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PHOSPHOLIPID COMPOSITION AND EXTERNAL LABELING OF AMINOPHOSPHOLIPIDS OF HUMAN En(a—) ERYTHROCYTE MEMBRANES WHICH LACK THE MAJOR SIALOGLYCOPROTEIN (GLYCOPHORIN A)

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Summary

Erythrocytes of the rare human blood group En(a—) lack the major sialoglycoprotein, glycophorin A, and the cell population heterozygous for the En(a) antigen contain half the normal amount of glycophorin A. With such cells we have studied whether glycophorin A influences the phospholipid composition and the availability of aminophospholipids to external labeling reagents. We here demonstrate that the amounts of all phospholipids are closely similar in normal and variant membranes. However, using the amino-reactive reagent trinitrobenzenesulfonate, we show that phosphatidylethanolamine is more easily labeled in intact En(a—) cells as compared to normal cells, whereas phosphatidylethanolamine shows an intermediate labeling in En(a) heterozygous cells.

Introduction

The human erythrocyte membrane has been extensively studied and is a good model for plasma membranes in general [1—4]. It is well established that the major portion of the red cell membrane lipids are arranged in a bilayer structure with their polar headgroups facing the aqueous milieu on both sides of the membrane [5—7]. The bulk of the lipids diffuse relatively freely in the horizontal plane of the membrane [8], while transmembrane movement of

Abbreviations: TNBS, 2,4,6-trinitrobenzene sulfonate, $NaCl/PO_4$, 0.15 M NaCl/0.01 M sodium phosphate, pH 7.4.

lipids is considerably more restricted [9]. Recent experiments indicate that exchange of phospholipids between the two leaflets of the lipid bilayer may occur [10—12].

As first proposed by Bretscher [13,14], the phospholipids of the human erythrocyte membrane are asymmetrically distributed in the two shells of the lipid bilayer. The choline-containing phospholipids are enriched in the outer part and the aminophospholipids in the inner part. This observation has been supported by numerous studies using a variety of non-penetrating probes [15—18], phospholipases [19,20] and phospholipid exchange proteins [20,21].

The reason for the lipid asymmetry is unknown. Because the lipid distribution is different in membranes from different sources [22,23], it is possible that membrane proteins are involved in generating or maintaining lipid asymmetry.

The major integral proteins of the human erythrocyte membrane are band 3 (for nomenclature see Ref. 24) and the sialoglycoprotein PAS 1 also known as the MN-glycoprotein and glycophorin A [25]. They are glycoproteins with the carbohydrate portions located on the external surface of the membrane [26—28]. Band 3 constitutes 20—25% of the membrane protein and may be involved in anion transport [29,30].

Glycophorin A is one of the most extensively studied membrane proteins and the large amount of information available on its structure makes it an attractive model for understanding structural-functional relationships. It contains 60% carbohydrate, including a large portion of the sialic acid of normal erythrocytes, and it carries the MN-antigens, and receptors for influenza virus and various lectins [25,31]. The amino acid sequence has been established [32] and it differs in the amino terminal portion depending on the MN-antigen activity [33,34]. It spans the membrane [35] and the hydrophobic amino acid sequence of 23 residues is most probably within the lipid bilayer. Glycophorin A evidently constitutes part of the intramembrane particles seen on freeze-fracturing [36,37] and it is present at the cell surface of basophilic normblasts and later stages of erythrocyte differentiation [38]. Its physiological function has remained unknown.

We and others recently showed that erythrocytes of the blood group En(a—) [39,40] totally lack glycophorin A [34,41—43]. We have taken advantage of this fact to study whether the absence of glycophorin A affects the phospholipid composition or the reactivity of aminophospholipids towards external labeling. The phospholipid composition of mutant membranes is not altered but by using the relatively non-penetrating amino-reactive reagent 2,4,6-trinitrobenzenesulfonate (TNBS) we here show that phosphatidylethanolamine is more reactive in En(a—) cells as compared to normal cell membranes. En(a) heterozygous cells, containing half the normal amount of glycophorin, show an intermediate labeling of phosphatidylethanolamine.

