Biochem. J. (1976) **153**, 75–78
Printed in Great Britain

Carbohydrate Composition of Lymphocyte Plasma Membrane from Pig Mesenteric Lymph Node

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(*Received* 9 *September* 1975)

Pig lymphocyte plasma membrane isolated from mesenteric lymph node contained 69 μg of carbohydrate/mg dry wt., which was made up of neutral sugar, amino sugar and sialic acid in the molar proportions 5:1.7:1. The neutral sugar comprised fucose, ribose, mannose, glucose, galactose and inositol (molar proportions 2:9:11:15:26:1), and the aminos ugar glucosamine and galactosamine (molar ratio 2:1). The ribose was most probably derived from RNA. All of the fucose and mannose and almost all of the glucosamine were associated with the membrane protein whereas the membrane lipid contained all of the inositol. The remaining sugars were distributed in various ratios between the protein and lipid fractions.

The cell-surface carbohydrate is believed to play an important role in determining the behaviour of eukaryotic cells (Hakomori, 1973; Roth, 1973; Nicolson, 1974). If this is the case, a detailed knowledge of the carbohydrate composition of the cell-surface membrane, and of the distribution of the carbohydrate between the lipid and protein fractions of the membrane is essential for the comprehension of cell behaviour at the molecular level. Some information on the carbohydrate compositions of the plasma membranes of rat ascites hepatoma cells (Shimizu & Funakoshi, 1970), human platelets (Barber & Jamieson, 1970), rabbit kidney cells (Quirk & Robinson, 1972) and rat intestinal cells (Kim & Perdomo, 1974) has been reported. The present paper describes the carbohydrate composition of pig lymphocyte plasma membrane and the relative distributions of the various sugars between the glycolipid and glycoprotein fractions.

Materials and Methods

Lymphocyte plasma membrane was isolated as described by Allan & Crumpton (1970) from homogenates of pig mesenteric lymph nodes by differential and sucrose-density-gradient centrifugation. Sucrose was removed from the purified membrane by dispersing three times in water with a tight-fitting glass homogenizer (Uniform; clearance 75–125 μm) and centrifuging at 30000rev./min for 30min (type 30 rotor; Beckman L2-65B). Two separate preparations of plasma membrane, each from a pool of six nodes, were used. The membrane protein and lipid fractions were separated by extracting the plasma membrane (10mg of protein/ml of water) with 19vol. of chloroform/methanol (2:1, v/v) at 20°C for 2h (Folch *et al.*, 1957). The protein residue was washed once with 10vol. of chloroform/methanol, and the lipid recovered from the organic phases was re-extracted with 30vol. of chloroform/methanol. After drying to constant weight *in vacuo* the lipid and protein fractions accounted for 50 and 45% respectively of the dry wt. of the whole membrane. The lipid fraction contained <1% of the leucine content of an equivalent amount of whole membrane and no fatty acids were detected by g.l.c. in the protein fraction.

Amino sugars were determined by ion-exchange chromatography after hydrolysis with 3M-toluene-*p*-sulphonic acid *in vacuo* at 100°C for 24h (Allen & Neuberger, 1973). Sialic acid was measured by the resorcinol method (Jourdian *et al.*, 1971); the thiobarbituric acid method (Warren, 1963) was not used because of interference by aldehyde groups present in the plasma membrane and the lipid fraction.

Neutral sugars were assayed by g.l.c. (Dutton, 1973; Clamp, 1974) after either methanolysis and trimethylsilylation (Sweeley & Tao, 1972) or acid hydrolysis, reduction and acetylation (Sloneker, 1972). *O*-Trimethylsilyl derivatives of the methyl glycosides were prepared and analysed as follows. Dried samples [1–2mg dry wt. containing a known amount (about 20 μg) of mannitol as the internal standard] were heated at 100°C for 2h under N_2 with methanolic HCl (0.5ml containing 0.5mmol of HCl). Fatty acids released during methanolysis were removed by extracting three times with hexane (Esselman *et al.*, 1972). The aqueous phase was

