Biochimica et Biophysica Acta, 443 (1976) 402–413

BBA 77403

ALKALI-LABILE OLIGOSACCHARIDES FROM GLYCOPROTEINS OF DIFFERENT ERYTHROCYTE AND MILK FAT GLOBULE MEMBRANES

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(Received January 8th, 1976)

SUMMARY

Phenol extraction of horse, sheep, cow, pig and human erythrocyte membranes and human milk fat globule membranes gave glycoprotein fractions, all of which were shown by gas chromatography to contain the reduced disaccharide β-D-galactosyl (1–3)-*N*-acetyl-D-galactosaminital after treatment with alkaline borohydride. Cow and pig erythrocyte membrane glycoproteins were found however to contain much lower amounts than the erythrocyte membrane glycoproteins of the other species tested. After gel filtration, a tetrasaccharide was isolated from horse and sheep glycoproteins containing the disaccharide plus two molecules of sialic acid. Periodate oxidation together with paper chromatography of alkaline degraded fragments showed these two molecules of sialic acid to be linked to positions C3 and C6 of the galactosyl and *N*-acetylgalactosamine residues respectively. Evidence was obtained for a similar structure from pig and cow erythrocyte glycoproteins and human milk fat globule membrane glycoproteins although the complete structure was not elucidated.

In all native glycoprotein fractions, the unsubstituted disaccharide β-D-galactosyl (1-3)-*N*-acetyl-D-galactosamine was found to be present to different extents.

Haemagglutination inhibition tests against human anti-T serum, *Arachis hypogoea* and *Vicia graminea* by desialylated glycoproteins showed the presence of the T-antigen, confirming the chemical findings. Inhibition was found to be proportional to the chemically detected amounts of disaccharide in each fraction. Evidence for a second carbohydrate chain in horse, sheep and human erythrocyte glycoproteins with a sialic acid substituted *N*-acetylgalactosamine residue as the terminal sequence was obtained using the agglutinin from *Helix pomatia*.

INTRODUCTION

Reductive alkaline cleavage of the glycoprotein from human erythrocyte membranes resulted in the release of a tetrasaccharide with the structure *N*-acetylneuraminyl(2-3)-β-D-galactosyl(1-3)-[*N*-acetylneuraminyl(2-6)]-*N*-acetyl-D-galactos-

aminitol. This structure was shown by Thomas and Winzler to be the major alkaline-labile oligosaccharide of the human erythrocyte membrane [1] and it is the structure required for blood group M and N activity [2]. Earlier work by Klenk and Uhlenbruck [3] had shown that the T-antigen (Thomsen-Friedenreich) is a cryptic receptor of human erythrocyte membrane glycoprotein and is normally masked by sialic acid. Subsequent investigations revealed that the immunodominant structure of the T-antigen is the alkaline-labile disaccharide β-D-galactosyl(1-3)-*N*-acetyl-D-galactosamine [4, 5], the asialo form of the tetrasaccharide isolated by Winzler.

The T-antigen has been reported to occur on the erythrocytes of a variety of species after neuraminidase treatment [6], and in humans it has clinical relevance in that it is responsible for erythrocyte panagglutination by naturally occurring serum antibodies after microbial infection [7, 8]. The presence of the free T-antigen has also been reported to occur on the surface of mammary carcinoma cells [9], thus distinguishing them from their normal counterpart.

We previously reported the presence on pig peripheral lymphocyte membrane of an alkaline-labile sialic acid-covered disaccharide of the structure β-D-galactosyl-(1-3)-*N*-acetyl-D-galactosamine [10] and an alkaline-labile tetrasaccharide, identical in structure to that reported by Winzler [1], on the membrane of the bovine milk fat globule [11]. The detection of the T-antigen on erythrocytes of different species suggested to us that the above disaccharide or tetrasaccharide may be present on the erythrocyte membranes of different species. In this paper we investigate this possibility as well as structures present on human milk fat globule membranes, in order to correlate chemical findings with previously known serological properties and determine whether this alkaline-labile structure is a common component of different types of membranes from different species.

MATERIALS AND METHODS

Materials

Animal blood was obtained from freshly slaughtered animals and prevented from clotting by addition of sodium citrate, (1 vol. 3.8 % sodium citrate solution to 5 vol. blood), human blood was obtained from outdated blood samples (Blood bank, University Clinic, Cologne).

