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THE SPHINGOMYELIN POOLS IN THE OUTER AND INNER LAYER OF THE HUMAN ERYTHROCYTE MEMBRANE ARE COMPOSED OF DIFFERENT MOLECULAR SPECIES

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Analyses of the fatty acid composition of the outer and inner pools of sphingomyelin in the human erythrocyte membrane revealed significant differences in molecular species composition of these two pools. The sphingomyelin in the inner monolayer, representing 15–20% of the total sphingomyelin content of this membrane, is characterized by a relatively high content (73%) of fatty acids, which have less than 20 carbon atoms, whereas these account for only 31% of the total fatty acids in the sphingomyelin in the outer leaflet. On the other hand, the ratio saturated/unsaturated fatty acids in the two pools is similar. Significant differences are also observed for the fatty acid composition of the sphingomyelin in human serum when compared to that in the outer monolayer of the corresponding red cell. These results are interpreted to indicate an (almost) complete absence of transbilayer movements of sphingomyelin molecules in the human erythrocyte membrane, whereas an exchange of this phospholipid between the red cell membrane and serum is either virtually absent, or affects only a minor fraction of the sphingomyelin in the outer membrane layer.

It has been firmly established that the four major phospholipid classes in the human erythrocyte membrane are distributed over both halves of the bilayer in a highly asymmetric fashion (see, for recent reviews, Refs. 1–4). The two choline-containing phospholipids dominate the outer leaflet where 76% of the phosphatidylcholine (PC) and 82% of the sphingomyelin (SM) is found, whereas 80% of the phosphatidylethanolamine (PE) and all of the phosphatidylserine (PS) is present in the inner half of the bilayer [5]. It is also known that the PC fraction of this membrane comprises over 20 different molecular species, different with respect to their fatty acyl constituents [6] and that these are randomly distributed over both halves of the bilayer [7]. Marinetti and Crain [8] similarly reported that the molecular species of PE molecules which are localized on the outer surface of the red cell membrane, are the same as those on the inner surface. Since for both PC [7,9] and PE [10] a topological asymmetry of their metabolism has been shown to exist in the erythrocyte membrane, a transbilayer equilibration must occur to achieve a random distribution of the various molecular species. This was indeed found for PC in rat erythrocytes [9,11–15] and more recently also in human red cells [16]. Although the half-time value (11–27 h) for transbilayer equilibration of PC in the human erythrocyte is rather low, the process may be significant when compared to the average life time (120 days) of this cell.

Accelerated transbilayer movements of PC have been shown to occur in normal human erythrocytes after cross-linking of their proteins with tetrathionate or diamide [17], as well as in sickled erythrocytes [18]. In both these entirely different

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cases, the abnormality of the cells appeared also to be expressed by changes in the accessibility of the amino-phospholipids (PE and PS) for exogenous probes [19–23]. Interestingly, the above mentioned changes are confined to the glycerophospholipids, since the availability of SM in the membrane of the intact abnormal cells (either chemically modified or sickled) to sphingomyelinase C is always identical to that in normal erythrocytes [20,23]. Furthermore, it is of interest to mention that in the plasma membrane of pro-erythroblasts, unlike the glycerophospholipids, SM already seems to have its ultimate asymmetric distribution found in the mature cell [24]. All taken together, this may indicate that the asymmetric distribution of SM in the erythrocyte membrane is maintained by interactions which are different from those which regulate the asymmetric distribution of the glycerophospholipids. It may be also suggested that SM experiences a transbilayer movement, if at all, which is considerably slower than that of PC. This prompted us to study the fatty acid compositions of both the outer and inner pools of SM in the human erythrocyte membrane.