Materials and Methods

Red blood cells. All erythrocytes were simultaneously obtained from the Finnish Red Cross Blood Transfusion Service, Helsinki. The En(a—) and normal cells were of blood group AB Rh+, and the En(a) heterozygous cells, from a

son of the En(a—) person were B Rh+. The labeling experiments were done in parallel within 2 weeks of blood donation.

Chemicals and isotopes. TNBS was from Sigma Chemical Co., St. Louis, MO, U.S.A., and was used without further purification. Glucose was purchased from J.T. Baker Chemicals B.V., Deventer, Holland. Sodium metaperiodate was from Merck AG, Darmstadt, F.R.G. Bovine serum albumin was obtained from Sigma. Tritated sodium borohydride (8.2 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, U.K., and handled as described [44].

Enzymes. Galactose oxidase with a specific activity of 130 units/mg protein was purchased from Kabi AB, Stockholm, Sweden. It displayed no protease or neuraminidase activities when measured as described previously [26]. *Vibrio cholerae* neuraminidase (500 units/ml) was from Behringwerke AG, Marburg-Lahn, F.R.G. It was free of protease activity. Bovine trypsin (3.5 units/mg protein) was from Merck AG, Darmstadt, F.R.G.

Radioactive labeling of cell surface proteins. Erythrocytes were labeled by the galactose oxidase method as described previously [26,28]. 1 ml of packed cells were washed three times in 10 ml of 0.15 M NaCl/0.01 M sodium phosphate (pH 7.4) ($NaCl/PO_4$) at 3000 rev./min for 10 min in a Sorvall SS-34 rotor and incubated with 5 units of galactose oxidase and 12.5 units of neuraminidase for 30 min at 37°C in 1 ml of Dulbecco's phosphate-buffered saline. The cells were then washed three times in $NaCl/PO_4$, 0.5 ml of $NaCl/PO_4$ was added and the cells were reduced with 0.5 mCi of NaB^3H_4/sample for 30 min at 22°C. After washing three times in $NaCl/PO_4$, the membranes were isolated as described below.

Labeling of cells with periodate/tritiated sodium borohydride was performed as described [45]. 0.5 ml of packed cells were washed three times with 10 ml of $NaCl/PO_4$ and incubated in 2 mM sodium metaperiodate in 2 ml of $NaCl/PO_4$ in the dark for 10 min at 0°C. The cells were then washed three times with 10 ml of $NaCl/PO_4$, suspended in 0.5 ml of $NaCl/PO_4$ and reduced with tritiated sodium borohydride as for the galactose oxidase-treated samples. After washing the membranes were isolated.

Treatment of cells with trypsin. 4 ml of packed normal erythrocytes were washed three times in 30 ml of $NaCl/PO_4$ and mixed with 0.4 ml of packed normal erythrocytes, labeled after treatment with periodate and NaB^3H_4, and incubated in $NaCl/PO_4$ with 0.1 mg/ml of trypsin for 30 min at 37°C. After incubation the cells were washed three times in $NaCl/PO_4$ and 0.5 ml of packed cells were removed and the membranes isolated. 50 μl of the isolated membranes were used for polyacrylamide gel electrophoresis. The rest of the cells were used for labeling with TNBS. Control cells were handled in the same way except that the trypsin treatment was omitted.

Isolation of cell membranes. Membranes were prepared by lysing cells at 0°C in 5 mM Tris-HCl buffer (pH 8.0) followed by centrifugation at 12 500 rev./min for 15 min in a Sorvall SS-34 rotor at 4°C. The membranes were washed with the same buffer until only traces of hemoglobin remained.