Human milk was collected fresh from the Frauenklinik of the University of Cologne and pooled.

The disaccharide β-D-galactosyl(1-3)-*N*-acetyl-D-galactosamine, isolated from human brain gangliosides (12), was a gift from Prof. W. Gielen (Pharmakologisches Institut der Universität, Köln).

Neuraminidase (500 units/ml) was obtained from Behringwerke Ltd. (Marburg). Monosaccharides for gas chromatography, *N*-acetyl-neuraminic acid, Dowex 50×8 and Dowex 1×8 were obtained from Serva Ltd. (Heidelberg). Sodium borohydride was purchased from Fluka Ltd. (Buchs, Switzerland).

Acrylamide, *N,N'*-methylenebisacrylamide, ammonium persulphate, sodium dodecyl sulphate and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were all purchased from Serva Ltd.

Agglutinin-containing extracts from *Helix pomatia*, *Arachis hypogoea*, *Vicia gramineα* as well as anti-T from human serum were prepared as described by Dahr et

al. [13]. A crude myxovirus B-Lee preparation was a gift from the late Prof. A. Gottschalk (Tübingen) and was centrifuged and membrane filtered before use.

Membrane filters (pore size 0.45 μm) were obtained from Millipore Ltd. (Bedford Mass. U.S.A.) and all other reagents for general use were purchased from Merck Ltd. (Darmstadt).

Glycoprotein isolation

Whole blood was centrifuged at 2000 $\times g$ for 15 min and the serum and buffy coat removed. The erythrocytes were washed three times with physiological saline (0.9 % w/v) and then haemolysed by the addition of 20 volumes of distilled water at 4 °C to the packed erythrocytes. The erythrocyte ghosts were allowed to sediment overnight at 4 °C, augmented by the addition of 5 ml of acetic acid (1.5 M) to 10 l of suspension. Milk fat globule membranes were isolated from 20 l of whole human milk as previously described [14]. The erythrocyte ghosts and milk fat globule membranes were resuspended in distilled water and centrifuged at 3000 $\times g$ for 30 min. The resulting pellet was again resuspended in distilled water and recentrifuged. This washing process was repeated three times. The washed pellet was then resuspended in physiological saline and extraction of the membranes carried out with 90 % phenol at 65 °C as described previously by Uhlenbruck et al. [15]. The glycoprotein solutions, after dialysis against distilled water, were membrane filtered and lyophilized. The lyophilized products were stored in a desiccator over $CaCl_2$ until required.

Chemical treatments

(1) *Desialylation of glycoproteins and oligosaccharides.* Desialylation of glycoproteins or oligosaccharides was carried out by mild hydrolysis (0.05 M H_2SO_4, 80 °C, 1 h) followed by neutralisation with Dowex 1 (OH^--form) resin. The resin was removed by centrifugation (2000 $\times g$ for 5 min) and washed three times with distilled water, following which the combined supernatant and washings were lyophilized. Incubation with neuraminidase was found to be equally as effective as acid hydrolysis.

(2) *Alkaline-borohydride treatment.* Alkaline borohydride treatment of glycoprotein samples was performed by dissolving glycoprotein (1–5 mg at a concentration of 1 mg/ml) in 0.05 M NaOH, containing 1.0 M $NaBH_4$ and incubating in the dark at 50 °C for 24 h. Trehalose was then added as an internal standard (30 μg) and excess borohydride destroyed by adjusting to pH 6.0 with Dowex 50 (H^+-form) resin until no more gas was evolved. After centrifugation (2000 $\times g$ for 5 min) the supernatant was removed and the resin washed three times with water. The combined supernatants and washings were lyophilized and boric acid removed by repeated evaporation with methanol.

(3) *Alkaline treatment.* Alkaline treatment of the glycoprotein samples was performed by dissolving the glycoprotein (5–10 mg) in water (5 ml) and adjusting to 0.01 M with a solution of 0.2 M barium hydroxide. The solution was incubated at 80 °C for 15 min, cooled and neutralised with 0.05 M H_2SO_4. The resulting precipitate was removed by centrifugation (2500 $\times g$, 10 min) and the supernatant dialysed against distilled water. The dialysate was lyophilized and used for paper chromatography [16, 17].