neutralized with Ag_2CO_3 and allowed to react with 50μl of acetic anhydride for 5h at 20°C. The precipitate was extracted twice with methanol and the methanol-soluble fraction was recovered by drying *in vacuo*. Trimethylsilylating reagent (25 or 50μl of Tri-Sil) was added and, after 3min, samples (2μl) were analysed on either a Pye series 104 (model 24) or a Perkin Elmer F11 chromatograph fitted with dual glass columns (200cm×0.3cm) containing 3% SE-30 on Diatomite C.A.W., and dual-flame ionization detectors. The initial temperature was held at 120°C for 30min and was then raised to 200°C at a rate of 1°C/min. Under these conditions the major mannose peak (91% of the total) was separated from the galactose peaks and it was possible to determine accurately small amounts of mannose in the presence of large amounts of galactose. Analysis of known amounts of authentic samples of various sugars showed that galactose, glucose, mannose, fucose and inositol were recovered in greater than 98% yield relative to the mannitol internal standard, that ribose was recovered in 62% yield and that deoxyribose could not be measured satisfactorily. The inositol present in the plasma membrane could not be measured by the above procedure since it overlapped the major peak of *N*-acetylglucosamine. Also, measurement of the fucose content of the membrane was precluded by a spurious peak eluted just before the fucose. Various results suggest that the spurious peak was caused by Tris which was added during the membrane preparation and which is known to bind to lipid (M. T. Flanagan, personal communication). Thus the area of the spurious peak was increased by the addition of Tris, and an authentic sample of Tris was eluted in an identical position.

In the case of the alditol acetate method, samples (1mg dry wt.) were hydrolysed with 1ml of 1M-H_2SO_4 under N_2 at 100°C for 3.5h and 150nmol of glucoheptose was then added as the internal standard. The hydrolysate was extracted three times with hexane to remove fatty acids (Esselman *et al.*, 1972) and neutralized with $BaCO_3$. Charged substances were next removed by eluting the neutralized hydrolysate from a column (1.5cm× 1.0cm) of Zeo-Karb 225 (H^+ form) with 4ml of water. This step was introduced to delete a spurious peak immediately preceding mannose that was most probably caused by Tris (see above). The free sugars were converted into their alditol acetates by reduction with $NaBH_4$ (3mg in 1ml of water) and by acetylation with 0.5ml of acetic anhydride at 100°C for 1.5h (Griggs *et al.*, 1971). The product was dissolved in 50μl of dichloromethane, and 2μl samples were analysed by using a Hewlett Packard model 5750 chromatograph equipped with dual stainless-steel columns (305cm×0.2cm) containing Gas Chrom Q (100–120 mesh) pre-coated with a mixture of 0.2% ethylene glycol adipate, 0.2% ethylene glycol succinate and 1.4% silicone XE-60, and dual-flame ionization detectors. N_2 was used as the carrier gas. The destruction of the individual sugars during hydrolysis was evaluated by analysing samples of plasma membrane that had been hydrolysed for 0.5, 1, 2, 3, 4, 8 and 16h and extrapolating to zero time. The following corrections were applied: galactose, glucose and mannose, 4%; inositol, 10%; fucose, 22%; ribose, 24%.

A comparison of the results obtained by the trimethylsilylation and alditol acetate methods showed that the former method generally gave higher values (about 25%) for the neutral sugars than the latter. It was assumed that the mannose, glucose and galactose contents revealed by trimethylsilylation represented the more accurate measurements. On the other hand, the amounts of fucose, ribose and inositol indicated by the alditol acetate method were adopted since difficulties were experienced in measuring these sugars by the trimethylsilylation procedure (see above).

Results and Discussion

The sugar compositions of the lymphocyte plasma membrane and of its protein and lipid fractions are summarized in Table 1. The membrane contained 69μg of carbohydrate/mg dry wt., which comprised 40μg of neutral sugars, 15μg of amino sugars and 14 μg of sialic acid. The neutral sugars included fucose, ribose, inositol, mannose, glucose and galactose. No other neutral sugars were detected, although some minor unidentified peaks which probably arose by degradation during hydrolysis were revealed especially by the alditol acetate method. The fucose, ribose and mannose of the membrane were associated exclusively with the protein fraction and all of the inositol was present in the lipid fraction, whereas the glucose and galactose were distributed between both fractions. The inositol was most probably derived exclusively from phosphatidylinositol and its amount was in reasonable agreement with independent measurements of this lipid [6nmol/mg dry wt. of membrane (G. M. Levis & M. J. Crumpton, unpublished observations)]. The ribose was presumably derived from RNA in which case it corresponded to 12μg of RNA/mg dry wt. of membrane. Direct measurements of the RNA content gave, however, two- to three-fold higher values (Allan & Crumpton, 1970). The association of glucose with the protein fraction is of questionable significance. Although this sugar is not a characteristic component of soluble glycoproteins, evidence is accumulating for the presence of strongly adsorbed, if not necessarily covalently bound, glucose in the surface membranes of various cell types including lymphocytes (Kim &