Labeling of cells and isolated membranes with TNBS. Red cells were washed three times in $NaCl/PO_4$ and 0.5 ml of packed cells or isolated membranes corresponding to 0.5 ml of packed cells, were incubated for indicated times at 22°C in 30 ml of 40 mM NaCl/120 mM $NaHCO_3$/5 mM glucose/1.5 mM TNBS/

pH 8.2) [15]. The pH of the suspensions remained at 8.1—8.2 during the incubation. After incubation the reaction was quenched with 0.1% bovine serum albumin in ice-cold $NaCl/PO_4$ and the samples immediately centrifuged at 4°C. Labeled cells were washed twice in $NaCl/PO_4$ after which the membranes were isolated. Labeled membranes were washed twice in ice-cold 5 mM Tris-HCl buffer. Trypsin-treated red cells were labeled with TNBS in the same way as control cells. After isolation the membranes were lyophilized.

Quantitation and identification of phospholipids. Lipids were extracted from lyophilized membranes with chloroform/methanol (2 : 1, v/v) according to Folch et al. [46] and the lower phases washed twice with theoretical upper phase. Phospholipids were separated by two-dimensional thin-layer chromatography on 20 × 20 cm silica gel plates (Kieselguhr 60, Merck AG, Darmstadt, F.R.G.) using chloroform/methanol/7 M ammonium hydroxide (65 : 20 : 4, by vol.) in the first direction and chloroform/acetone/methanol/acetic acid/water (50 : 20 : 10 : 10 : 5, by vol.) in the second direction [47]. In the first dimension the plates were run twice with drying in between. Labeled phosphatidylethanolamine and phosphatidylserine were recognized by their intense yellow colours. Unlabeled phospholipids were visualized by brief exposure to iodine vapor. Phospholipid spots were scraped from the plates and the lipids quantitated after phosphorus determination [48].

Isolation of phosphatidylethanolamine and phosphatidylserine and labeling of phospholipids with TNBS in vitro. 50 ml of packed normal red cell membranes were lyophylized and the lipids extracted. Part of the lipid extract containing 0.8 mg phosphorus was taken for isolation of phosphatidylethanolamine and phosphatidylserine by preparative two-dimensional thin-layer chromatography. Purified erythrocyte phosphatidylethanolamine and phosphatidylserine (3 μg phosphorus or approximately 75 μg phospholipid) were labeled with 1.5 mM TNBS in 1 ml of chloroform/methanol (1 : 1, v/v) containing 0.11 ml of 5% $NaHCO_3$ (pH 8.2) for the times indicated at 22°C. The reaction was terminated by removing excess TNBS by partition [46] and the lower phases washed with theoretical upper phase. The extent of derivatization was determined by absorption at 337 nm using purified *N*-2,4,6-trinitrophenyl phosphatidylethanolamine and *N*-2,4,6-trinitrophenyl phosphatidylserine as standards. The standard trinitrophenyl phosphatidylethanolamine and trinitrophenyl phosphatidylserine were obtained by labeling isolated pure phosphatidylethanolamine and phosphatidylserine from red cells with TNBS in vitro.

Polyacrylamide gel electrophoresis. Electrophoresis in the presence of sodium dodecyl sulfate was done according to Laemmli [49] on 8% acrylamide cylindrical gels. After completion of the electrophoresis the gels were sliced with a 2 mm slicer and the radioactivities determined in a Wallac-LKB Liquid Scintillation counter 81000 after solubilization with NCS solubilizer (Amersham/Searle, Arlington Heights, IL, U.S.A.) [26]. The efficiency for tritium was 37%.

Results

Labeling of erythrocytes by the galactose oxidase-NaB^3H_4 technique

When studied by polyacrylamide gel electrophoresis, band 3 and the sialo-

glycoproteins PAS 1—3 were the major labeled glycoproteins of normal erythrocytes seen after treatment with neuraminidase and galactose oxidase followed by reduction with NaB^3H_4 (Fig. 1A). The glycophorin A dimer peak (PAS 1) [50] was reduced in En(a) heterozygous cells (Fig. 1B) and absent from En(a—) membranes (Fig. 1C). The PAS 2 peak which includes the monomer of glycophorin A was strongly reduced in En(a—) membranes.