(4) *Periodate oxidation.* Periodate oxidation of native or desialylated glyco-

proteins was carried out by dissolving the sample in 0.02 M sodium acetate buffer (2 mg/ml, pH 4.5) containing 0.01 M sodium periodate. The reaction was cooled in ice and left in the dark at 4 °C for 24 h after which, excess borohydride was added to reduce any remaining periodate. The excess borohydride was then destroyed by adjusting to pH 6.0 with 2.0 M acetic acid followed by dialysis overnight against distilled water and lyophilisation. It was later found however, that dialysis directly after periodate treatment brought about the same result.

Analytical procedures

(1) *Sialic acid.* Total sialic acid was released by acid hydrolysis (0.1 M H_2SO_4, 1 h at 80 °C) and assayed by the method of Aminoff [18] using *N*-acetylneuraminic acid as a standard. Free sialic acid was assayed directly without prior hydrolysis.

(2) *Hexoses and hexosamines.* Hexoses were determined colorimetrically by the method of Dische and Danilchenko [19], using D-galactose as a standard.

Individual hexoses and hexosamines were determined by gas chromatography of their trimethylsilyl derivatives, after acid hydrolysis (3 M HCl, 4 h at 100 °C) and reacetylation as described previously [11]. Samples were run on columns of 3 % SE-30 and OV-17 on Gas-Chrom Q using a temperature programme of 125–230 °C at 4 °C/min.

(3) *Disaccharides.* Reduced disaccharides released by alkaline borohydride treatment were examined by gas chromatography, as previously described [20], after removal of sialic acid by mild acid hydrolysis. Reduced disaccharides were measured as above using an isothermal programme of 250 °C.

(4) *Sephadex chromatography.* Glycoprotein samples (10 mg) that had been treated with alkaline borohydride and freed of boric acid, were dissolved in water (2 ml) and applied to a column of Sephadex G-25 (1 × 100 cm) previously equilibrated with 0.1 M acetic acid. The column was eluted with 0.1 M acetic acid at 4 °C and fractions were collected and assayed for sialic acid and hexose.

(5) *Paper chromatography.* Products from alkaline hydrolysis of membrane glycoproteins were run on Schleicher-Schüll paper No. 2043b Mgl (Schleicher-Schüll Ltd. Dassel, G.F.R.) in *n*-butan-1-ol/pyridine/water (6 : 4 : 3, v/v). The spots were developed with Ehrlich spray [17] and standards of *N*-acetylneuraminic acid with orcinol spray [21].

Alkaline-labile oligosaccharides, released in a reduced form by alkaline borohydride, were run in ethyl acetate/pyridine/acetic acid/water (5 : 5 : 1 : 3, v/v) using the descending technique. Neutral and reduced sugars were visualised by alkaline silver nitrate [21] and sialic acid-containing compounds by a periodate-thiobarbituric acid method. The paper was sprayed on a horizontal surface with an acidic solution of $NaIO_4$ (100 ml H_2SO_4, 0.025 M containing 0.5 g $NaIO_4$) and left at room temperature for 15 min before drying. This was followed by spraying with a solution of sodium arsenite (2 g sodium arsenite dissolved in 100 ml of 0.5 M HCl), leaving for 10 min and then drying. To develop the colour the paper was sprayed a third time (6 g thiobarbituric acid dissolved in 100 ml 0.5 M NaOH) and heated for 10 min at 100 °C.

(6) *Sodium dodecyl sulphate polyacrylamide gel electrophoresis.* Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed on 10 % acrylamide gels using the discontinuous system of Laemmli [22]. Samples (50–100 μg) were applied to the top of a spacer gel, Pyronin G being used as a marker. After electrophoresis the

gels were stained for protein with Coomassie blue [23] or for carbohydrate by periodate-Schiff's reagent [24].

(7) *Immunological assays.* Haemagglutination tests were performed using the serial dilution system of Cooke Ltd. (Cooke Instruments, Zollikon, Switzerland). Desialylated erythrocytes were prepared from outdated blood (Blood bank, University Medical Clinic, Cologne) by incubating a 2 % suspension (20 ml) in phosphate buffered saline with 200 μl of neuraminidase solution for 1 h at 37 °C. Cells were then washed with phosphate-buffered saline and 2 % suspensions used for assays.

