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Amphiphile-induced vesiculation in aged hereditary spherocytosis erythrocytes indicates normal membrane stability properties under non-starving conditions

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Summary

Aged HS erythrocytes with a defined primary defect in band 3 protein or ankyrin were incubated with amphiphiles (detergents) at sublytic concentrations (37°C, 60 min) or glucose-starved (37°C, 24 h). In line with previous studies, the release of AChE (exovesicles) from HS erythrocytes during glucose-starvation was significantly higher (11%) compared to that from control erythrocytes (1%). Control and HS cells responded, however, similarly to amphiphile-treatment (non-starving conditions). Amphiphiles induced similar types of shape alterations and a similar amount of AChE release (14–15%). Furthermore, the size and shape of amphiphile-induced exo- and endovesicles released from control and HS erythrocytes were similar. The results suggest that the stability properties of the membrane are not seriously disturbed in aged HS erythrocytes under non-starving conditions.

Keywords: Hereditary spherocytosis, erythrocyte, vesiculation, membrane skeleton-lipid bilayer interaction, detergent, ATP depletion.

Abbreviations: HS, hereditary spherocytosis; AChE, acetylcholinesterase; ATP, adenosine triphosphate; C12-zwittergent, 3-(dodecyldimethylammonio)-1-propanesulphonate; C12-maltoside, dodecyl *D*-maltoside; TEM, transmission electron microscopy; HE, hereditary elliptocytosis; DIDS, 4,4′-diisothiocyano-2,2′-stilbene disulphonate

Introduction

HS is a heterogeneous group of disease, i.e. mutations in genes coding for different membrane proteins associated with the membrane skeleton may be involved (Mohandas and Chasis 1993, Delaunay 1995, Delaunay *et al.* 1996). A deficiency of the defective protein, as well as in associated skeleton proteins, is usually detected (Agre *et al.* 1985, Mohandas and Chasis 1993, Eber *et al.* 1996, Cho *et al.* 1998). HS erythrocytes are characterized by a more or less pronounced reduced osmotic resistance and cellular deformability, and an abnormal, sometimes spherocytic, shape. It is thought that an increased tendency of HS erythrocytes to shed exovesicles, resulting in a reduced surface area to volume ratio, is partially the reason to these properties (Mohandas and Evans 1994, Inaba *et al.* 1996), and that the increased tendency for vesiculation is due to decreased membrane skeleton-lipid bilayer interactions (Palek and Jarolim 1993, Mohandas and Evans 1994). HS and HE erythrocytes from the same blood donors as in the present study were recently shown to have a normal aminophospholipid flippase activity, phospholipid scramblase activity and transmembrane phosphatidylserine distribution (de Jong *et al.* 1999).

This study was undertaken in order to examine and compare vesiculation induced by amphiphiles in HS erythrocytes, having defined genetic defects in band 3 or ankyrin (table 1), with that occurring in normal erythrocytes (Hägerstrand and Isomaa 1989, 1991, 1992). Band 3, aided by ankyrin, is the major integral protein linking the plasma membrane and the membrane skeleton in human erythrocytes (Nakao 1990, Low *et al.* 1991). Since the content of band 3 and/or that of ankyrin and spectrin is reduced by various degrees in the studied HS erythrocytes (Eber *et al.* 1996), it was assumed that these abnormalities would, due to decreased membrane skeleton-lipid bilayer interactions, markedly affect the membrane perturbation of membrane intercalated amphiphiles. Glucose starvation of erythrocytes was studied in a comparative purpose, since HS erythrocytes subjected to ATP depletion have been reported to shed exovesicles more readily than normal erythrocytes (Reed and Swisher 1966, Weed and Bowdler 1966, Snyder *et al.* 1978).

