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Lipid organization in erythrocyte membrane microvesicles

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The aminophospholipids of microvesicles released from human erythrocytes on storage or prepared from erythrocyte ghosts by shearing under pressure are susceptible to the action of 2,4,6-trinitrobenzenesulphonic acid. The aminophospholipids of the former vesicles are also susceptible to attack by phospholipase A_2. Under the same conditions, the aminophospholipids of erythrocytes undergo little reaction. This suggests that the phospholipids in microvesicle membranes are more randomly distributed than those in erythrocyte membranes. Measurements have also been made of the ability of filipin to react with the cholesterol of sealed and unsealed erythrocyte ghosts and of microvesicles prepared from them. From the initial rates of reaction, it was concluded that there is no preferential transfer of cholesterol molecules from one side of the bilayer to the other during the formation of the microvesicles.

The pinching off of small vesicles from cell surface projections such as microvilli or filopodia appears to be a common phenomenon (Trams *et al.*, 1981). The process has been most studied in erythrocytes, which shed microvesicles following a change of shape from the normal smooth, biconcave disc to the spiculated echinocyte. This change occurs as the cells age (Bessis & Mandon, 1972; Rumsby *et al.*, 1977; Lutz *et al.*, 1977) but it can be induced by raising their internal calcium content (Allan *et al.*, 1976). The microvesicles are of uniform size (100–150nm in diameter) and can easily be isolated from blood which has been stored for several weeks (Shukla *et al.*, 1978*a*). Their lipid content is very similar to that of the original erythrocytes and, although they contain haemoglobin, they are depleted of extrinsic membrane proteins such as spectrin and actin (Allan *et al.*, 1976; Lutz *et al.*, 1977; Shukla *et al.*, 1978*b*). Haemoglobin-free microvesicles of similar size may be prepared from erythrocyte ghost membranes either by exposing them to ultrasonic vibrations or by shearing them in a press (Schrier *et al.*, 1971; Lin & Macey, 1978).

Larger vesicles of up to 1 μm in diameter may also be obtained from erythrocyte ghosts by using more gentle homogenization methods. Their membranes may be in either right-side-out or inside-out orientation, depending on the conditions used (Steck & Kant, 1974).

The results of a great many investigations have established that, in erythrocytes and their ghosts, the phospholipids are asymmetrically arranged in the membrane (Op den Kamp, 1979) but studies on the larger erythrocyte vesicles described above have given conflicting results (Kahlenberg *et al.*, 1974; Haest, 1982). The cholesterol of such vesicles appears to be uniformly distributed between the two sides of the membrane (Blau & Bittman, 1978). In these vesicles, as in erythrocytes, the diameter is large compared with the thickness of the membrane so there is little difference between the area of the inner surface and that of the outer surface of the membrane. In the smaller microvesicles, there is an appreciable difference between the areas of the two surfaces of the membrane, and because of the possible effects of membrane curvature on lipid distribution (Scott & Green, 1980; Lange *et al.*, 1981) we have now studied the lipids of these structures in more detail.

Materials and methods

Materials

Human blood was obtained from the Department of Haematology, Royal Liverpool Hospital. Erythrocyte ghosts were prepared by the method of Dodge *et al.* (1963) and, when required, were resealed as described by Shukla *et al.* (1978*c*). Microvesicles were isolated from the plasma of blood which had been stored at 4°C for 5–8 weeks

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(Shukla *et al.*, 1978*a*). We refer to these as erythrocyte microvesicles. Other microvesicles were obtained from erythrocyte ghost membranes by shearing them at 500lbf/in^2 (3450kPa) (Schrier *et al.*, 1971). This gave a preparation of microvesicles rather smaller in size than those obtained from whole erythrocytes. Although there were few larger ones present most of them were about 100nm in diameter. We refer to these as ghost microvesicles.

2,4,6-Trinitrobenzenesulphonic acid was supplied by BDH Chemicals, Poole, Dorset, U.K., phospholipase A_2 from *Naja naja* venom (EC 3.1.1.4) was supplied by Sigma Chemicals, Kingston-upon-Thames, Surrey, U.K. and filipin was from the Upjohn Company, Kalamazoo, MI, U.S.A. Solutions of the latter were routinely checked spectrophotometrically for purity (Blau & Bittman, 1977).

Methods

Phospholipid accessibility to external reagents was assessed using both trinitrobenzenesulphonic acid and phospholipase A_2. With the former, the reaction with aminophospholipids was carried out using the conditions of Roseman *et al.* (1975) as described by Shukla *et al.* (1980). The centrifugation was increased when microvesicles were used, to 20000*g* for 30min. For phospholipase A_2 experiments, cells or microvesicles containing 0.7–1mg of phospholipid were incubated in 20ml of 0.87% NaCl (adjusted to pH7.4 with Tris buffer) for 1h at 37°C in a shaking water bath in the presence of enzyme (10i.u.) and $CaCl_2$ (10mM).