RESULTS

Analysis of glycoprotein fractions

Glycoprotein fractions from horse, sheep and pig erythrocyte membranes showed patterns on sodium dodecyl sulphate gel electrophoresis similar to those obtained from human erythrocyte membrane glycoprotein. Several bands were present over a large molecular weight range, most of which stained for both protein and carbohydrate. Whether these represent different glycoproteins or different aggregation products, which is very possible [25], was not determined. Glycoprotein fractions from cow erythrocyte membranes and human milk fat globule membranes did not reveal distinct bands, although the glycoprotein fraction from cow contained a large molecular weight staining region (Fig. 1).

The carbohydrate analysis of the different glycoprotein fractions is shown in Table I. It can be seen that human, sheep and horse erythrocyte glycoprotein fractions form a group in that they possess relatively higher amounts of *N*-acetyl-galactosamine.

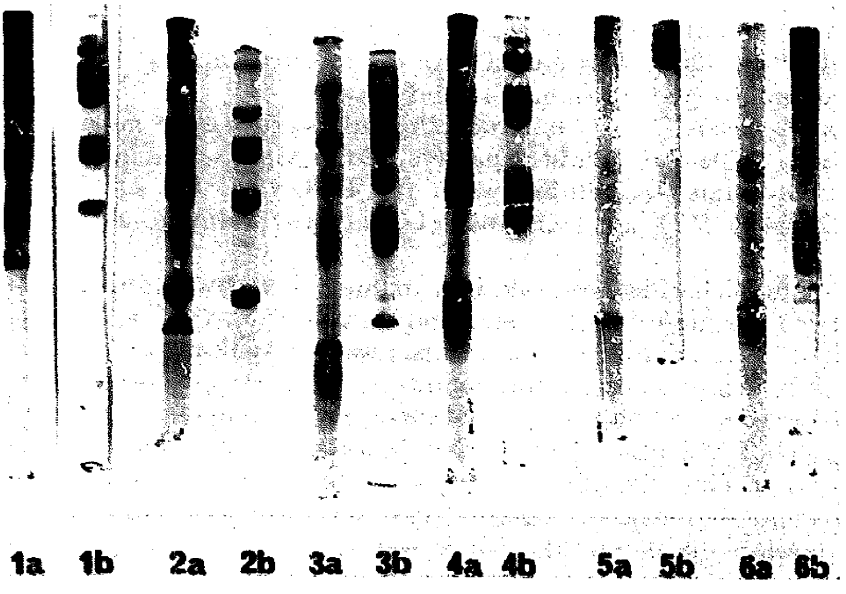

Fig. 1. Sodium dodecyl sulphate gel electrophoresis patterns of the glycoprotein fractions using 10 % acrylamide containing gels. Samples (75 μg) of lyophilized glycoprotein from (1) human erythrocyte, (2) horse, (3) sheep, (4) pig, (5) cow, (6) human milk fat globule membranes were prepared as described by Dahr et al. [23]. Gels were stained for protein with Coomassie brilliant blue (a) and for carbohydrate with periodate-Schiff reagent (b).

TABLE I

MONOSACCHARIDE COMPOSITION OF GLYCOPROTEIN FRACTIONS

Sialic acid is calculated as *N*-acetylneuraminic acid. Variation was less than 10 % of each value.

Monosaccharide (μmol/100 mg glycoprotein)	Erythrocytes					Milk fat globule membranes
	Horse	Sheep	Cow	Pig	Human	Human
Fucose	1.?	4.3	3.7	4.9	3.7	24.4
Mannose	1.7	1.1	1.1	7.8	6.3	15.6
Galactose	28.9	35.0	105.6	37.8	28.9	55.6
Glucose	6.6	6.1	13.9	8.3	2.2	45.6
N-acetyl-galactosamine	17.6	38.9	34.8	13.6	20.8	8.6
N-acetyl-glucosamine	5.0	11.3	76.0	19.9	12.7	34.4
Sialic acid	33.2	33.5	42.3	14.2	48.7	5.2

The other fractions from pig and cow erythrocyte membranes and human milk fat globule membranes are relatively higher in *N*-acetylglucosamine (Table I).

Alkaline borohydride treatment of glycoprotein fractions

(1) *Identification of disaccharide.* The lyophilized glycoprotein fractions which had been treated with alkaline borohydride, desialylated and assayed gas chromatographically, showed a single peak on columns of SE-30 and OV-17, corresponding to a standard of β-D-galactosyl(1-3)-*N*-acetyl-D-galactosaminitol. This reduced disaccharide was present in all the fractions tested, although those from cow and pig erythrocytes and human milk fat globules contained low amounts.