Phospholipid composition of red cell membranes and isolation of labeled aminophosphatide derivatives

The phospholipid compositions of the erythrocyte membranes were determined after extraction with chloroform/methanol and isolation by two-dimensional thin-layer chromatography. Table I shows that the phospholipid contents were similar in normal and En(a—) variants and not significantly different. Trinitrophenyl phosphatidylethanolamine and trinitrophenyl phosphatidylserine were clearly separated from other lipids (Fig. 2A and B) and were identified using purified trinitrophenyl phosphatidylethanolamine and trinitrophenyl phosphatidylserine as standards (data not shown).

Reactions of phosphatidylethanolamine and phosphatidylserine with TNBS in vitro

Isolated phosphatidylethanolamine reacted fast with TNBS whereas the reaction with phosphatidylserine was slower (Fig. 3). After 30 min the reaction was complete for both lipids.

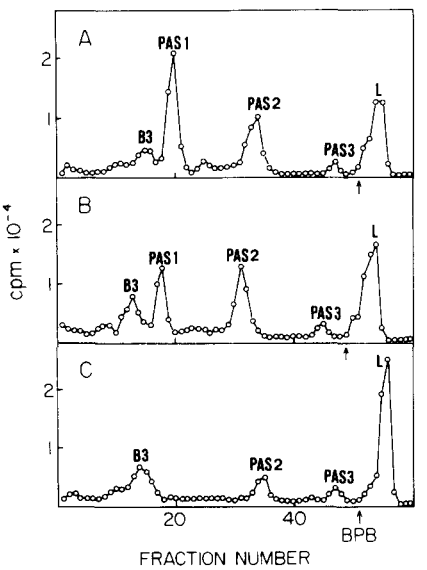

Fig. 1. Polyacrylamide gel electrophoresis patterns of erythrocyte membranes labeled by the galactose oxidase method. (A) normal cells. (B) En(a) heterozygous cells. (C) En(a—) cells. B3, band 3. The sialoglycoproteins PAS 1—3 are indicated. PAS 1, glycophorin A dimer; PAS 2, predominantly glycophorin A monomer; L, lipid peak. The arrows indicate the positions of the bromophenol blue marker dye.

TABLE I

PHOSPHOLIPID COMPOSITIONS OF NORMAL, En(a) HETEROZYGOUS AND En(a—) ERYTHROCYTE MEMBRANES

Values are given in per cent of total phospholipids analyzed. Means of three experiments ± S.E.

Phospholipids	Normal erythrocytes	En(a) heterozygous erythrocytes	En(a—) erythrocytes
Phosphatidylcholine	33.0 ± 0.8	31.9 ± 1.2	35.3 ± 1.6
Phosphatidylethanolamine	27.1 ± 0.9	26.4 ± 0.5	28.2 ± 0.2
Sphingomyelin	27.3 ± 0.2	27.4 ± 0.3	24.2 ± 0.7
Phosphatidylserine	10.5 ± 0.4	11.9 ± 0.4	9.4 ± 0.6
Phosphatidylinositol	2.1 ± 0.2	2.4 ± 0.5	2.5 ± 0.5
Choline phosphatides/ aminophosphatides	1.60	1.55	1.58

Labeling of intact cells with TNBS

Normal, En(a) heterozygous and En(a—) cells were incubated in parallel for indicated times with TNBS, the membranes isolated and the lipids extracted. After separation on thin-layer chromatography, the labeled lipid derivatives were visualized and the percentages of derivatized aminophospholipids determined by phosphorus analysis. Fig. 4A shows that for each incubation time, phosphatidylethanolamine was most efficiently labeled in En(a—) cells, less so in En(a) heterozygous cells and least in normal cells. After 12 h of incubation