Results

HS erythrocytes

Morphology of untreated erythrocytes. Thin sections (and light microscopy) indicated a discoid shape of control erythrocytes (figure 1(*a*)) and a varying non-axisymmetric shape of HS erythrocytes (figure 1(*b*) (patient JT)). The shape of erythrocytes in different HS-samples varied, being slightly (patients BB, LF, FLF, FG) (not shown) or markedly (JT) irregular. In samples where erythrocytes had only a slightly irregular shape, many cells were nearly discoid but lacked the invaginated centre (not shown). Only a few spherocytic erythrocytes and no large cell fragments were seen. Endovesicles (invaginations) occurred frequently (JT (figure 1(*b*)), LF, FLF) or sparsely (BB, FG) in HS erythrocytes (untreated). Endovesicles have previously been observed in erythrocytes from splenectomized patients (Holroyde and Gardner 1970, Sills *et al.* 1988).

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Table 1. Characteristics of defects in HS and HE erythrocytes.

HS erythrocytes:	
Band 3 defects (dominant):	
JT:	Missense mutation in exon 13, codon 518 (Arg > Cys) (R518C)
BB:	Deletion (of methionine) in exon 18, codon 663 or 664 (delM663)
LF:	Frameshift in exon 11, codon 419 (1369delC)
FLF*:	As LF
Ankyrin-1 defect (recessive):	
FG:	Defect in exon 41 (5703 (+16 C > T)) + missense mutation in (−108 T > C)
HE erythrocytes:	
CH:	Spectrin alpha Lely + spectrin alpha 230 mutations
SK:	Spectrin alpha Lely + spectrin alpha Lyon mutations

* FLF = father to LF.
Note: Patients JT, LF and FLF were splenectomized, while BB and FG were not. Patients CH and SK were not splenectomized.
Adapted from Eber *et al.* (1996).

Amphiphile-effects on cell morphology and exovesiculation. Control erythrocytes and HS erythrocytes responded in a similar way to amphiphile treatment. The echinocytogenic amphiphiles C12-zwittergent (263 μM, 10 min) and C12-maltoside (40 μM, 10 min) induced at high sublytic concentrations spiculated (sphero-echinocytic) shapes and exovesiculation, while the stomatocytogenic amphiphile chlorpromazine (200 μM, 30 min) induced invaginated (sphero-stomatocytic) shapes and endovesiculation (figures 1(*c*)–(*f*) (JT)). The amount of AChE release, used to detect exovesicle release (Lutz *et al.* 1977, Snyder *et al.* 1978), was similar (14–15%) in HS samples (BB, LF, FLF, FG) and in control erythrocytes (table 2(*a*)) following incubation with the echinocytogenic amphiphile C12-zwittergent (263 μM, 30 min). TEM studies showed that exovesicles released from HS erythrocytes had a largely similar morphology and size as those released from control cells (see Hägerstrand and Isomaa 1989, 1992), i.e. they were mainly spherical in case of C12-zwittergent (figures 1(*c*) and (*d*) insets) and mainly tubular in the case of C12-maltoside (not shown), The endovesicle morphology and pattern in HS erythrocytes (figures1 (*e*) and (*f*)) were similar to in control cells.

Effect of glucose starvation on cell morphology and exovesiculation. During 24 h incubation at 37°C in the presence of glucose, neither control nor HS erythrocytes (BB, JT, LF, FLF, FG) released AChE (exovesicles) to the supernatant. This is also indicated by the non-echinospherocytic shape of control (figure 1(*g*)) and HS erythrocytes (figure 1(*j*)) (JT)) following incubation. However, during 24 h incubation at 37°C in the absence of glucose, the AChE release was 1% for control cells and 11% for HS erythrocytes (table 2(*a*)). Such prolonged incubations without glucose lead to ATP depletion (Lutz *et al.* 1977, Snyder *et al.* 1978, Ferrell and Huestis 1984). The total AChE activity was similar in control and HS erythrocyte samples. TEM studies showed that haemoglobin containing exovesicles released from control (figure 1(*h*)) and HS erythrocytes (figure 1(*k*)) during energy depletion were mostly spherical, but also tubular (figures 1(*i*) and (*l*), respectively). The diameter of spherical and tubular exovesicles collected following energy depletion of control and HS erythrocytes was about 2.5 times that of exovesicles induced by amphiphile treatment. It can be noted that incubation of HS cells (JT) with glucose (figure 1(*j*)) leads to a partial recovery from the irregular shape (figure 1(*b*)).