The amount of the unsubstituted alkaline-labile disaccharide was determined by omitting the desialylation step prior to the gas-chromatographical analysis. The total and the unsubstituted disaccharide content of each fraction is presented in Table II.

TABLE II

β-GALACTOSYL(1-3)-*N*-ACETYLGALACTOSAMINE CONTENT OF GLYCOPROTEIN FRACTIONS

Values in brackets are the percentage of disaccharide recovered after periodate oxidation against an untreated control.

Disaccharide (μmol/100 mg glycoprotein)	Erythrocytes					Milk fat globule membranes
	Horse	Sheep	Cow	Pig	Human	Human
Total disaccharide	19.3	18.3	1.0	2.3	20.3	4.3
Unsubstituted disaccharide	3.8	8.3	0.3	1.8	2.3	1.5
Substituted disaccharide	14.7 (76 %)	10.8 (58 %)	0.7 (70 %)	0.4 (17 %)	18.4 (92 %)	1.3 (30 %)

(2) *Sephadex chromatography of horse and sheep alkaline-labile oligosaccharides.* Apart from the human erythrocyte membrane glycoprotein, horse and sheep glycoprotein fractions contained the highest amounts of substituted disaccharide (Table II). These two fractions therefore were subjected to Sephadex gel chromatography after alkaline borohydride treatment, in order to isolate alkaline-labile oligosaccharides. After passage through Sephadex G-25, sheep erythrocyte glycoprotein showed the presence of three peaks (Fig. 2). A similar pattern was observed with horse glycoprotein. Tubes from each peak were pooled and lyophilized.

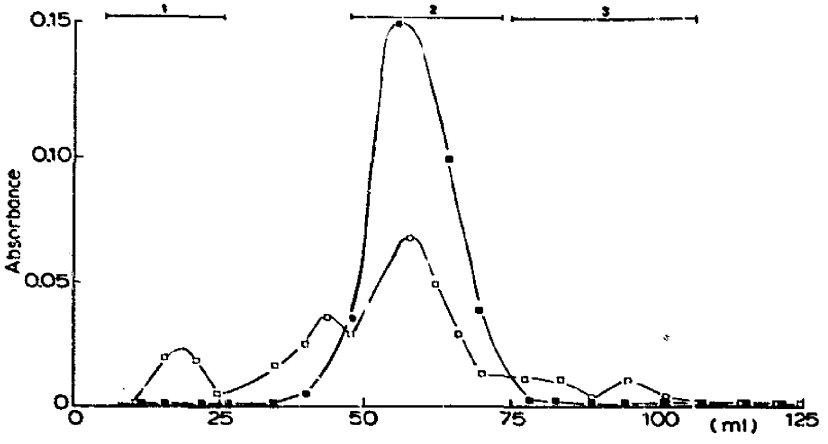

Fig. 2. Fractionation on Sephadex G-25 of sheep erythrocyte membrane glycoprotein fraction after alkaline borohydride treatment. The boric acid free products were dissolved in water (2 ml) and applied to a column (1 × 100 cm), equilibrated and eluted with 0.1 M acetic acid at 4 °C with a flow rate of 10 ml/h. Samples of the fractions (1 ml) were analysed for hexose $A_{420\,nm}$, —□—; sialic acid $A_{550\,nm}$, —■—. Fractions were pooled as indicated.

The lyophilized product of peak 2 was dissolved in water (2 ml) and after passing through a column of Sephadex G-15 the elutate showed the presence of two peaks (Fig. 3 shown for sheep erythrocyte glycoprotein). Fractions were pooled and the major peak (fraction 2A) lyophilized and assayed for free and bound sialic acid and β-D-galactosyl(1-3)-*N*-acetyl-D-galactosaminitol. The ratio of sialic acid to disaccharide was found to be approximately 2 : 1 in both horse and sheep glycoprotein (1.93 : 1 for sheep, 1.90 : 1 for horse). No unsubstituted disaccharide or sialic acid was present in this fraction, and when a total hydrolysis of this fraction was carried out, the only monosaccharides detected by gas chromatography were galactose and *N*-acetylgalactosaminitol in a ratio of 1 : 1. Fraction 2B from Fig. 3 was found to contain small amounts of unsubstituted disaccharide only. There was no evidence for a peak containing a trisaccharide.