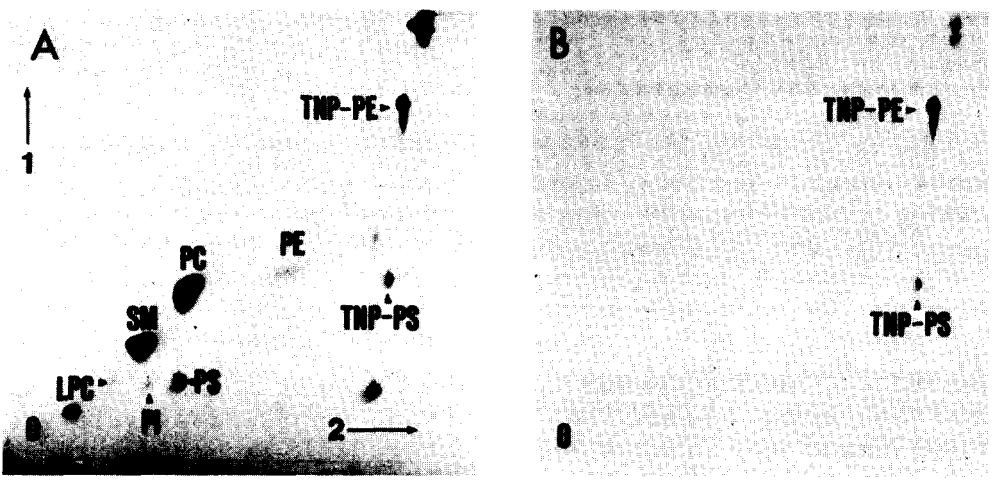

Fig. 2. Two-dimensional thin-layer chromatography patterns of phospholipids from normal red cell membranes and trinitrophenyl phosphatidylethanolamine and trinitrophenyl phosphatidylserine. (A) Total phospholipids from TNBS-labeled membranes visualized after exposure to iodine vapor. (B) Trinitrophenyl derivatives of aminophosphatides from TNBS-labeled membranes; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin; PI, phosphatidylinositol; LPC, lysophosphatidylcholine; TNP-PE, trinitrophenyl phosphatidylethanolamine; TNP-PS, trinitrophenyl phosphatidylserine; O, origin. 1, first dimension of chromatography (chloroform/methanol/7 M ammonium hydroxide, 65 : 20 : 4, v/v); 2, second dimension of chromatography (chloroform/acetone/methanol/acetic acid/water, 50 : 20 : 10 : 10 : 5, v/v). Phospholipids corresponding to 20 μg of lipid phosphorus were chromatographed in (A) and (B).

