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A BLOOD GROUP Sd^a-ACTIVE PENTASACCHARIDE ISOLATED FROM TAMM-HORSFALL URINARY GLYCOPROTEIN

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SUMMARY. Human Tamm-Horsfall urinary glycoprotein from an individual of the blood group Sd(a+) phenotype was tritium-labelled by treatment with galactose oxidase and sodium boro[^{3}H]hydride and was then digested with endo-β-galactosidase. A series of dialysable, labelled fragments was released from which a pentasaccharide was isolated that strongly inhibited the agglutination of Sd(a+) red cells by human anti-Sd^a serum and hence contained the Sd^a determinant structure. Reduction, methylation analysis and sequential exo-glycosidase digestion established the structure of the pentasaccharide as :

GalNAcβ(1→4)[NeuAc(2→3)]Galβ(1→4)GlcNAcβ(1→3)Gal

INTRODUCTION. Tamm-Horsfall (T-H) glycoprotein has been much studied with respect to its chemical composition, physical properties and distribution in normal and pathological kidneys [see 1] but little information is available on the structure of the oligosaccharide chains. The blood group Sd^a character is a genetic marker carried on human T-H glycoprotein and this character is associated with the presence of N-acetylgalactosamine in Sd(a+) T-H preparations [2]; a finding in agreement with earlier indirect observations suggesting that this sugar is the immunodominant sugar in the Sd^a determinant on the red cell surface [3,4]. A disaccharide GalNAcβ(1→4)Gal, isolated from the degradation products of Sd^a-active T-H glycoprotein was believed to constitute a terminal non-reducing structure on the oligosaccharide chains, but was not active in Sd^a haemagglutination inhibition tests [5]. We now report the isolation of a serologically active pentasaccharide fragment from the endo-β-galactosidase digestion products of an Sd^a-active T-H glycoprotein.

MATERIALS AND METHODS. Sodium boro[^{3}H]hydride (14.9 Ci/mmol) was purchased from Amersham International Ltd., U.K. and sodium borodeuteride from Aldrich Chemical Co. Ltd., U.K. The ion exchange resin AG1 X4, 200-400 mesh (acetate form) was obtained from Bio-Rad Laboratories Ltd., U.K. Jack bean β-hexosaminidase and Clostridium perfringens neuraminidase were purchased from Sigma

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Chemical Co., U.K., Vibrio cholerae neuraminidase from Koch-Light Laboratories Ltd., U.K. and Escherichia freundii endo-β-galactosidase from Miles Laboratories Ltd., U.K. Endo-β-galactosidase from Bacteroides fragilis [6] was a gift from Drs P. Scudder and T. Feizi, MRC Clinical Research Centre, Harrow, U.K. Samples of the glycosphingolipid GM_2 (GalNAcβ(1→4)[NeuAcα(2→3)]Galβ(1→4)Glc-Cer) were kindly supplied by Professors K. Sandhoff, University of Bonn, F.D.R. and T. Yamakawa, University of Tokyo, Japan. Dolichos biflorus lectin and Glycine soja lectin were the gifts of Drs G.W.G. Bird, Birmingham University, U.K. and P. Tippett, MRC Blood Group Unit, London, U.K., respectively.

T-H glycoprotein, isolated from the urine of an Sd(a+) individual by the salt precipitation procedure [7] was freed from lipid and tritium-labelled as described earlier [5] except that sialic acid was not removed from the glycoprotein before labelling.

Paper chromatography was carried out on Whatman No. 40 paper in solvent a (ethyl acetate-pyridine-water, 2:1:1 by vol.), solvent b (ethyl acetate-pyridine-water, 10:4:3, by vol.) or solvent c (ethyl acetate-pyridine-acetic acid-water, 5:5:1:3, by vol.). Radioactive spots were detected on a radiochromatogram scanner and standard sugars were visualised with alkaline silver nitrate reagent. Thin layer chromatography was performed on pre-coated Merck HPTLC silica gel 60 plates (B.D.H., U.K.) developed with solvent d (ethanol-butan-1-ol-pyridine-water-acetic acid (100:10:10:30:3, by vol.) [8] and the sugars were detected with orcinol reagent. Paper electrophoresis was carried out on Whatman 3MM paper in pyridine-acetate buffer pH 5.4 at 70 volts/cm with picric acid as a marker.