Osmotic resistance. HS erythrocytes (LF, FLF and FG) had a lower resistance against hypotonic stress than control cells (table 3). The lower resistance against hypotonic stress may be due to a smaller surface area:volume ratio reported in HS cells (de Jong *et al.* 1999).

Activity of the anion exchanger. $^{32}PO_4^{3-}$-efflux experiments indicated a slightly reduced out-transport of $^{32}PO_4^{3-}$ from HS erythrocytes (LF, FLF, FG) (table 4). At 60 min, HS cells had released 13% less $^{32}PO_4^{3-}$ than control cells. The anion efflux was blocked by DIDS (10μM) in both control and HS cells. A decreased anion efflux in HS cells may be due to a deficiency of the anion pump. HS erythrocytes are generally thought to be depleted in band 3 (Delaunay 1995, Cho *et al.* 1998), and band 3 was reported to be reduced to 93% of normal in erythrocytes from FG (Eber *et al.* 1996). A reduction of band 3 may possibly be due to a release of band 3 enriched exovesicles (see Allan *et al.* 1976, Lutz *et al.* 1977).

HE erythrocytes

HE erythrocytes (patients CH and SK) were ellipsoidal (not shown). Erythrocytes from the patient SK with a spectrin 230 + spectrin Lely mutation had broad protrusions and large fragments occurred. Endovesicles were rarely (CH) or frequently (SK) observed in HE erythrocytes. The AChE released upon amphiphile-treatment and energy depletion (table 2(*b*)) was similar to control cells. HE erythrocytes had a largely similar sensitivity to osmotic swelling as control erythrocytes (not shown).

Discussion

Previous studies have indicated that exovesiculation upon ATP depletion occurs more readily in HS erythrocytes than in normal erythrocytes (Reed and Swisher 1966, Weed and Bowdler 1966, Snyder *et al.* 1978). In line with these reports, this study revealed that HS erythrocytes released a substantial amount of AChE upon 24 h incubation (37°C) without glucose, while control erythrocytes did not. The reason of the higher sensitivity of HS erythrocytes to ATP depletion is not fully known. It is thought that the defects in the membrane skeleton and its associated proteins make the HS erythrocyte membrane more leaky, because HS erythrocytes have an increased passive permeability to monovalent cations (Joiner *et al.* 1995, de Franceschi *et al.* 1997, Delaunay *et al.* 1999). A higher ATP consumption, in order to maintain the ion gradients, may lead in a shorter time period to ATP depletion in HS erythrocytes than in normal erythrocytes under glucose starving conditions. Secondary effects of such an energy depletion may lead to membrane

instability and vesiculation, because ATP is required to maintain the normal discoid erythrocyte shape (Nakao *et al.* 1960), probably by feeding a continuous phosphorylation of phosphoinositides (Ferrell and Huestis 1984, Bütikofer *et al.* 1989a, Backman *et al.* 1998). ATP depletion causes a dephosphorylation of phosphoinositides located in the inner membrane leaflet, leading to their splitting into phosphatidylinositol and diacylglycerol. This leads to a diminished area of the inner membrane leaflet, and thereby to echinocytosis and exovesiculation via the bilayer couple mechanism (see below). However, it has been suggested that other mechanisms must additionally be involved (Björk *et al.* 1997, Backman *et al.* 1998). Band 3, which is deficient in HS erythrocytes (Eber *et al.* 1996) and has an increased lateral and rotational mobility in HS erythrocytes with a primary ankyrin defect (Cho *et al.* 1998), is regarded important for the functional state of phosphoinositides (Gascard *et al.* 1993). It can be speculated that the tendency for exovesiculation in HS erythrocytes subjected to energy depletion is affected by an impaired interaction between the band 3/ankyrin complex and phosphoinositides.