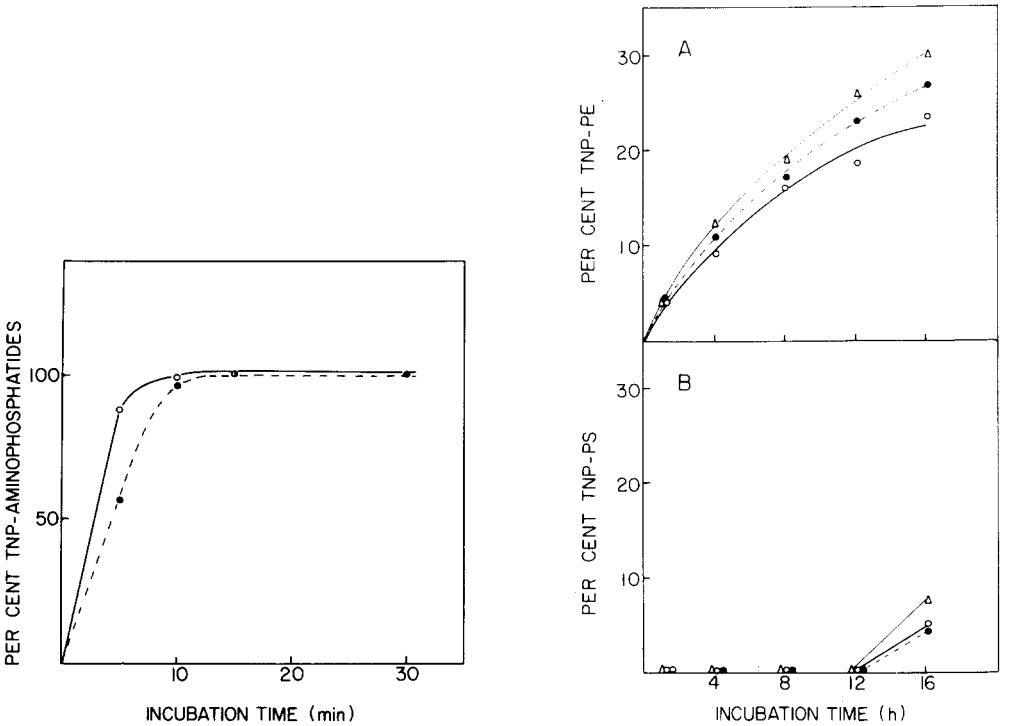

Fig. 3. Labeling of phosphatidylethanolamine and phosphatidylserine with TNBS in vitro. Phosphatidylethanolamine and phosphatidylserine corresponding to 3 μg of lipid phosphorus were incubated for indicated times in 1 ml of chloroform/methanol (1 : 1, v/v) containing 1% $NaHCO_3$, pH 8.2, at 22°C. After partition with water trinitrophenyl phosphatidylethanolamine and trinitrophenyl phosphatidylserine were determined by their absorption at 337 nm. ○———○, trinitrophenyl phosphatidylethanolamine; ●- - - - - -●, trinitrophenyl phosphatidylserine.

Fig. 4. Labeling of phosphatidylethanolamine and phosphatidylserine in intact erythrocytes with TNBS. Washed red ells were incubated with 1.5 mM TNBS for indicated times in bicarbonate buffer, pH 8.2, at 22°C. (A) Formation of trinitrophenyl phosphatidylethanolamine from phosphatidylethanolamine. (B) Formation of trinitrophenyl phosphatidylserine from phosphatidylserine. ○———○, normal cells; ●- - - - - -●, En(a) heterozygous cells; △- - - - -△, En(a—) cells. Results are expressed as per cent trinitrophenyl aminophosphatides of total aminophosphatides.

about 19% of phosphatidylethanolamine was labeled in normal cells, 23% in En(a) heterozygous cells and 26% in En(a—) cells. No phosphatidylserine was labeled using an incubation time of up to 12 h but at 16 h some phosphatidylserine became labeled (Fig. 4B). At this time hemolysis began to occur. The labeling values were means of two independent determinations and the differences between corresponding samples were less than 1% for all cells.

Treatment of intact normal cells with trypsin and subsequent labeling with TNBS

Because the external portion of glycophorin A in normal cells can be cleaved by trypsin, and such cells partially resemble En(a—) cells, we studied the labeling of trypsinized cells with TNBS. The degree of degradation of surface

proteins by trypsin was determined by polyacrylamide gel electrophoresis of periodate/NaB^{3}H$_4$-labeled normal cells (Fig. 5A) and it showed complete cleavage of the PAS 1 peak (glycophorin A dimer) and reduction of the PAS 2 peak (mainly glycophorin A monomer) whereas PAS 3 evidently remained unchanged (Fig. 5B). However, trypsin treatment did not affect the labeling of phosphatidylethanolamine by TNBS (Fig. 6).

Labeling of isolated red cell membranes with TNBS

There was no difference in the extent of labeling of phosphatidylethanolamine and phosphatidylserine in isolated normal, En(a) heterozygous and En(a—) membranes. The labeling of phosphatidylethanolamine was almost complete (92%) after 3 h of incubation (Fig. 7A) whereas only 64% of phosphatidylserine had reacted (Fig. 7B). All values were means of two independent determinations. The differences between duplicate samples were less than 1%.

