Biochem. J. (1976) **155**, 701–703
Printed in Great Britain

Abnormal Carbohydrate Composition of the Major Penetrating Membrane Protein of En(a–) Human Erythrocytes

By MICHAEL J. A. TANNER,* ROBERT E. JENKINS,* DAVID J. ANSTEE† and JOHN R. CLAMP‡
**Department of Biochemistry and ‡Department of Medicine, University of Bristol, Bristol BS8 1TD, U.K., and †South West Regional Blood Transfusion Centre, Southmead, Bristol BS10 5ND, U.K.*

(*Received* 5 *March* 1976)

The major penetrating membrane glycoprotein (band 3) was isolated from En(a–) and normal human erythrocytes. The two proteins differed only in carbohydrate composition. Band 3 from En(a–) erythrocytes contained greater amounts of galactose and *N*-acetylglucosamine. The loss of the sialoglycoprotein sialotetrasaccharides in the En(a–) cell is not compensated by the appearance of these units in band 3 of En(a–) erythrocytes.

We have previously shown that a human erythrocyte variant, the homozygous En(a–) erythrocyte, lacks the major erythrocyte sialoglycoprotein (PAS 1) (Tanner & Anstee, 1976). The remaining major penetrating glycoprotein (band 3; Steck, 1974) of these cells is also modified, and migrates with a higher apparent molecular weight on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis than the corresponding protein of normal erythrocyte membranes (Tanner & Anstee, 1976). There is no clinical abnormality known to be associated with the En(a–) condition, even though the major sialoglycoprotein is lacking from these cells. This raises questions about the functional role of the oligosaccharides that are bound to the sialoglycoprotein. Since the electrophoretic mobility of band 3 is also altered in En(a–) cells, it was important to determine whether this alteration in band 3 of En(a–) erythrocytes reflects the transfer of the oligosaccharides normally associated with the sialoglycoprotein to band 3, so allowing the normal functional expression of the sialoglycoprotein oligosaccharides. In this paper we show that, although bands 3 of En(a–) and normal erythrocytes differ in carbohydrate composition, the predominant oligosaccharide components of the erythrocyte sialoglycoprotein (the *O*-glycosidically linked sialotetrasaccharides) are not found in band 3 of En(a–) erythrocytes.

Materials and Methods

Whole blood from a homozygous En(a–) donor (G. W.; Furuhjelm *et al.*, 1973; Tanner & Anstee, 1976) was kindly provided by the late Dr. U. Furuhjelm and Dr. J. Leikola, Finnish Red Cross Blood Transfusion Service, Helsinki. The methods used for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and preparation of peptide 'maps' have been described (Boxer *et al.*, 1974). Carbohydrates were measured by a g.l.c. method (Clamp, 1974), modified by the introduction of the second internal standard at a later stage in the technique. In this modification arabinitol (100nmol) in methanol is added to the supernatant after transfer to a clean flask and before flash-evaporation.

Band 3 was prepared by the following method. 'Ghosts' were prepared from packed washed erythrocytes [100ml of normal erythrocytes or 14ml of En(a–) cells] as described by Tanner & Boxer (1972). The 'ghosts' were extracted with 7vol. of ice-cold 0.1M-NaOH containing 10mM-2-mercaptoethanol as described by Steck (1972). The insoluble material was washed with diluted iso-osmotic phosphate buffer, pH7.4 (Tanner & Boxer, 1972), suspended in 1vol. of the same solution and made 10% in sodium dodecyl sulphate, 50mM in Tris/HCl, pH7.5, 1mM in EDTA (disodium salt) and 2mM in phenylmethanesulphonyl fluoride. The mixture was heated at 100°C for 5min, cooled, and the clear solution desalted on a column (27cm×10cm) of Sephadex G-50 equilibrated with 1% sodium dodecyl sulphate/1mM-EDTA (disodium salt), pH8.0. The desalted solution was concentrated by pressure dialysis over an Amicon PM-10 membrane to a volume of about 20ml. The concentrate was applied to a Sephadex G-150 column (80cm×7.5cm) equilibrated with 1% sodium dodecyl sulphate/1mM-EDTA, pH8.0. The fractions containing band 3 were detected by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, pooled and then concentrated by pressure dialysis. The concentrate was dialysed against water, 40% (v/v) methanol and then water again, and was finally freeze-dried. The procedure used for the preparation of band 3 of En(a–) erythrocytes was similar to the above, except that the pellet remaining after NaOH extraction of the 'ghosts' was solubilized and applied directly to a column (130cm×2.5cm) of Sephadex G-150 equilibrated with 1% sodium dodecyl sulphate/1mM-EDTA, pH8.0.

Band 3 was labelled with ^{125}ICl as follows. ICl (Macfarlane, 1963), containing 3.3 ng-atoms of I/ml, was allowed to equilibrate for a few minutes on ice with 0.25 mCi of $Na^{125}I$ (carrier-free). Then 0.25 mg of the protein in 0.2 ml of 1% sodium dodecyl sulphate in 0.2 M-Na_2HPO_4 was added. After 3 min on ice, the reaction was stopped with 5 μl of 1 M-ascorbic acid, and the labelled protein was exhaustively dialysed before the preparation of peptide 'maps'.

