Sera of i subjects have the capacity to synthesize the branched GlcNAcβ(1⟶6)[GlcNAc(β1⟶3)]Gal... structure

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1. INTRODUCTION

Anti-I antibodies recognize the branched Galβ(1⟶4)GlcNAcβ(1⟶6)[Galβ(1⟶4)GlcNAcβ(1⟶3)]Galβ(1⟶4)GlcNAc... structure whereas anti-i antibodies react with the linear $[Gal\beta(1\longrightarrow4)GlcNAc\beta(1\longrightarrow3)]_n$ sequence (see citations in [1,2]). Even before the immunodominant structures of I and i antigens became known one of us predicted that a hypothetical *I* gene should specify an *N*-acetylglucosaminyltransferase which transfers the amino sugar in a β(1⟶6) linkage to D-galactopyranosyl residues of type 2 glycans [3,4]. Consequently in subjects with a hereditary deficiency of I antigen in erythrocytes; i.e., in subjects with i phenotype the enzyme should be either missing or defective. Our recent finding of UDP-GlcNAc:Gal-R,β(1⟶6)-*N*-acetylglucosaminyltransferase activity in human serum [5] gave us the opportunity to test the hypothesis. We have succeeded for the first time in demonstrating the biosynthesis of the branched structure, but under the experimental conditions employed its formation was similar in both I and i sera.

2. MATERIALS AND METHODS

Samples of sera of two i subjects (E.L., S.L.) were obtained by courtesy of Ms M. Grabowska and Ms H. Budzińska of the Regional Blood Banks in Kielce and Łódź, respectively. E.L. erythrocytes were typed as i, A_1B, Rh+, and those of S.L. individual as i, O, Rh−. Control sample 3 was a mixture of sera from 10 donors of different ABO blood groups, and control serum 4 was obtained from a W.N. donor with I, A_1, Rh+ blood phenotype. Other reagents were the same as in [5]. The enzymic transfer of *N*-acetyl-D-glucosamine to lactose labelled with ^{14}C in galactopyranosyl residue was perfomed as in [5] but the incubation times were prolonged to 72 h. The reaction mixtures contained the following components in a total volume of 100 μl: TES (pH 7.0), 10 μmol; $MnCl_2$, 2 μmol; ATP, 0.6 μmol; NaN_3, 0.5 μmol; UDP-*N*-acetyl-D-glucosamine, 3.7 μmol; [^{14}C]-Galβ(1⟶4)Glc, 7.8×10^5 cpm, 2 nmol; serum, 50 μl.

The reaction products were deproteinized and deionized as in [5] and then freed from lactose by chromatography on Whatman 1 paper in ethyl acetate/pyridine/water (12:5:4, v/v). For autoradiography the chromatograms were exposed to X-ray film (ORWO DK-5) at room temperature for times specified in the figures. Hydrolysis of the reaction products with β-*N*-acetylhexosaminidase from *Turbo cornutus* was performed as in [5]. For methylation analysis aliquots of each reaction product were mixed with 2-L-fucosyllactose, and reduced with sodium borohydride. The reduced

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products were then methylated and processed to partially methylated alditol derivatives as in [5]. Thereafter a standard mixture of partially methylated galactitol acetates containing: 2,3,4,6-tetra-*O*-methyl-; 2,3,6-tri-*O*-methyl-; 2,4,6-tri-*O*-methyl-; 2,3,4-tri-*O*-methyl-; 2,3-di-*O*-methyl-; 2,4-di-*O*-methyl-; 2,6-di-*O*-methyl-; 3,4-di-*O*-methyl-; 3,6-di-*O*-methyl-; 4,6-di-*O*-methyl-; and 6-*O*-methyl-galactitol derivatives was added to each sample. The resulting mixtures were then separately subjected to preparative gas chromatography as in [5] on a 200 × 0.4 cm glass column packed with 3% ECNSS-M on Gas Chrom Q and in two instances also on a column of similar dimensions but packed with 3% OV-225 on Gas Chrom Q. The two columns were operated isothermally at 180 and 170°C. Vapours of individual peaks were collected and counted for radioactivity as in [5]. Partially methylated standards of galactitol acetates were prepared by undermethylation of methyl α-D-galactoside (Koch-Light) under two sets of conditions when 50 and 100% of the theoretical amounts of sodium methyl sulfinyl carbanion were present in the reaction mixtures. After derivatization to partially methylated galactitol acetates the samples were pooled and subjected to preparative gas chromatography on a 3% ECNSS-M column. Individual peaks were collected and