Measurements of filipin binding to membranes were made in a Durrum–Gibson model D-110 stopped-flow spectrophotometer as described by Blau & Bittman (1978).

The extent to which the ghost microvesicles were sealed was estimated from measurement of the glyceraldehyde-3-phosphate dehydrogenase activity in the presence and absence of Triton X-100 (Steck & Kant, 1974). Haemolysis was estimated from the haemoglobin absorbance at 418nm after removal of cells or microvesicles by centrifugation. Complete haemolysis was taken as the value obtained upon diluting the cells or microvesicles with distilled water.

Lipid extraction and determination of phospholipid and cholesterol were as previously described (Wharton *et al.*, 1980). Individual classes of phospholipid were separated by t.l.c. on silica gel H plates with chloroform/methanol/acetic acid/water (25:15:14:2, by vol.) as developing solvent.

Table 1. *Labelling of erythrocytes and microvesicles with trinitrobenzenesulphonic acid*

The reaction was carried out as described in the Materials and methods section. Results are means of two or three determinations, with the ranges in parentheses.

Preparation	Trinitrobenzenesulphonic acid-reactive aminophospholipid (% of total)
Erythrocytes	8.4 (6.1–10.3)
Erythrocyte microvesicles	59.7 (55.6–63.7)
Ghost microvesicles	58.2 (55.8–61.9)

Results

Phospholipid distribution in microvesicles

Trinitrobenzenesulphonic acid reacts with amino groups and hence the only phospholipids which react with the probe are phosphatidylethanolamine and phosphatidylserine. The results obtained with erythrocytes and microvesicles are shown in Table 1. Although on prolonged incubation more of the phosphatidylethanolamine of intact erythrocytes will react with the reagent, the values given in Table 1 agree with those obtained by others using incubations of similar length (Gordesky & Marinetti, 1973; Haest & Deuticke, 1975). With microvesicles from both cells and ghosts, the results are very different. There is a greatly increased reaction of lipid amino groups, suggesting that in the time of the reaction much of the phosphatidylethanolamine and/or phosphatidylserine is exposed to the reagent at the outer surface of the membrane.

The second probe of phospholipid distribution, phospholipase A_2, has been used by several workers to study phospholipid distribution in intact erythrocytes and their ghosts (Op den Kamp, 1979; van Deenen, 1981). With both cells and their microvesicles, there is appreciable phospholipid breakdown, although there is little release of phospholipid or of haemoglobin from either (Table 2). The pattern of glycerophospholipid breakdown seen with whole cells confirms the findings of earlier workers in that only phosphatidylcholine is attacked to any appreciable degree. In contrast to this, all three phospholipids studied are readily attacked in the microvesicles. The differences in phosphatidylcholine breakdown between cells and microvesicles are not statistically significant ($P > 0.1$), whereas aminophospholipid degradation is very much greater in the latter. Shukla *et al.* (1978*a*) also reported that these vesicles are more easily attacked by phospholipase C than are erythrocytes.

The results obtained with both the enzyme and the chemical reagent are in agreement and indicate that there are major differences in membrane

Table 2. *Effect of phospholipase A_2 on erythrocytes and microvesicles*

Experimental details are given in the Materials and methods section. Results are means of three experiments (±S.E.M.).

Preparation	Haemoglobin released (%)	Phospholipid release (%)	Phospholipid hydrolysis (%)		
			Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidylserine
Erythrocytes	3.2±0.5	16.5±3.5	36.0±14.8	3.7±5.1	4.0±3.1
Erythrocyte microvesicles	0	16.5±2.5	53.3±9.6	42.7±10.1	45.3±5.7

organization between the original erythrocytes and the microvesicles derived from them.

Cholesterol distribution

Because of the problems raised by the absorption of light by the haemoglobin in the microvesicles isolated from stored blood, erythrocyte ghost microvesicles were used for these determinations.