Amphiphiles are thought to induce shape alterations and vesiculation in erythrocytes by intercalating preferentially into one membrane monolayer, thereby expanding this monolayer relative to the other (Sheetz and Singer 1974, 1976). This leads to echinocytic or stomatocytic shapes, and, from the formed spikes and invaginations, respectively, vesicles may be shed (Hägerstrand and Isomaa 1989, 1992, Kralj-Iglic *et al.* 2000). Although the membrane skeleton may have a passive role in amphiphile-induced shape alterations (Mohandas *et al.* 1983, Sheetz 1983), the properties of the membrane skeleton and the skeleton-bilayer interactions may affect the shape and plasma membrane stability of human erythrocytes (Haest *et al.* 1980, Lange *et al.* 1982, Sheetz 1983, Low *et al.* 1991, Iglic *et al.* 1995, Iglic 1997, Hägerstrand *et al.* 2000), and the size of released exovesicles (Mohandas and Evans 1994, Iglic *et al.* 1998, Iglic and Hägerstrand 1999). When the bilayer is not directly supported by the skeleton, e.g. due to a reduction in the number of linkages between the bilayer and the skeleton or in the skeleton density, this can lead to loss of cohesion between skeleton and bilayer and consequently to increased susceptibility for shedding of membrane vesicles (Haest 1982, Palek 1987, Mohandas and Evans 1994, Iglic and Hägerstrand 1999). The stability of the membrane, in order to prevent vesiculation, can only be warranted when the bilayer interacts with the skeleton at many sites (Haest 1982, Mohandas and Evans 1994). At such conditions, i.e. when the connection between the skeleton and the bilayer is strong, vesiculation is less favourable, presumably because of accumulated shear energy of the membrane skeleton in the budding region of a supposed vesicle. Namely, it has been shown that the shear energy of the membrane skeleton in the membrane protrusion (bud) strongly increases during the budding process, in the case that the skeleton is not dissociated from the membrane bilayer (Iglic and Hägerstrand 1999).

An intact membrane skeleton may also stabilize erythrocyte shape by maintaining a homogeneous lateral distribution of membrane integral proteins (Sheetz 1983, Kralj-Iglic *et al.* 1996). Besides, alterations in the structure (Gimsa and Ried 1995) and abundance (Khodadad and Weinstein 1983, Liu *et al.* 1991) of membrane embedded proteins may also affect erythrocyte shape (Kralj-Iglic *et al.* 1996), as well as the vesiculation process (Hägerstrand *et al.* 1999, Kralj-Iglic *et al.* 1999, 2001).

The present study showed that HS and control (normal) erythrocytes responded in a similar way to amphiphile treatment, i.e. the type of shape alterations and the size and shape of released exovesicles were similar. Also, the amount of AChE release was similar. However, the relevance of this observation is uncertain, since e.g. the kinetics of vesicle shedding and the AChE density in exovesicles are not known. The release of many small exo- and endovesicles (invaginations) from amphiphile-treated HS erythrocytes indicates the presence of relatively strong skeleton-lipid bilayer ('vertical') interactions over the whole erythrocyte membrane, similar to those of the normal erythrocyte membrane. Namely, in the case of a substantial decrease in the skeleton-bilayer interactions over the erythrocyte surface, the size of released vesicles would be much larger (Mohandas and Evans 1994, Bobrowska-Hägerstrand *et al.* 1998, Iglic *et al.* 1998). In both normal and HS erythrocytes, a substantial decrease in skeleton-bilayer interactions (membrane stability) may occur at ATP depletion conditions, since this study indicates that the exovesicles released following glucose-starvation were larger than those induced by amphiphile-treatment. This observation also suggests that exovesicle size (and shape alteration) due to ATP depletion is not only determined by an asymmetric expansion of the bilayer leaflets, but is also influenced by weakened or altered membrane skeleton-lipid bilayer interactions. This influence is apparently more pronounced in HS than in normal erythrocytes, since more AChE was shed from HS erythrocytes.