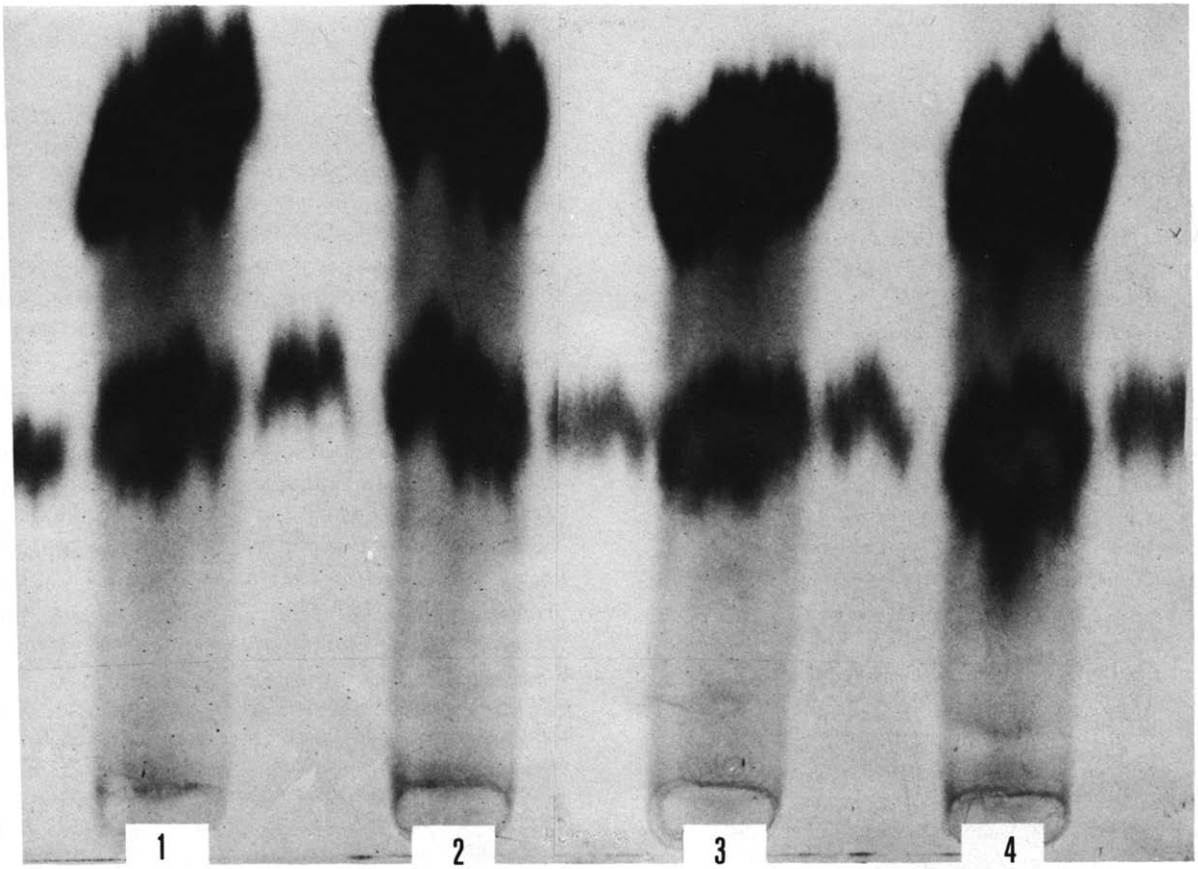
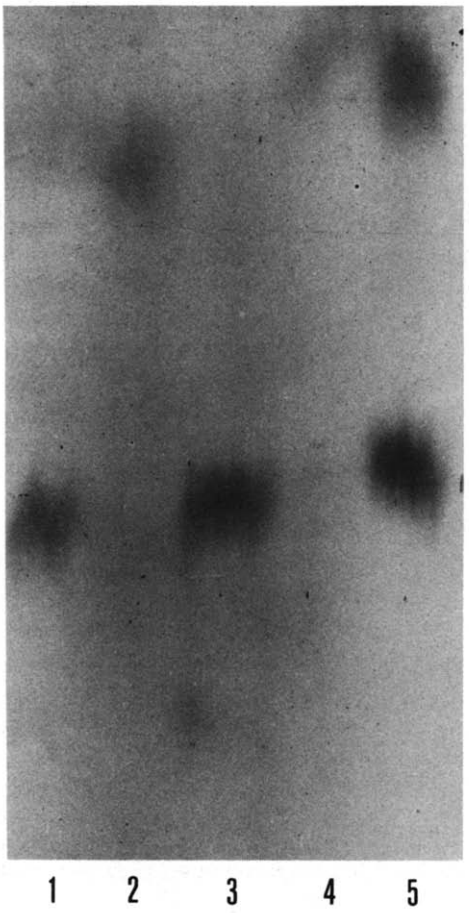