Before beginning studies with microvesicles, it was confirmed by using liposomes of phosphatidylcholine and cholesterol that the initial rate of change of absorbance (dA/dt) was linear with cholesterol concentration over the range 10–80 μM when a concentration of 10 μM-filipin was used (Blau & Bittman, 1977). These authors used the initial rate of the cholesterol reaction with filipin to assess the distribution of cholesterol in erythrocyte membranes by comparing the rates obtained with sealed and unsealed erythrocyte ghosts. To make a similar assessment of the distribution in microvesicles, attempts were made to unseal them using a variety of detergents. However, with all compounds tested there was a biphasic effect so that at low concentrations, the detergents increased the rate of reaction but at higher concentrations, they decreased it. It was therefore decided to adopt the method used by Blau & Bittman (1978) for larger membrane vesicles, i.e. to compare the results obtained with microvesicles with those obtained with unsealed erythrocyte ghost membranes containing the same amount of cholesterol. To establish that the ghosts were unsealed, samples were also compared with ghosts that had been sealed. It can be seen (Fig. 1) that the results obtained with sealed and unsealed ghosts are in agreement with those of Blau & Bittman (1978) in that the rate of reaction with unsealed ghosts is about twice (2.17-fold) that obtained with sealed ghosts, suggesting that the cholesterol is fairly evenly distributed across the bilayer. With the microvesicles (Fig. 2), there is a substantially lower initial rate of reaction for a given level of cholesterol than for the unsealed ghost membranes. The values obtained indicate that in the microvesicle preparation 61% of the cholesterol reacts rapidly with filipin. The glyceraldehyde-3-phosphate dehydrogenase determinations indicated that 19–23% of the microvesicles were unsealed. When corrected for this, the filipin

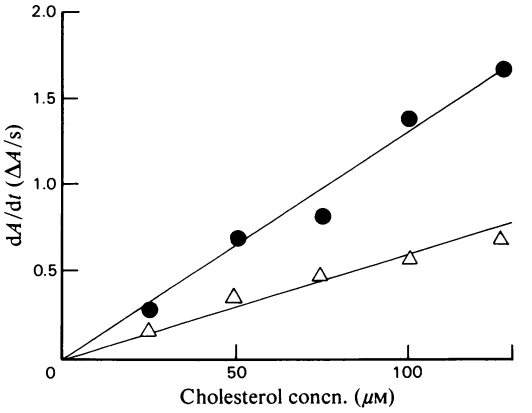

Fig. 1. *Reaction of filipin with cholesterol of sealed and unsealed erythrocyte ghosts*

Observations were made as described in the text. ●, Unsealed erythrocyte ghosts; △, sealed erythrocyte ghosts.

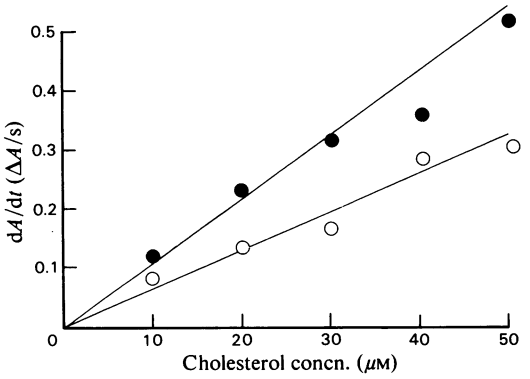

Fig. 2. *Reaction of filipin with cholesterol of erythrocyte microvesicles and unsealed erythrocyte ghosts*

Observations were made as described in the text. ○, Erythrocyte membrane microvesicles; ●, unsealed erythrocyte ghosts.

results suggest that 51% of the membrane cholesterol is in the outer leaflet of the bilayer. Thus as in the erythrocyte, cholesterol is uniformly distributed in the microvesicle membrane.

Discussion

The use of both phospholipase A_2 and trinitrobenzenesulphonic acid for the determination of phospholipid distribution in membranes can be criticized on the grounds that their products might perturb the membrane under study and the latter may penetrate it. However, with erythrocytes, all phospholipases and trinitrobenzenesulphonic acid give similar results and these agree with those obtained using the non-penetrating and non-perturbing phospholipid transfer proteins, so in this system their use appears to be valid (Op den Kamp, 1979; Haest *et al.*, 1981; van Deenen, 1981).

Two types of microvesicle have been used for the assessment of phospholipid distribution. Those released from erythrocytes spontaneously are sealed structures that retain their haemoglobin and the results obtained with both trinitrobenzenesulphonic acid and the much larger phospholipase A_2 molecule are similar. Schrier *et al.* (1971), who first described the preparation of microvesicles from ghost membranes by shearing, reported that these are right-side out and impermeable to *p*-chloromercuribenzoate, a molecule of similar size to trinitrobenzenesulphonic acid. However, our glyceraldehyde-3-phosphate dehydrogenase activity measurements suggested that about 20% of the ghost microvesicles were unsealed. This does not affect the conclusion that in these, as in the erythrocyte microvesicles, the aminophospholipids, phosphatidylethanolamine and phosphatidylserine, are much more accessible to external reagents than they are in intact erythrocytes. Phospholipase A_2 digestion did not reveal any significant difference in phosphatidylcholine accessibility between erythrocyte vesicles and whole cells (Table 2) and preliminary results indicate that this is also true for ghost microvesicles.