Hexosamines and hexosaminitols were measured as previously described [9]. For analysis of hexoses and hexitols, oligosaccharide samples were hydrolysed in 2N trifluoroacetic acid for 2h at 100°C and examined by GLC as their trimethylsilyl derivatives. Sialic acid was estimated by the Warren procedure [10] or on an amino acid analyser [11]. Samples for the latter method were subjected to methanolysis with 1.5N HCl in methanol for 18h at 80°C, and the neutralised solutions were loaded directly onto the column of the analyser. Elution was carried out at 85°C with 0.35N sodium citrate buffer pH 5.09 containing 0.3M boric acid and 15% ethanol. NeuAcα(2→6)Galβ(1→4)GlcNAc, kindly supplied by Dr A. Corfield, University of Bristol, U.K., was included as a standard. Methylation analysis was carried out as described previously [9].

RESULTS. Endo-β-galactosidase treatment of tritium-labelled Sd^a-active T-H glycoprotein. Preliminary experiments with endo-β-galactosidase from E. freundii [12,13] demonstrated the release of dialysable, radioactive fragments from the T-H glycoprotein. Paper chromatography of the diffusible material revealed a radioactive component (B) (Fig. 1a) that on elution with water was found to be inhibitory in Sd^a haemagglutination tests. Fraction B was separated by paper electrophoresis into an uncharged labelled component (BN) and a charged Sd^a-active labelled fraction (BA) which migrated with an $R_{picrate}$ 0.50 (Fig. 1b). Complete acid hydrolysis of BA, followed by re-N-acetylation and paper chromatography demonstrated that all the radioactivity in the oligosaccharide was associated with N-acetylgalactosamine.

Experiments carried out under the same conditions with the endo-β-galactosidase from B. fragilis [6] yielded a series of diffusible products which on

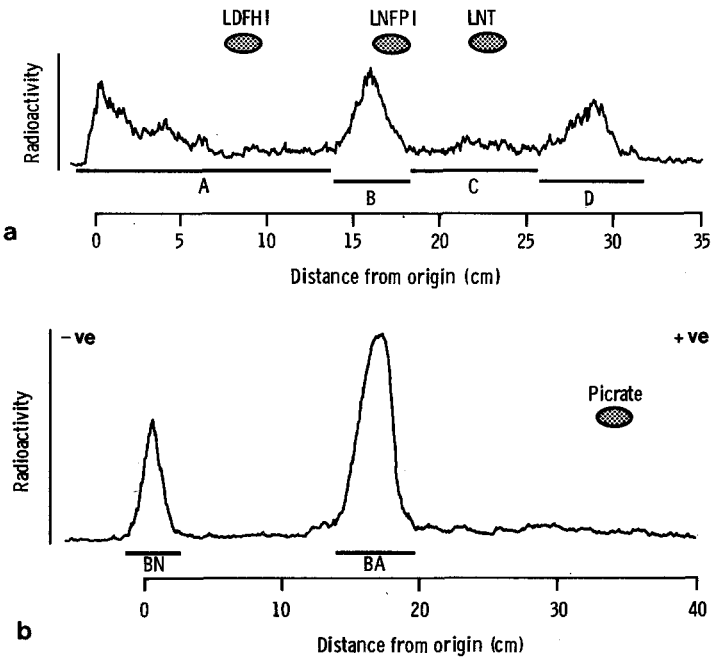

Fig. 1. Digestion of Sd^a-active T-H glycoprotein with E. freundii endo-β-galactosidase. T-H glycoprotein (100 mg, 14 x 10^6 c.p.m.) incubated 64h at 37° with 0.02 unit E. freundii β-galactosidase in 2 ml 0.01M sodium acetate buffer pH 5.9. The digest was dialysed against distilled water and the diffusate concentrated.
(a) Paper chromatogram of diffusible products developed in solvent a for 40h and scanned for radioactivity. Standards : LDFHI = lacto-difucohexaose I; LNFPI = lacto-N-fucopentaose I; LNT = lacto-N-tetraose. The bars indicate the regions of the papers which were eluted.
(b) Fraction B eluted with water from the chromatogram, concentrated, and subjected to paper electrophoresis as described in Materials and Methods. Paper scanned for radioactivity.

chromatography, and subsequent electrophoresis of Fraction B, gave radioactivity scans that were virtually identical with those obtained when the glycoprotein was digested with the E. freundii enzyme. Fraction BA isolated from either of the digests migrated as a single spot on paper chromatography in three different solvents with lacto-N-tetraose (LNT) as reference standard; solvent a, R_{LNT} 0.82; solvent b, R_{LNT} 0.66; solvent c, R_{LNT} 0.64. Orcinol staining of TLC plates developed in solvent d revealed, for both oligosaccharide fractions, a strong band with R_f value 0.55 and a barely discernible minor band (R_f 0.60).