Table 1. *Carbohydrate composition of pig lymphocyte plasma membrane, and of the protein and lipid fractions of the membrane*

The compositions of the protein and lipid fractions are expressed relative to the whole membrane. The quoted values were derived from duplicate analyses of two different membrane preparations (variation ±5%). A value of 0 signifies that none was detected. Neutral sugars were identified on the basis of their coincidence with authentic samples. Experimental details are given in the Materials and Methods section.

Sugar	Composition (nmol/mg dry wt.) Plasma membrane	Lipid	Protein	Amount recovered in protein and lipid (% of plasma membrane)
Fucose*	7	0	5	71
Ribose*	32	0	32	100
Inositol*	4	4	0	100
Mannose†	40	0	33	83
Glucose†	52	31	13	85
Galactose†	91	21	60	89
Glucosamine‡	45	1	45	102
Galactosamine‡	22	7	14	96
Sialic acid§	45	20	24	98
Total (μg/mg dry wt. of membrane)	69	18	46	93

* Measured by the alditol acetate method.

† Measured by the trimethylsilylation method.

‡ Probably occurs in the membrane as the *N*-acetyl derivative.

§ Measured as *N*-acetylneuraminic acid but probably occurs mainly as *N*-glycollylneuraminic acid (see the Results and Discussion section).

Perdomo, 1974; Droege *et al.*, 1975). The amino sugars of the membrane comprised glucosamine and galactosamine in an approximate molar ratio of 2:1. Essentially all the glucosamine was associated with the protein whereas 64% of the galactosamine was located with the protein and 32% with the lipid. The membrane contained 14μg of sialic acid/mg dry wt. which was distributed almost equally between the protein and lipid. Although the sialic acid is expressed as *N*-acetylneuraminic acid, it is probably mainly *N*-glycollylneuraminic acid. This view is based on the observations that the gangliosides of the pig lymphocyte plasma membrane (G. M. Levis, G. P. Evangelatos & M. J. Crumpton, unpublished work) and the glycoprotein of pig erythrocyte stroma (Klenk & Uhlenbruck, 1958) contain primarily *N*-glycollylneuraminic acid.

A comparison of the carbohydrate composition of the pig lymphocyte plasma membrane with those reported for the surface membranes of other mammalian cells (Shimizu & Funakoshi, 1970; Barber & Jamieson, 1970; Quirk & Robinson, 1972; Kim & Perdomo, 1974) shows some interesting differences and similarities. Thus the preferential association of glucosamine with the protein fraction of the lymphocyte membrane is shared by the plasma membranes of rat ascites hepatoma (Shimizu & Funakoshi, 1970), rat intestine brush border (Quirk & Robinson, 1972) and rat liver (Evans & Gurd, 1971). The lymphocyte membrane contained much more carbohydrate (154μg/mg of protein) than the rat ascites hepatoma membrane (77μg/mg of protein; Shimizu & Funakoshi, 1970), and its molar proportions of neutral sugar, amino sugar and sialic acid (5:1.7:1) differed markedly from those of the intestine brush-border membrane (36:9:1; Kim & Perdomo, 1974). However, perhaps the most striking difference concerned the proportion of the total carbohydrate located in the protein fraction of the lymphocyte membrane (67%) compared with the erythrocyte membrane in which apparently 93% of the carbohydrate is associated with the protein (Steck & Dawson, 1974). The relevance of the above differences is not clear, but the results are consistent with the suggestion that the carbohydrate composition of the cell surface is characteristic of the cell type.

We thank Dr. Helen Muir for some preliminary g.l.c. carbohydrate analyses of the lymphocyte membrane. A. K. A. is grateful to the M.R.C. for support from a project grant.

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