The increased accessibility of aminophospholipids in the microvesicles could be because of an increase in their rate of transfer from one side of the bilayer to the other (flip-flop) or to a randomization of phospholipid distribution between the two sides of the bilayer. The flip-flop rate is normally very slow in erythrocytes, with a half-time of a few hours (Op den Kamp, 1979). It is increased under several conditions (Franck *et al.*, 1983; Schrier *et al.*, 1983; Bergmann *et al.*, 1984) but the fact that all three phospholipids are hydrolysed by phospholipase A_2 to about the same extent suggests that randomization of the lipids in the bilayer has occurred.

It has been established that when erythrocytes are deprived of their energy supply, their lipid asymmetry is lost (Haest & Deuticke, 1976; Shukla *et al.*, 1978*a*; Loyter *et al.*, 1979). Shukla *et al.* (1978*a*) demonstrated that increased accessibility of aminophospholipids to phospholipases occurs as the erythrocyte ages *in vitro* and is associated with the transition from the normal smooth biconcave disc to the spiculated spheroechinocyte, although some changes can be detected before any obvious shape change is seen (Shukla & Hanahan, 1982). Thus loss of the asymmetric distribution occurs in the parent cell before the microvesicles are released. If this change had been a result of strain imposed on the cell as it changed shape, then the lipids would have reverted to their original distribution in the released microvesicles. The results show that this is not the case. Microvesicles and spiculated regions of spheroechinocytes have a high degree of curvature and it has been suggested that such regions are associated with an increased rate of flip-flop (Franck *et al.*, 1983). The high degree of curvature itself is not responsible for maintaining the altered phospholipid distribution, because after the release of microvesicles the erythrocytes become smooth and spherical (spherocytes) but the aminophospholipids remain accessible to external reagents (Shukla *et al.*, 1978*a*).

Several workers have proposed that the asymmetric distribution of phospholipids seen in the normal erythrocyte is maintained by association of the aminophospholipids with the membrane skeleton proteins on its cytoplasmic surface (Haest *et al.*, 1978; Marinetti & Crain, 1978; Franck *et al.*, 1982). As mentioned in the introduction, microvesicles have lost most of their spectrin and so the present finding that lipid asymmetry is also lost is consistent with this proposal. Haest (1982) cites unpublished data which show that this may also be true of the larger vesicles prepared from erythrocyte ghosts.

The measurements of cholesterol distribution were made using ghost microvesicles. Although cholesterol flip-flop in modified erythrocytes may be rapid (Lange *et al.*, 1984), measurement of the reaction of cholesterol with filipin is made on the millisecond time scale. Thus, in contrast to the phospholipid determinations, flop-flop during the period of measurement need not be considered.

The finding that cholesterol is fairly uniformly distributed between the two leaflets of the erythrocyte ghost membrane (Fig. 1) agrees with the results of Blau & Bittman (1978) obtained by the same methods and with those of Lange & Slayton (1982). Other workers (Fisher, 1976; Enomoto &

Sato, 1977; Hale & Schroeder, 1982) have suggested that more of the cholesterol is in the outer leaflet of the membrane, although Fisher (1976) found that the distribution was variable.

Lange *et al.* (1981) have proposed that one function of cholesterol, particularly in plasma membranes where it is present in high concentration, derives from its ability to transfer from one side of the bilayer to the other more rapidly than phospholipids. Thus sterol molecules would preferentially transfer to compensate for changes in the relative areas of the two surfaces of the membrane when cells change shape. We have previously attempted to obtain evidence for such a hypothesis using lymphocytes which can be converted to smooth and microvillous forms (Scott & Green, 1980). No significant differences in cholesterol distribution were found as the cells changed shape, but the expected changes were small and could have escaped detection by the methods used. The present results show that when erythrocyte ghost membranes are made to form tightly-curved microvesicles by shearing, there is also no preferential movement of cholesterol across the bilayer. It can be calculated that in a microvesicle of 100nm diameter, the area of the outer surface of the membrane bilayer will be about 16% bigger than that of the inner surface. Cholesterol provides about 22% of the area of the erythrocyte membrane (Cooper *et al.*, 1975) so if only cholesterol molecules had transferred across the bilayer to compensate for this difference, nearly 70% of the total sterol would have ended up in the outer leaflet. The fact that this does not occur suggests that if there is preferential transfer of cholesterol in intact cells it is probably necessary because of the association of some phospholipids with membrane skeletal proteins rather than because of the inherent differences in flip-flop rates of sterols and phospholipids.

Note added in proof

It has recently been reported that phospholipid asymmetry is retained in the membranes of microvesicles released from fresh erythrocytes by treatment with Ca^{2+} plus A23187 [Raval, P. J. & Allan, D. (1984) *Biochim. Biophys. Acta* **772**, 192–196].

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