Taken together, these results indicate that there is no major differences in the physical properties of the lipid bilayer and the membrane skeleton-lipid bilayer interactions between aged HS and normal erythrocytes under non-starving conditions. These results are in line with the concept that decreased membrane mechanical stability is not a consistent feature of HS erythrocytes (Mohandas and Chasis 1993), as, for example, the membrane shear modulus of HS erythrocytes may be unaltered relative to the normal erythrocyte membrane (Mohandas *et al.* 1980). Since the membrane shear modulus is solely due to the membrane skeleton and, therefore, strongly depends on the skeleton-bilayer interactions, one may deduce that the skeleton-bilayer interactions were not seriously disturbed in the HS erythrocytes used in the study of Mohandas *et al.* (1980). It should be noted that the HS erythrocytes examined in this study were aged. An alteration of the membrane stability properties, in the direction of a normalization, may have occurred during the life span and handling of the HS erythrocytes due to shedding of (destabilizing) membrane area and/or components. However, although the membrane stability properties appear to be largely normal under non-starving conditions, the aged HS cells show an increased sensitivity to ATP depletion, possibly due to dysfunctional membrane barrier properties.

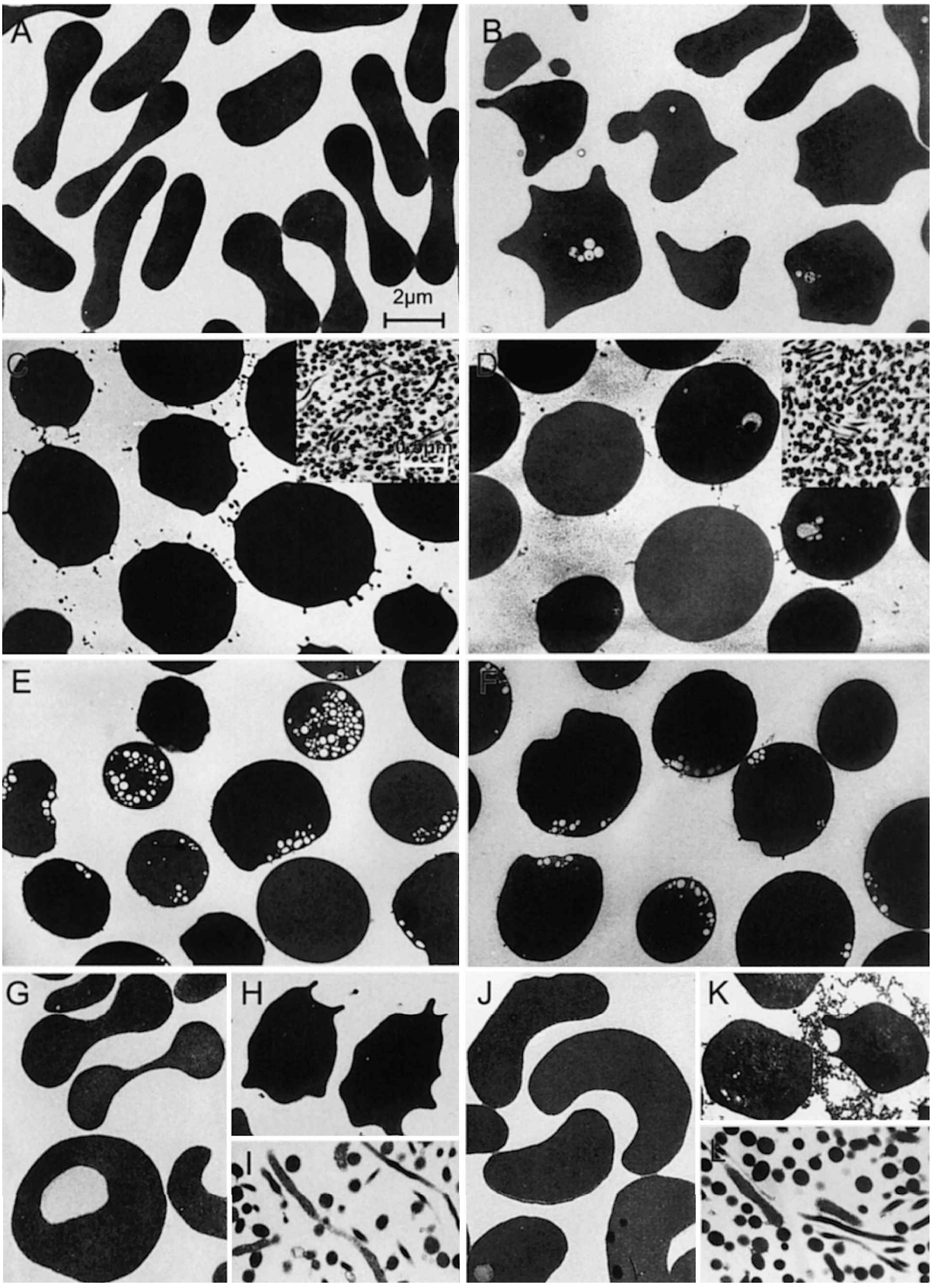

Figure 1. Thin sections of amphiphile treated and energy depleted control and HS (patient JT) erythrocytes. Untreated (*a*) control and (*b*) HS erythrocytes. (*c*) control (normal) and (*d*) HS erythrocytes treated with the echinocytogenic amphiphile C12-zwittergent (263 μM, 10 min).

Experimental procedures

C12-maltoside was purchased from Fluka (Buchs, Switzerland), C12-zwittergent from Calbiochem (San Diego, CA, USA) and chlorpromazine hydrochloride from Sigma (St. Louis, MO, USA).

HS blood from five patients and HE blood from two patients, with defined mutations (table 1), as well as blood from healthy relatives (control), were drawn, after informed consent, by venipuncture into heparinized tubes in Göttingen, Germany, and sent on ice by courier post to Åbo, where experiments were performed within 30 h, i.e. within 4 days after the blood was drawn. The erythrocytes were washed three times in the buffer containing 145 mM NaCl, 