(3) *Paper chromatography of alkaline-borohydride treated glycoproteins.* Paper chromatography of alkaline-borohydride products from horse, sheep and human erythrocyte membrane glycoproteins gave a thiobarbituric acid-positive spot. An R_F value of 0.20, relative to lactose, was found which was identical to that of the tetrasaccharide isolated from horse and sheep glycoproteins by column chromatography. Treatment of this spot with neuraminidase or weak acid and rechromatography

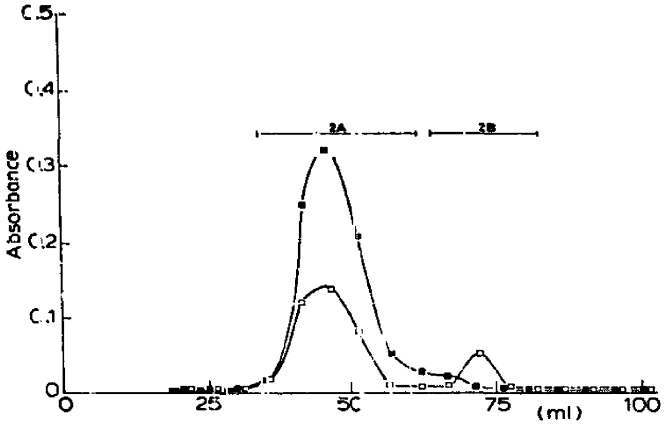

Fig. 3. Fractionation on Sephadex G-15 of fraction 2 from Sephadex G-25 (Fig. 2). Lyophilized fraction 2 was dissolved in water (3 ml) and applied to a column (1 × 100 cm) of Sephadex G-15, equilibrated and eluted with 0.1 M acetic acid at 4 °C with a flow rate of 10 ml/h. Samples of the fractions (1 ml) were analysed for hexose $A_{420\,nm}$, —□—; sialic acid $A_{550\,nm}$, —■—. Fractions were pooled as indicated.

revealed a spot identical to that of β-galactosyl(1-3)-*N*-acetylgalactosaminitol, which could also be identified, after elution from the paper, by gas chromatography. This was the only spot found after alkaline borohydride treatment of desialylated glycoproteins.

Periodate treatment of glycoprotein fractions

Native glycoproteins that had been treated with periodate, alkaline borohydride reduction and desialylation were examined by gas chromatography for the presence of the disaccharide β-galactosyl(1-3)-*N*-acetylgalactosaminitol. The amounts of disaccharide obtained after periodate treatment of glycoproteins is expressed as a percentage of the untreated control in which periodate was absent (Table II). Samples that were desialylated prior to periodate oxidation were found to contain no reduced disaccharide, this being completely destroyed during the reaction.

Alkaline treatment of glycoprotein fractions

Alkaline treatment of all the glycoprotein fractions tested followed by paper chromatography gave a spot with an R_F value of 0.09, staining with direct Ehrlich reagent at room temperature, which is known to be specific for chromogens [26]. The products from pig and cow erythrocyte glycoproteins and human milk fat globule membrane glycoproteins however, showed weaker staining spots. The spot corresponded to that obtained from alkaline treatment of human erythrocyte membrane glycoprotein. This zone has previously been identified from chromatographs of alkaline treated bovine salivary mucoprotein as 6-O-sialyl-chromogen, which had arisen from 6-O-sialyl-*N*-acetylgalactosamine [16].

Elution of this spot from the chromatogram, followed by treatment with neuraminidase and rechromatography, revealed the presence of a zone corresponding to sialic acid and a zone corresponding to chromogen 1 [16]. Alkaline treatment of the desialylated glycoproteins revealed chromogen 1 as the only spot.

Immunological assays

The inhibition titres between the different glycoprotein fractions and the agglutinins outlined in Materials and Methods are shown in Table III. It can be seen that the strongest inhibitors of human anti-T serum, *A. hypogoea* and *V. graminea* agglutinins are human, horse and sheep erythrocyte glycoproteins. Cow erythrocyte and human milk fat globule membrane glycoproteins had no detectable inhibitory activity against these agglutinins and the pig erythrocyte fraction fell intermediate between these two groups.