Fig.1. Autoradiography of the reaction products of enzymic transfer of *N*-acetyl-D-glucosamine to lactose labelled in galactopyranosyl residues employing i and I sera as enzyme sources. The products were synthesized, deproteinized, deionized and subjected to paper chromatography, and autoradiography. Paper chromatograms were exposed to X-ray films for 48 h. Lanes 1, 2 and 4 represent reaction products synthesized with E.L. (i), S.L. (i) and W.N. (I) serum samples, respectively; lane 3, reaction products obtained with a sample of pooled sera of 10 donors. Single bands in between numbered lanes represent a product of enzymic transfer of *N*-acetyl-D-[^{14}C]glucosamine to lactose obtained as in [5]. The upper, intense band in lanes 1–4 is lactose.

then mixed together in roughly equimolar proportions. Identities of each isolated peak were confirmed before pooling by GLC–MS technique using a 5995A Hewlett-Packard mass spectrometer equipped with 12 m long capillary-column made of fused silica and coated with methylsilicone. The column was operated at 160°C. Mass spectra were recorded at an ionization potential of 70 eV and a temperature of ion source of 148°C.

3. RESULTS

Fig.1 shows autoradiograms of the reaction products of enzymic transfer of *N*-acetyl-D-glucosamine to radioactive lactose using I and i sera as enzyme source. The radioactive bands migrating below lactose were separately isolated from each sample and counted for radioactivity. Yields varied from 50000–60000 cpm for different sera.



Aliquots of the pooled reaction product 1 + 2 and of the reaction product 3 + 4 were subjected to enzymic hydrolysis with *β-N*-acetylhexosaminidase. As shown by fig.2 the products were decomposed with the liberation of radioactive lactose. The remaining portions of the reaction products were separately subjected to methylation analysis employing preparative gas chromatography (see table 1). The product derived label was recovered in 2,4,6-tri-*O*-methylgalactitol, 2,3,4-tri-*O*-methylgalactitol and 2,4-di-*O*-methylgalactitol. Thus the penultimate galactopyranosyl residues of the reaction products were 3-*O*, 6-*O*, and 3,6-di-*O* substituted with residues of *N*-acetyl-D-glucosamine, respectively. The results were similar for all i and I sera examined.