Larger scale preparations of oligosaccharide Fraction BA. In order to further characterise the Sd^a-active fragment BA, the digestion of T-H glycoprotein (1.7g) was carried out on a larger scale with the endo-β-galactosidase (0.4

unit) from B. fragilis. After incubation for 64h at 37° the digest was dialysed exhaustively against water and the combined diffusates were concentrated and subjected to paper chromatography and paper electrophoresis as in the smaller scale experiments. Fraction BA was desalted on a Sephadex G25 column (60 x 1 cm) to yield 4.4 mg of oligosaccharide. TLC showed the strong orcinol-staining band and the trace of a faster moving component noted in the earlier preparations. This minor contaminant was removed by repeating the paper electrophoresis step, dividing the radioactive peak BA and eluting only the half comprising the trailing edge of the peak. The eluted fraction was strongly Sd^a-active, gave a single band on TLC, and a single radioactive spot on paper chromatography in solvents a, b and c.

Characterisation of product. The oligosaccharide BA (500 μg) was reduced with $NaB[^3H]_4$ (2 mCi), and sodium borodeuteride (5 mg). Paper chromatography of the desalted product in solvent a gave a single radioactive peak, R_{LNT} 0.75. Sugar analysis revealed the presence of N-acetylgalactosamine, N-acetylglucosamine, galactose, galactitol and sialic acid in the molar ratios of 0.93:1.0:1.06:0.81:0.94; hence the compound is a pentasaccharide with galactose at the reducing terminal. Sialic acid was determined on the amino acid analyser [11] with NeuAcα(2→6)Galβ(1→4)GlcNAc as standard. The value obtained by the Warren method [10] was lower but paper electrophoresis revealed that the hydrolysis conditions used in this procedure gave incomplete release of sialic acid from the pentasaccharide. Similar findings were earlier reported for the release of N-acetylneuraminic acid from GM_2 [14]. To obtain a sialic acid-free product for methylation analysis the reduced pentasaccharide (350 μg) was treated for 2h at 80°C with 0.1N H_2SO_4 and unchanged pentasaccharide was removed by passage through an AG1 resin column. Paper chromatography of the column eluate in solvent a showed one major tetrasaccharide component, R_{LNT} 1.08, which was recovered. Methylation analysis of the reduced pentasaccharide yielded 3,4,6-tri-O-methylgalactosamine, 2,6-di-O-methylgalactose, 3,6-di-O-methylglucosamine and 1,2,4,5,6-penta-O-methylgalactitol. In the methylation products of the asialo-compound the 2,6-di-O-methylgalactose was replaced by 2,3,6-tri-methyl-

galactose demonstrating that in the pentasaccharide, the sialic acid is linked to the 3-O position of galactose. The identities of the neutral sugar methyl ethers obtained from the reduced pentasaccharide and from the asialo-compound were confirmed by Dr A.M. Lawson (Clinical Research Centre, Harrow, U.K.) by GLC-mass spectrometry. The reduced pentasaccharide was resistant to the action of V. cholerae or C. perfringens neuraminidases and Jack bean β-hexosaminidase. The sialic acid-free tetrasaccharide was, however, susceptible to the β-hexosaminidase; the sequence and anomeric linkages of the sugars were therefore determined by sequential exo-glycosidase digestion of this oligosaccharide. The products were examined by paper chromatography in solvent a. The tetrasaccharide (40 μg) treated with β-hexosaminidase (1.75 units, pH 4, 18h, 37°C) was 80% hydrolysed and yielded N-acetylgalactosamine and a radioactive trisaccharide R_{LNT} 1.52. This trisaccharide was 70% hydrolysed by Jack bean β-galactosidase (130m units, pH 4, 18h, 37°C) to give galactose and a radioactive disaccharide, R_{Lac} 1.13, which when treated with β-hexosaminidase (0.6 units, pH 5.5, 24h, 37°C), was hydrolysed completely to N-acetylglucosamine and [^{3}H]galactitol.

The results of methylation analysis and sequential exo-glycosidase digestion thus enabled the following structure to be assigned to the pentasaccharide :

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GalNAcβ(1→4)Galβ(1→4)GlcNAcβ(1→3)Gal
              ↑2,3
            NeuAc
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Serological activity of oligosaccharide BA. The pentasaccharides isolated from E. freundii or B. fragilis endo-β-galactosidase digestion products of Sd^a-active T-H glycoprotein were equally active when tested in haemagglutination inhibition tests with two human anti-Sd^a sera (Table 1). The sialic acid-free tetrasaccharide did not give detectable inhibition indicating that both N-acetylgalactosamine and sialic acid are required for binding with the Sd^a antibody. Liposomes prepared from two specimens of the glycolipid GM_2 were without inhibitory activity with the human anti-Sd^a reagents.