Thoroughly washed erythrocytes, derived from freshly drawn human blood, were incubated under gentle agitation at 37°C in four volumes of 150 mM NaCl/0.25 mM $MgCl_2$/10 mM Tris-HCl, pH 7.4, and using 6 IU of highly purified sphingomyelinase C (*Staphylococcus aureus*) per 1.0 ml packed cells [5]. After 1 h, the cells were collected by centrifugation for 5 min at 2500 × *g*. Haemolysis of the cells (determined as described in Ref. 25), did not exceed 2%. Sphingomyelinase C activity was arrested, as previously described [5], by the addition of an excess EDTA to the residual cells, and the lipids were subsequently extracted according to Rose and Oklander [26]. The non-lytic hydrolysis of SM, determined according to standard procedures [5,27], always was within the range of 80 to 85%. Non-degraded SM, representing the fraction of this phospholipid in the inner monolayer of the red cell membrane, was recovered as follows: Extracted lipids were separated by thin-layer chromatography according to Skipski et al. [28]. The position of the SM was localized by inspection of the Rhodamine 6G stained chromatogram under ultraviolet light, using pure SM (spotted on the same plate) as reference. The SM containing spot was scraped from the plate and the SM extracted by treating the silica gel three times with 15 ml chloroform/methanol (1 : 9, v/v) and once with 10 ml methanol. The ceramides produced by the non-lytic treatment of the red cells with sphingomyelinase and therefore representing the SM fraction in the outer monolayer, were recovered in a similar way, using silica gel G plates and chloroform/ methanol/ acetic acid (20 : 1 : 0.15, v/v) as developing system. Reference ceramides were prepared by exhaustive digestion of pure bovine brain SM (Koch-Light Laboratories Ltd., Colnbrook, U.K.) with sphingomyelinase C. A sufficiently large area was scraped from the plate to avoid any loss of particular ceramide species. Chromatograms were dried and lipid extracts concentrated under a nitrogen atmosphere to prevent oxidation of unsaturated fatty acids. The fatty acids of the SM and ceramides were converted into their methyl esters by treatment of the lipids with boron trifluoride (14% in methanol) according to the method of Morrison and Smith [29]. The methyl esters were analyzed on a Hewlett Packard Becker 419 gas-liquid chromatograph with a flame ionization detector. Composition of the fatty acid samples was calculated from the peak areas.

Sphingomyelin carries only one fatty acid per molecule. Since the second aliphatic chain is part of the long chain base which, in case of SM in the human erythrocyte membrane, is fixed in length and composition [30], the fatty acid composition directly reflects the molecular species composition of this phospholipid. Table I shows the molecular species composition of the outer and inner pools of SM in the human erythrocyte membrane, which results have been obtained from duplicate analyses on blood samples of four different donors. The difference in molecular species composition between these two fractions is most obvious. The inner fraction, comprising 15–20% of the total SM complement of the membrane, is characterized by a relatively high content (73%) of fatty acids containing less than 20 carbon atoms, whereas the fatty acids with 20 or more carbon atoms dominate the molecular species in the outer pool of SM (Table I). Interestingly, the total amounts of saturated and unsaturated fatty acids in both pools are essentially identical (Table I). From the known

TABLE I

MOLECULAR SPECIES COMPOSITION OF SPHINGOMYELIN IN THE OUTER AND INNER LAYER OF THE HUMAN ERYTHROCYTE MEMBRANE AND IN HUMAN SERUM

Fatty acid	Percentage				
	Erythrocyte				Serum [d]
	Outer layer [a]	Inner layer [a]	Total membrane		
			Calculated [b]	Literature [c]	
16:0	23.8±0.6	38.9±4.4	26.5±0.9	26.6±2.7	40.6±1.2
18:0	5.5±0.1	13.0±4.1	6.8±0.7	6.4±0.9	10.5±0.6
18:1	1.5±0.8	15.0±2.0	3.9±0.8	–	3.8±0.4
18:2	0.4±0.4	5.8±2.0	1.3±0.6	–	0.7±0.1
20:0	2.1±0.1	–	1.7±0.1	2.1±0.4	3.8±0.3
20:4	1.5±0.1	1.0±0.4	1.4±0.1	1.6±0.4	–
22:0	12.0±0.9	6.8±0.9	11.0±0.7	10.7±1.0	17.9±1.4
24:0	26.0±1.2	9.0±2.8	23.0±1.1	25.6±3.3	10.1±0.5
24:1	27.2±0.6	10.6±4.2	24.4±0.9	27.0±1.8	12.6±1.2
Total fatty acids with less than 20 carbon atoms	31.2±1.1	72.7±6.6	38.5±1.5	33.0±2.8	55.6±4.4
Total fatty acids with 20 carbon atoms or more	68.8±1.6	27.3±5.1	61.5±1.6	67.0±3.9	44.4±1.9
Total saturated fatty acids	69.4±1.6	67.7±6.7	69.0±1.7	71.4±4.5	82.9±2.0
Total unsaturated fatty acids	30.6±1.1	32.4±5.1	31.0±1.3	28.6±1.8	17.1±1.3

[a] Means ± S.D. from duplicate analyses on the erythrocytes obtained from four different blood samples ($n = 8$).