4. DISCUSSION

Our results clearly show that a prolonged incubation of human serum with UDP-GlcNAc and lactose in the presence of Mn^{2+} results not only in the formation of GlcNAcβ(1⟶3)Galβ(1⟶4)-Glc, and GlcNAcβ(1⟶6)Galβ(1⟶4)Glc trisaccharides [5] but also of GlcNAcβ(1⟶6)[GlcNAc β(1⟶3)]Galβ(1⟶4)Glc branched tetrasaccharide. We did not notice this in [5], most probably because the linkage analysis was performed only after 24 h incubation. No difference has been observed between the biosynthesis of the branched tetrasaccharide in I and i sera. This finding apparently contradicts our earlier results on the deficiency of the branched structure in glycoconjugates isolated from i erythrocytes [6,7]. Two ex-

Fig.2. Hydrolysis of the isolated reaction products with *β-N*-acetylhexosaminidase. The products isolated from respective regions of paper chromatograms (see fig.1) were digested with *β-N*-acetylhexosaminidase and then subjected to paper chromatography and autoradiography. The paper chromatogram was then exposed to X-ray films for 196 h. Lanes 1 and 2, pooled products 1 + 2 (about 5000 cpm) incubated with heat inactivated enzyme (5 min at 100°C) and with the active enzyme respectively; lanes 3 and 4, pooled products 3 + 4 (about 5000 cpm) incubated with the heat inactivated and active enzyme, respectively; lane 5, mixture of radioactive lactose and of a product of *N*-acetyl-D-[^{14}C]glucosamine transfer to lactose obtained as in [5]. Numbering of samples as in fig.1.

planations of this discrepancy are possible. Firstly, UDP-GlcNAc: Gal-R,β-6-*N*-acetylglucosaminyltransferase of i sera may have a higher K_m value towards one of its substrates than the enzyme of I sera. Different K_m values of *A*-gene specified transferase of *N*-acetyl-D-galactosamine (EC 2.4.1.40) towards 2-L-fucosyllactose as recorded in sera of subjects with A_1 and A_2 blood groups (see citations in [8]) is a good example of this possibility. Clarification of this point would require a substrate specific for GlcNAcβ(1⟶6)Gal linkage formation presumably containing at least two linear *N*-acetyllactosamine units. Otherwise enzyme deficiency in i subjects may be restricted to the hemopoetic tissue with serum activity largely unaffected.

Recently a number of reports on the occurrence and properties of β(1⟶3)- and β(1⟶6)-transferases of *N*-acetylglucosamine [9,10] or of β(1⟶3)-*N*-acetylglucosaminyltransferase alone

Table 1

The distribution of radioactivity (cpm) in a mixture of partially methylated galactitol acetates including those derived from methylated products of transfer of *N*-acetylglucosamine to lactose labelled in D-galactopyranosyl residue as catalysed by sera of i and I subjects

Positions of OCH_3 groups	3% ECNSS-M		3% OV-225	3% ECNSS-M		3% OV-225
	No.1	No.2	No.1+2	No.3	No.4	No.3+4
2,3,4,6-	2744	2346	2309	1262	1601	2572
2,4,6-	6545[a] (5223)	6408[a] (4924)	5960[a] (5179)	2832[a] (2217)	4367	7872[a] (6560)
2,3,6- + 3,4,6-	152[a] (1322)	161[a] (1484)	140[a] (922)	85[a] (605)	201	132[a] (1350)
2,3,4-	1374[b] (924)	709[b] (635)	887[b] (834)	515[b] (359)	1113[b] (718)	1513[b] (1018)
2,6- + 4,6-	220[b] (410)	62[b] (136)	138[b] (191)	45[b] (201)	78[b] (474)	241[b] (736)
3,6-	174	26	56	24	44	67
6-	0	0	58	30	45	57
2,3-	129[c] (177)	82[c] (109)	144	48[c] (98)	120[c] (140)	177
2,4-	713[c] (665)	595[c] (568)	579	313[c] (283)	352[c] (332)	790
3,4-	109	60	28	32	41	0
Average radioactivity in between specified derivatives	138 (0–211)	92 (0–141)	114 (0–170)	56 (0–81)	99 (0–182)	148 (0–190)