Human red cells which react very strongly with anti-Sd^a reagents are also agglutinated by lectins from Dolichos biflorus and Glycine soja seeds [15].

Table 1. Serological activity of oligosaccharide BA isolated from endo-β-galactosidase digestion products of Sd^a-active T-H glycoprotein.

Inhibiting substance	Minimum amount (μg/10 μl) giving complete inhibition of agglutination		
	Human anti-Sd^a	Dolichos biflorus	Glycine soja
Sd^a-active T-H glycoprotein	0.02	3	6
Endo-β-galactosidase products :			
Total diffusate	50	-	-
Oligosaccharide BA	2	6	2
Asialo-oligosaccharide BA	>200	-	-

The reactivity of Sd(a++) cells with these lectins was inhibited by the pentasaccharide isolated from T-H glycoprotein (Table 1).

DISCUSSION. The susceptibility of T-H glycoprotein to digestion by two endo-β-galactosidases of the type that hydrolyse keratan sulphate [12,13,6] reveals for the first time the occurrence of internal β-Gal-GlcNAc linkages in the oligosaccharide chains of this macromolecule. The Sd^a pentasaccharide fragment released by the enzymes constitutes a structure which has not been described hitherto. The terminal non-reducing disaccharide unit GalNAcβ(1→4)Gal occurs in O-linked chains of trout egg glycoprotein [16] and in the ganglioside GM_2 [14] but it has not been found on other asparagine-linked oligosaccharide chains. The terminal branched trisaccharide assembly, GalNAcβ(1→4)[NeuAcα-(2→3)]Gal, in the Sd^a pentasaccharide is apparently identical with that in GM_2 and it is therefore surprising that liposomes prepared from the ganglioside were not Sd^a active. We have not yet confirmed that the sialic acid in the pentasaccharide is unsubstituted N-acetylneuraminic acid but the available evidence does not suggest that this molecule carries additional substituents.

The nature of the molecules carrying Sd^a on red cells is unknown but there is evidence to suggest that the antigen called Cad is in fact a highly reactive form of Sd^a [17]. Recently Cad specificity on human red cell membranes was shown to be associated with sialoglycoproteins and the presence of an abnormally high N-acetylgalactosamine content in these glycoproteins led to a tentative structure being proposed for the Cad determinant [18] which resembles,

but is not identical with, the structure now established for the Sd^a determinant in T-H glycoprotein. Clarification of the precise relationship between Sd^a and Cad specificities at the immunochemical level must await further characterisation of the Cad determinant.

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REFERENCES

1. Fletcher A.P. (1972) in Glycoproteins : Their composition, structure and function (Gottschalk A., ed.) 2nd edn., pp. 892-908.
2. Soh C.P.C., Morgan W.T.J., Watkins W.M. and Donald A.S.R. (1980) Biochem. Biophys. Res. Commun., 93, 1132-1139.
3. Sanger R., Gavin J., Tippett P., Teesdale P. and Eldon K. (1971) Lancet i, 1130.
4. Bird G.W.G. and Wingham J. (1971) Vox Sang., 20, 55-61.
5. Donald A.S.R., Soh C.P.C., Watkins W.M. and Morgan W.T.J. (1982) Biochem. Biophys. Res. Commun., 104, 58-65.
6. Scudder P., Uemura K., Dolby J., Fukuda M.N. and Feizi T. (1983) Biochem. J. (In Press)
7. Tamm I. and Horsfall F.L. (1950) Proc. Soc. Exp. Biol. Med., 74, 108-114.
8. Veh R.W., Michalski J., Corfield A.P., Sander-Wewer M., Gies D. and Schauer R. (1981) J. Chromatog., 212, 313-322.
9. Donald A.S.R. (1981) Eur. J. Biochem., 120, 243-249.
10. Warren L. (1959) J. Biol. Chem., 234, 1971-1975.
11. Donald A.S.R. (Unpublished)
12. Kitamikado M. and Ueno R. (1970) Bull. Jap. Soc. Sci. Fish, 36, 1175-1180.
13. Fukuda M.N. (1981) J. Biol. Chem., 256, 3900-3905.
14. Svennerholm L. (1963) J. Neurochem., 10, 613-623.
15. Mollison P.L. (1983) Blood Transfusion in Clinical Medicine, 7th edn., p.471, Blackwell, Oxford.
16. Inoue S., Iwasaki M. and Matsumura G. (1981) Biochem. Biophys. Res. Commun., 102, 1295-1301.
17. Race R.R. and Sanger R. (1975) Blood Groups in Man, 6th edn., pp. 399-400, Blackwell, Oxford.
18. Cartron J.P. and Blanchard D. (1982) Biochem. J., 207, 497-504.