TABLE III

HAEMAGGLUTINATION INHIBITION OF AGGLUTININS BY NATIVE AND DESIALYLATED GLYCOPROTEINS

Values are expressed as the reciprocal of their inhibition titres. Values represent the minimum amount of inhibitor required to bring about inhibition of four agglutination doses of agglutinin. Glycoprotein samples at a concentration of 5 mg/ml and disaccharide of 2.5 mg/ml were used against: 1. Desialylated human erythrocytes of group O. 2. Desialylated human erythrocytes of group NN or MN. 3. Human erythrocytes of group A. 4. Human erythrocytes of group O.

Glycoprotein fraction	Source of Agglutinin									
	Human anti-T[1]		*A. hypogoea*[1]		*V. graminea*[2]		*H. pomatia*[3]		*Myxovirus*[4]	
Human	ø*	2^{7}*	ø	2^{10}	ø	2^{5}	ø	2^{8}	2^{10}	2^{6}
Horse	ø	2^{6}	2^{0}	2^{9}	ø	2^{5}	ø	2^{7}	2^{4}	ø
Sheep	ø	2^{4}	ø	2^{8}	ø	2^{3}	ø	2^{9}	2^{3}	ø
Pig	ø	ø	ø	2^{5}	ø	2^{4}	ø	ø	2^{6}	ø
Cow	ø	ø	ø	ø	ø	ø	ø	ø	2^{1}	ø
Human milk fat globule membrane	ø	ø	ø	ø	ø	ø	2^{5}	2^{5}	2^{1}	ø
βGal(1-3)GalN		2^{3}		2^{5}		ø		ø		N.T.

* Left hand column represents native glycoproteins, right hand column represents desialylated glycoproteins. βGal(1-3)GalN, β-D-galactosyl(1-3)-*N*-acetyl-D-galactosamine; N.T., not tested.

Only human milk fat globule membrane glycoprotein was found to inhibit *H. pomatia* agglutinin in its native form, although human, horse and sheep erythrocyte glycoproteins were inhibitory after desialylation. Myxoviral receptors were present on all glycoproteins tested and in every case this receptor was destroyed after desialylation, except human erythrocyte glycoprotein where it was substantially reduced although not completely abolished.

DISCUSSION

Carbohydrate analysis of the different glycoprotein fractions revealed that horse, sheep and human erythrocyte membrane glycoproteins possessed relatively high amounts of *N*-acetyl-galactosamine and, after alkaline borohydride treatment and desialylation, relatively high amounts of the disaccharide β-D-galactosyl(1-3)-*N*-acetyl-D-galactosaminitol. Glycoproteins from pig and cow erythrocytes and human milk fat globule membranes, on the other hand, showed higher amounts of *N*-acetyl-

glucosamine and lower amounts of the above disaccharide. This probably represents a difference in the amounts of alkali-labile and alkali-stable chains within the different membrane glycoproteins. The amounts of unsubstituted disaccharide found in the native glycoproteins corresponded to the percentage of disaccharide destroyed by periodate, with the exception of human milk fat globule membrane glycoprotein where an exact correlation was not possible.

The different amounts of disaccharide present in the glycoprotein reflected their ability, after desialylation, to inhibit the haemagglutination activity of human anti-T serum and *A. hypogoea*, which are specific for the disaccharide β-galactosyl-(1-3)-*N*-acetylgalactosamine (4, 27). *V. graminea* was also inhibited and as this lectin is known to be blood group N specific as well as requiring the above disaccharide [28], it can be stated that the serological evidence confirms the chemical evidence for the presence of the above disaccharide.

The disaccharide β-galactosyl(1-3)-*N*-acetylgalactosamine is seemingly widespread in nature, occurring in many non-membrane proteins such as pig submaxillary gland glycoprotein [29], fetuin [30] and "antifreeze" glycoprotein from Antarctic fish [20] as well as on other membranes such as human erythrocyte membrane glycoprotein [1]. We have also shown it to be present on bovine milk fat globule membrane [11] and on pig lymphocyte plasma membrane [10]. In most cases it is further substituted by sialic acid or fucose, although the T-antigen has been serologically detected on certain mammary tumour cell surfaces [9].