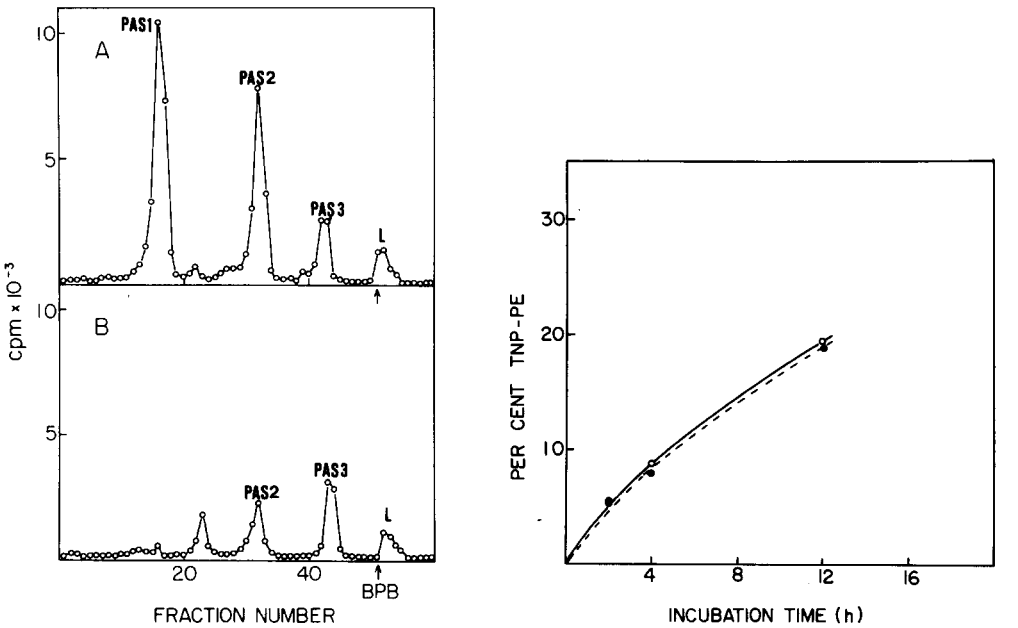

Fig. 5. Polyacrylamide gel electrophoresis patterns of periodate/NaB^{3}H$_4$-labeled normal and trypsin-treated membranes. (A) Normal erythrocytes. (B) Normal erythrocytes after treatment with trypsin. The labeled proteins are indicated as in Fig. 1.

Fig. 6. Labeling of phosphatidylethanolamine in intact normal erythrocytes and trypsin-treated normal erythrocytes with TNBS. Red cells were incubated with 1.5 mM TNBS for indicated times in bicarbonate buffer, pH 8.2, at 22°C. o———o, normal cells; •- - - - -•, trypsin-treated cells. Results are expressed as per cent trinitrophenyl phosphatidylethanolamine of total phosphatidylethanolamine. Values are means of two independent determinations.