Numbering of serum samples is the same as in fig.1 and 2. [a–c] Values corrected for incomplete separation of peaks by the triangle method. The determined count values are given below in parentheses. For sample 4 the whole 'theoretical' 2,4,6-tri-*O*-methylgalactitol peak was collected and hence no correction was made. Uncorrected count values are given for 2,3-di-*O*-methylgalactitol and 2,4-di-*O*-methylgalactitol for samples 1 + 2, and 3 + 4 because the two derivatives are well separated on the 3% OV-225 column. 2,3,6-Tri-*O*-methylgalactitol + 3,4,6-tri-*O*-methylgalactitol, and 2,6-di-*O*-methylgalactitol + 4,6-di-*O*-methylgalactitol are represented by single count values respectively because the derivatives of each pair are not separated on either column employed. Radioactivity recovered in 2,3,4,6-tetra-*O*-methylgalactitol is due to a contamination of the reaction products with lactose

[11–13] have appeared. Yates and Watkins [11] have not found $\beta(1\longrightarrow6)$-*N*-acetylglucosaminyltransferase activity with lactose as an acceptor in human serum but the linkage analysis was performed on a product obtained under different experimental conditions, namely at a lower UDP-GlcNAc concentration. In none of these reports was the formation of the branched structure observed.

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REFERENCES

[1] Hakomori, S. (1981) Semin. Hematol. 18, 39–62.
[2] Feizi, T. (1981) Immunol. Commun. 10, 127–156.
[3] Kościelak, J. (1977) in: Human Blood Groups (Mohn, J.F., Plunkett, R.W., Cummingham, R.K. and Lambert, R.M. eds) pp.143–149, Karger, Basel.
[4] Kościelak, J., Miller-Podraza, H., Krauze, R. and Piasek, A. (1976) Eur. J. Biochem. 71, 9–18.
[5] Zieleński, J. and Kościelak, J. (1983) FEBS Lett., in press.
[6] Kościelak, J., Zdebska, E., Wilczyńska, Z., Miller-Podraza, H. and Dzierzkowa-Borodej, W. (1979) Eur. J. Biochem. 96, 331–337.
[7] Zdebska, E., Magnuska, A., Kuśnierz, G., Seyfried, H. and Kościelak, J. (1980) FEBS Lett. 120, 33–36.
[8] Watkins, W.M. (1980) Adv. Hum. Genet. 10, 1–136.
[9] Van den Eijnden, D.H., Winterwerp, H., Smeeman, P. and Schiphorst, W.E.C.M. (1983) J. Biol. Chem. 258, 3435–3437.
[10] Basu, M., Chon, H.C., Brown, T. and Basu, S. (1983) in: Glycoconjugates, Proc. 7th Int. Symp. Glycoconjugates, Lund-Ronneby, July 17–23 (Chester, M.A., Heinegard, D., Lundblad, A. and Svensson, A. eds) p.773, Rahms and Lund.
[11] Yates, A.D. and Watkins, W.M. (1983) in: Glycoconjugates, Proc. 7th Int. Symp. Glycoconjugates, Lund-Ronneby, July 17–23 (Chester, M.A., Heinegard, D., Lundblad, A. and Svensson, A. eds) pp.762–763, Rahms and Lund.
[12] Piller, F. and Cartron, J.P. (1983) in: Glycoconjugates, Proc. 7th Int. Symp. Glycoconjugates, Lund-Ronneby, July 17–23 (Chester, M.A., Heinegard, D., Lundblad, A. and Svensson, A. eds) pp.764–765, Rahms and Lund.
[13] Van de Eijnden, D.H. and Schiphorst, W.E.C.M. (1983) in: Glycoconjugates, Proc. 7th Int. Symp. Glycoconjugates, Lund-Ronneby, July 17–23 (Chester, M.A., Heinegard, D., Lundblad, A. and Svensson, A. eds) pp.766–767, Rahms and Lund.