negative individuals: Evidence of chain extension as a result of reduced fucosyltransferase competition, *Vox Sang* **67**, 387–96 (1994).
29 Brockhausen I, Biosynthesis of O-glycans of the N-acetylgalactosamine-α-Ser/Thr linkage type. In *Glycoproteins*, edited by Mountreuil J, Vliegenthart JFG, Schachter H (Elsevier Science, Amsterdam, 1995), pp. 201–59.

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