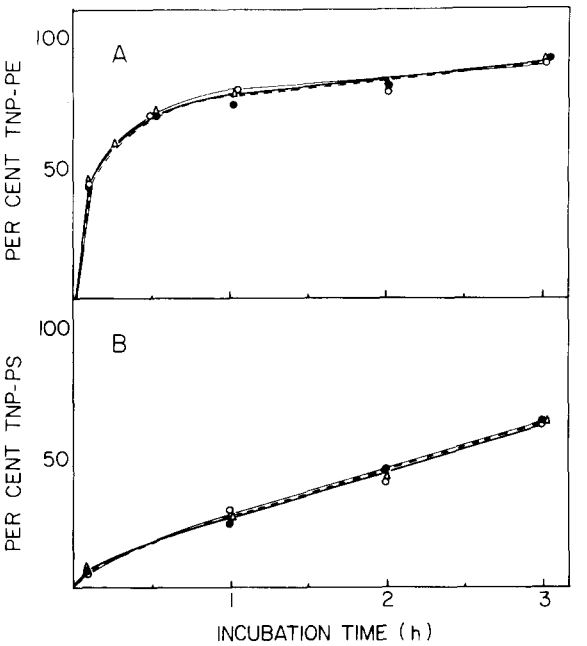

Fig. 7. Labeling of phosphatidylethanolamine and phosphatidylserine with TNBS in isolated erythrocyte membranes. Isolated membranes were incubated with 1.5 mM TNBS for indicated times in bicarbonate buffer, pH 8.2, at 22°C. (A) Formation of trinitrophenyl phosphatidylethanolamine from phosphatidylethanolamine. (B) Formation of trinitrophenyl phosphatidylserine from phosphatidylserine. ○———○, normal red cell membranes; ●- - - - -●, En(a) heterozygous red cell membranes; △- - - - -△, En(a—) red cell membranes. Results are expressed as per cent trinitrophenyl aminophosphatides of total aminophosphatides.

Discussion

It is remarkable that the absence of the major sialoglycoprotein from En(a—) red cells is compatible with apparently normal erythrocyte functions because the individuals with this rare genetic abnormality are perfectly healthy and for example do not show any reticulocytosis [39,40]. We have earlier shown that the glycolipid composition of En(a—) membranes is normal [42] and as shown in this report also the phospholipid composition is not changed. The glycolipids are located exclusively in the outer bilayer leaflet of red cell membranes [27] and are more easily oxidized by galactose oxidase in En(a—) membranes than in normal red cell membranes [42].

By using the non-permeable amino group-reactive reagent TNBS we studied the reactivities of the aminophospholipids phosphatidylethanolamine and phosphatidylserine in En(a—), En(a) heterozygous and normal erythrocyte membranes. This probe has been extensively used by Gordesky and coworkers [15,16] who have studied in detail its reactions with the phospholipids of the human erythrocyte. Their results with normal erythrocytes were closely similar to those we obtained. They found that approximately 20% of phosphatidylethanolamine reacted in intact cells after prolonged incubation but no phosphatidylserine. They also showed that hemoglobin was poorly labeled after

12 h and we have confirmed that the labeling of hemoglobin in intact normal and En(a) variants was identical and less than 1% of that obtained after hemolysis (data not shown). After incubation for 12 h 19% of phosphatidylethanolamine was derivatized in normal cells, 23% in En(a) heterozygous cells and 26% in En(a—) cells. The more efficient labeling of phosphatidylethanolamine in intact En(a—) cells as compared to normal cells was probably not simply due to the reduced negative surface charge of En(a—) cells [40] or shielding by the external part of glycophorin A in normal cells, because trypsin treatment of normal cells which removed most of glycophorin A did not affect the labeling. Shielding of phosphatidylethanolamine by glycophorin A was also almost certainly ruled out by the identical labeling of this lipid in isolated membranes.

Isolated phosphatidylethanolamine and phosphatidylserine reacted fast with TNBS in vitro, more slowly in isolated membranes and much more slowly in intact cells. This indicates that only part of both phosphatidylethanolamine and phosphatidylserine both in intact cells and membranes is readily available for labeling. Lipid tightly bound to membrane proteins is known and this lipid may be less available for external reagents. Jost et al. [51] for example showed by spin-labeling techniques that cytochrome *c* oxidase incorporated into lipid bilayers contained a portion of tightly bound lipid which was not mobile in the plane of the membrane.

The more efficient labeling of phosphatidylethanolamine in En(a) variant cells is not understood and the results must be interpreted with caution. It could be due to an enrichment of this aminophospholipid in the outer half of the lipid bilayer or due to shielding by that part of glycophorin A closely associated with the lipid bilayer. Other unknown reasons are not excluded. At present there is no way of designing experiments which would give definite answers. In this connection it is interesting to note that purified glycophorin A, when incorporated into liposomes, was able to alter the distribution of phosphatidylcholine [12]. Important is, however, that the absence of glycophorin A does not lead to changes in the amounts of individual phospholipids but there are membrane changes reflected in the reaction of TNBS with phosphatidylethanolamine.

Acknowledgements

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