[b] Calculated from the relative amounts of each fatty acid in the SM fractions in the outer and inner monolayer, using a ratio of 82:18 as the distribution of this phospholipid over both these leaflets.

[c] Data taken from Dodge and Phillips [31].

[d] Means ± S.D. from duplicate analyses on the serum of two different blood samples ($n = 4$).

distribution of SM over both halves of the bilayer and the molecular species composition of each of the two pools given in Table I, one can easily calculate the overall molecular species composition of the entire SM complement of the membrane. These calculated values appear to be in good agreement with those published by Dodge and Phillips [31] for the fatty acid composition of the SM in the human red cell membrane (Table I), which indicates that the analytical data obtained for the two separate pools are reliable. Thus, SM seems to occupy a very special place among the phospholipids in the erythrocyte membrane, in that its inner and outer pools are composed of different molecular species, whereas the various molecular species of PE [8] and PC [7] are distributed randomly among both halves of the bilayer.

Although the transbilayer movement of PC in the human erythrocyte membrane is rather slow ($t_{1/2}$ is 11–27 h, Ref. 16), this process is still fast in relation to the life-time of this cell of 120 days and also fast compared to the renewal of the erythrocyte PC by exchange with serum PC or acylation of lyso-PC, which has a rate of 1% h^{-1} [32,33] and a half-time of about 100 h [34], respectively. Hence, it was suggested [16] that the translocation of PC in this membrane is fast enough to abolish any differences in PC species composition of the inner and outer pools generated by these two different renewal processes and that it will be, therefore, responsible to maintain a random distribution of the various molecular species of PC over the two monolayers. Conversely, the pronounced differences which are shown to exist in the molecular species which constitute the outer and inner pools of SM, seem to indicate that the transbilayer

movement of this phospholipid in the human erythrocyte membrane is extremely slow, or even totally absent.

Reed [32] has shown that, similar to PC, also whole molecules of SM are exchanged between the erythrocyte membrane and the serum lipoproteins, with a turnover rate of approximately 1% per hour for human cells. As a consequence of this process and the absence of any transbilayer movement of SM in the erythrocyte membrane, it seems plausible to expect that the molecular species composition of the SM in the outer layer of the membrane should reflect that of the SM in the serum. However, the fatty acid composition of SM isolated as described above from lipid extracts derived from human plasma according to Folch et al. [35], shows significant differences not only when compared to that of the total SM complement of the human red cell membrane, but even more pronounced when compared to that of the SM fraction located in the outer leaflet (Table I). This surprising discrepancy might be explained by the fact that according to Reed's observations [32] only 30% of the SM in the human red cells, which is equivalent to only 36% of the SM in the outer membrane leaflet, appears to take part in this exchange process. Hence, two thirds of the SM in the outer membrane layer is not in equilibrium with the SM in the serum, which may account for the observed differences in molecular species composition of these two pools. It is essential to note here that Crain and Zilversmit [15] observed a complete exchangeability of SM in intact rat erythrocytes during an in vitro incubation of these cells with unilamellar vesicles in the presence of a non-specific exchange protein from beef liver. It should be realized, however, that in this particular erythrocyte species all of the SM is located in the outer monolayer of the membrane [33,15].

In summary, the orientation of the SM molecules in the bilayer of the erythrocyte membrane can be characterized by the following features which appear to be specific for this non-glycerophospholipid: (i) a transbilayer movement of these molecules may be virtually absent, as is suggested by the considerable difference observed in the molecular species composition of the pools in either half of the bilayer; (ii) the transbilayer distribution of this phospholipid is altered neither in chemically modified normal cells [20], nor in sickled erythrocytes [23]; (iii) the transbilayer distribution of this phospholipid found in mature (murine) erythrocytes already exists in the corresponding (pro)erythroblasts [24]. It is tempting to speculate that all these observations point to a highly static orientation of (at least the majority of) the SM molecules in the erythrocyte membrane, a situation which may already exist at one of the earliest stages of the biogenesis of the cell. In this respect, SM thus seems to occupy a very special place among the phospholipids in the red cell membrane. This immediately raises the intriguing question by which kind of specific interactions with other membrane constituents this phenomenon is governed. The answer to this question must await further studies.

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