Results and Discussion

Band 3 was isolated from normal and En(a–) erythrocyte 'ghosts'. Fig. 1 shows the results of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the purified proteins. Both preparations contain a single major component which retains the characteristic diffuse banding pattern of this 'ghost' component.

The extent of the similarity of the polypeptide chains of the proteins from the two sources was assessed by labelling the isolated proteins with ^{125}ICl. The proteins were digested with thermolysin and the products separated on peptide 'maps'. The radioautographs of the two peptide 'maps' were indistinguishable, showing that, at the resolution of this technique, the polypeptide chains of the two proteins are the same. This, together with our earlier observation that the pattern of tyrosine labelling of the extracellular region of band 3 of En(a–) and normal erythrocytes is the same (Tanner & Anstee, 1976), suggests that both the amino acid sequence and organization of the polypeptide chain of band 3 in En(a–) cells is probably the same as it is in normal erythrocytes.

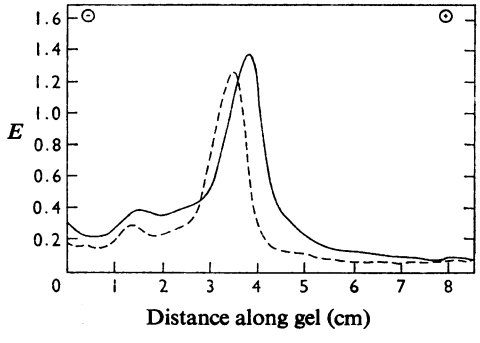

Fig. 1. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of purified band 3 from normal and En(a–) erythrocytes*

Scans at 550 nm of parallel gels containing 5.5% acrylamide, stained with Coomassie Blue: ——, 50 μg dry wt. of normal band 3; – – – –, 50 μg dry wt. of band 3 of En(a–) erythrocytes. The minor slower-moving component, which is present in each preparation, has an apparent mol.wt. of 175000 and 190000 in the normal and En(a–) erythrocytes respectively. It probably represents a trace of an aggregated dimer of band 3.

The carbohydrate content of band 3 from normal and En(a–) erythrocytes is given in Table 1. It is clear that band 3 from En(a–) erythrocytes differs from that of normal erythrocytes in its content of galactose and *N*-acetylglucosamine. The increased carbohydrate content of band 3 of En(a–) erythrocytes (equivalent to an increased mol.wt., of about 7000) is of the same order as the difference in apparent molecular weight of band 3 of En(a–) and normal erythrocytes on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Tanner & Anstee, 1976). Using the data in Table 1 we can calculate that band 3 carries approx. 18% of the carbohydrate of the normal erythrocyte 'ghost' (see Tanner & Boxer, 1972), whereas in the En(a–) cells band 3 carries the equivalent of 26% of the carbohydrate of the normal erythrocyte. It is clear that the increased carbohydrate content of band 3 of En(a–) erythrocytes does not simply compensate in a gross way for the substantial loss of cell-surface carbohydrate (30–45%) resulting from the absence of the sialoglycoprotein.

Band 3 is involved in the anion-transport system of human erythrocytes (Cabantchik & Rothstein, 1974). In view of the alteration in band 3 of En(a–) erythrocytes the anion-transport characteristics of normal and En(a–) erythrocytes were compared. Measurements made using the chloride electrode (Halestrap, 1976) showed that the formate-induced efflux of chloride from En(a–) cells was unimpaired and could be completely blocked by incubation of En(a–) cells with 4,4′-di-isothiocyanodistilbene-2,2′-disulphonate, a specific inhibitor of erythrocyte anion transport (Cabantchik & Rothstein, 1974). En(a–) erythrocytes appear to have a normal anion-transport system.

Table 1. *Carbohydrate content of band 3 from normal and En(a–) erythrocytes*

The values for normal band 3 are means ± S.D. of determinations on four preparations. The protein contents of the samples were determined by the method of Lowry *et al.* (1951). Molecular weights of 93000 and 100000 were used for band 3 from normal and En(a–) erythrocytes respectively in calculating the data.

	Carbohydrate (mol/mol of protein) in band 3 from:	
Sugar	Normal	En(a–)
Fucose	4±1	5
Mannose	7±1	8
Galactose	24±4	39
N-Acetylglucosamine	25±5	44
N-Acetylgalactosamine	4±1	4
Sialic acids	5±3	5
% of carbohydrate	15	21

The erythrocyte sialoglycoprotein (PAS 1) carries two types of oligosaccharide chains. A large number (16) of small, serine- or threonine-linked (*O*-glycosidically linked), sialotetrasaccharides are present (Tomita & Marchesi, 1975). The structure of these is known and they each contain one *N*-acetylgalactosamine, one galactose and two sialic acid residues (Thomas & Winzler, 1969). The remaining carbohydrate is probably present in one larger oligosaccharide chain of the asparagine-linked (*N*-glycosidically linked) type and these characteristically contain *N*-acetylglucosamine and mannose (Tomita & Marchesi, 1975).