Alkaline-labile fragments of horse and sheep erythrocyte glycoproteins were shown, after purification on Sephadex gels, to contain a tetrasaccharide which possessed two molecules of sialic acid linked to the reduced disaccharide β-galactosyl-(1-3)-*N*-acetylgalactosaminitol. Treatment by periodate showed that galactose could be either monosubstituted by sialic acid at C3 or disubstituted. *N*-acetylgalactosamine was shown, by chromogen formation in an alkaline medium followed by paper chromatography, to be substituted at C6 by sialic acid [16, 11]. As no alkali-labile *N*-acetylgalactosamine was detected the sialyl-chromogen must have originated from the alkaline-labile tetrasaccharide and in order to fit the periodate oxidation data the second molecule of sialic acid must be linked to the C3 position of galactose in the tetrasaccharide. This structure, shown in Fig. 4, was further supported by paper chromatography of alkaline borohydride treated glycoproteins. In view of the known specificity of neuraminidase [31], the sialyl linkages are shown as α in Fig. 4.

This structure is identical to that isolated from human erythrocyte membrane by Winzler [1] and recently shown to be present on bovine milk fat globules [11] and rat brain glycopeptides [32].

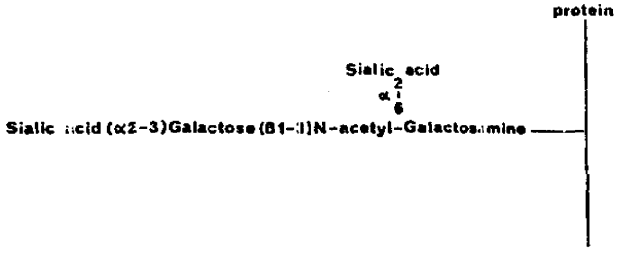

Fig. 4. Proposed structure of alkali-labile tetrasaccharide.

Glycoproteins from pig and cow erythrocyte membrane and from human milk fat globule membrane were also shown to contain an alkali-labile oligosaccharide in which the C6 position of *N*-acetylgalactosamine is substituted by sialic acid. As intact alkali-labile oligosaccharides were not isolated from these fractions, it is not known if the galactosyl moiety is substituted by one or more sialic acid residues, although the total amounts of sialic acid and galactose present suggest that the galactose residue is only monosubstituted and hence would be at position C3 to satisfy periodate oxidation data.

Horse, sheep and human erythrocyte glycoprotein fractions also showed evidence for a second sialic acid substituted carbohydrate chain, as the agglutinin from *H. pomatia* was inhibited only after desialylation. This would suggest a terminal *N*-acetylgalactosamine residue masked by sialic acid. Alkaline borohydride treatment followed by desialylation and gas chromatography failed to detect the presence of an *N*-acetylgalactosaminitol peak and so it is not known whether this carbohydrate group is linked directly to protein or is the terminal sequence of an alkali-stable chain.

The inhibition of myxoviral haemagglutination was not found to be proportional to the amount of sialic acid present and other factors as well as sialic acid content must be important such as topographical distribution and molecular weight.

It is of interest to note that human erythrocyte membrane glycoprotein is very different from human milk fat globule membrane glycoprotein, the former possessing high amounts of alkali-labile oligosaccharides and the T-antigen, after desialylation, whereas the latter possesses very little. The bovine erythrocyte, by contrast, exhibits the opposite relationship. Previous work [11] showed the bovine milk fat globule membrane glycoprotein to possess relatively high amounts of alkali-labile material (and the T-antigen after desialylation) whereas the results in this paper show very little to be present on bovine erythrocytes. Previous reports have shown bovine erythrocytes to be devoid of T-antigen [4], but the results presented here show that chemically the immunodominant disaccharide can be detected if only in low amounts, presumably too low to be detected by normal haemagglutination inhibition tests.

The results presented in this paper show that chemical findings on alkali-labile oligosaccharides can be correlated with known serological properties of cells and in view of the existence of the T-antigen on tumour cells [9], these methods may prove to be useful in determining the chemical structures of antigens associated with different, as well as malignant cells.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 68/D1). R.A.N. is an awardee of the Alexander von Humboldt Stiftung.

Thanks are expressed to Dr. Krüger (Frauenklinik, University of Cologne) for supplying us with samples of human milk and to technicians Frau Dorit Karduck and Fräulein Gisela Peters for excellent technical assistance.

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