The additional sugars found on band 3 of En(a–) erythrocytes (Table 1) are *N*-acetylglucosamine and galactose, and these are found both in *N*-glycosidically linked units and *O*-glycosidically linked units, as, for example, in blood-group glycoproteins. These additional monosaccharides might therefore be incorporated by the extension of an existing oligosaccharide unit into normal band 3 or by the creation of new *N*-glycosidically linked units not usually present in band 3. It is not clear whether this change contributes to the viability of the En(a–) erythrocyte. The sialic acid and *N*-acetylgalactosamine contents of band 3 of En(a–) erythrocytes remain unchanged (Table 1). Thus the loss of the sialotetrasaccharides, the major oligosaccharide constituents of the sialoglycoprotein, is not compensated for by the appearance of these units in band 3 of En(a–) erythrocytes. This is consistent with evidence that the lectin from *Maclura aurantiaca*, which is thought to bind to the *N*-acetylgalactosamine moiety of these sialotetrasaccharides (Dahr *et al.*, 1975), does not bind to band 3 of En(a–) erythrocytes (Tanner & Anstee, 1976). Further, no new component which binds this lectin appears in En(a–) erythrocyte 'ghosts', although two periodate-staining components present in similar amounts in both En(a–) and normal cells (PAS 3 and a component in the PAS 2 band) do bind this lectin (Tanner & Anstee, 1976). If the *Maclura aurantiaca* lectin is detecting in these components sialotetrasaccharides that are the same as those present on the sialoglycoprotein, then this alternative source of the sialotetrasaccharide may account for the apparent clinical normality of En(a–) homozygotes.

Cell surfaces carry substantial amounts of membrane-bound carbohydrate, but little is known about the function of these defined oligosaccharide structures. Although the loss of the sialoglycoprotein and its associated sialotetrasaccharides does not appear to be detrimental to the viability of the En(a–) erythrocyte, the rarity of the En(a–) condition (Race & Sanger, 1975) suggests that the presence of this protein confers some advantage to the erythrocyte. This may result from the general properties of the carbohydrate rather than from specific functional roles for the individual oligosaccharides associated with the sialoglycoprotein. Thus, although a proportion of the carbohydrate present on cell surfaces may well be involved in specific chemical interactions (such as cell–cell interactions; Roseman, 1970), it seems possible that the role of much of the carbohydrate on cell surfaces may be a more general one. The nature of this general role is difficult to define, however. The presence of substantial amounts of carbohydrate at the cell surface may provide a thermodynamic barrier against cell fusion and vesicularization of the plasma membrane, and thus contribute to the maintenance of the long-term integrity and stability, which is characteristic of mammalian plasma membranes. The suggestion that the carbohydrates at the cell surface may interact by hydrogen-bonding to form a lattice over the entire cell surface (Bretscher, 1973) is an attractive one. Apart from strengthening the hydrophilic barrier, this hydrogen-bonded network would be expected to impose restraints on the structure of cell-surface carbohydrates and could give rise to the defined and relatively limited variety of saccharide sequences that are found at the cell surface.

We are grateful to Dr. A. P. Halestrap for carrying out the anion-transport experiments. We thank Mrs. Diana Barker for her skilful assistance and the late Dr. U. Furuhjelm and Dr. J. Leikola for gifts of En(a–) blood. We thank Dr. G. H. Tovey, Director, South West Blood Transfusion Centre, Bristol, for his help and encouragement. R. E. J. was the recipient of an S.R.C. studentship. The work was supported in part by the Medical Research Council.

References

Boxer, D. H., Jenkins, R. E. & Tanner, M. J. A. (1974) *Biochem. J.* **137**, 531–534

Bretscher, M. S. (1973) *Science* **181**, 622–629

Cabantchik, Z. I. & Rothstein, A. (1974) *J. Membr. Biol.* **15**, 207–226

Clamp, J. R. (1974) *Biochem. Soc. Symp.* **40**, 3–16

Dahr, W., Uhlenbruck, G. & Bird, G. W. G. (1975) *Vox Sang.* **28**, 133–148

Furuhjelm, U., Nevanlinan, H. R. & Pirkola, A. (1973) *Vox Sang.* **24**, 545–549

Halestrap, A. P. (1976) *Biochem. J.* **156**, 193–207

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Macfarlane, A. S. (1963) *J. Clin. Invest.* **42**, 346–348

Race, R. R. & Sanger, R. (1975) *Blood Groups in Man*, pp. 463–474, Blackwell Press, Oxford

Roseman, S. (1970) *Chem. Phys. Lipids* **5**, 270–289

Steck, T. L. (1972) in *Membrane Research* (Fox, C. F., ed.), pp. 71–93, Academic Press, New York

Steck, T. L. (1974) *J. Cell Biol.* **62**, 1–19

Tanner, M. J. A. & Anstee, D. J. (1976) *Biochem. J.* **153**, 271–277

Tanner, M. J. A. & Boxer, D. H. (1972) *Biochem. J.* **129**, 333–347

Thomas, D. B. & Winzler, R. J. (1969) *J. Biol. Chem.* **244**, 5943–5946

Tomita, N. & Marchesi, V. T. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2964–2968