5 mM KCl, 4 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1 mm $MgSO_4$, 1 mM $CaCl_2$ with or without (prior to energy depletion experiments) 10 mM glucose (pH 7.4). Cells were kept at 4°C or at room temperature until used. The cell density in the samples was 1.65×10^8 cells/ml ($\sim$1.5% hematocrit). Erythrocytes were incubated under gentle mixing with amphiphiles at high sublytic concentrations at 37°C for 60 min (with glucose), as previously described (Isomaa *et al.* 1987), or at 37°C for 24 h with or without glucose. Prolonged incubations without glucose lead to ATP depletion (see Lutz *et al.* 1977, Snyder *et al.* 1978, Ferrell and Huestis 1984). Following incubation, erythrocyte samples were studied by light microscopy, whereby they, and exovesicles isolated from the cell-free supernatant (Hägerstrand and Isomaa 1989), were prepared for TEM by a standard protocol (Hägerstrand and Isomaa 1989). Osmotic resistance was monitored as release of haemoglobin from cells in a graded series of buffer/water. AChE is a membrane enzyme frequently used to monitor exovesicle-release from human erythrocytes (Lutz *et al.* 1977, Billington and Coleman 1978, Bütikofer *et al.* 1989b, Hagelberg and Allan 1990, Hägerstrand and Isomaa 1994). AChE activity was monitored in the cell-free supernatant according to the method of Ellman (1961), as previously described (Hägerstrand and Isomaa 1989). Efflux of $^{32}PO_4^{3-}$ from pre-loaded erythrocytes was studied as previously described (Isomaa *et al.* 1986). Exactly 10 μM DIDS was used to inhibit anion efflux.

Table 2. AChE release from human erythrocytes following amphiphile treatment and energy depletion.

	(a)		*(b)*	
	Control	HS	Control	HE
C12-zwitt. 263 μM	14 $\pm$ 4 (3)	15 $\pm$ 4 (5)	18 (2)	17 $\pm$ 6 (3)
Energy depletion	1 $\pm$ 1 (4)	11 $\pm$ 5 (5)	2 (2)	2 $\pm$ 1 (3)

Values are % AChE in the cell-free supernatant (following incubation) of the total AChE activity in the sample. Erythrocytes were amphiphile-treated with C12-zwittergent (263 μM, 10 min, with glucose) or energy depleted by incubated for 24 h at 37°C without glucose.
HS erythrocytes were from patients BB, JT, LF, FLF and FG. HE erythrocytes were from patients MH and SK.

Table 3. Osmotic resistance (hemoglobin release) of control and HS erythrocytes.

	Buffer (B)	80% B	70% B	60% B	50% B	Water
Control (n = 2)	100	100	100	99	83	31
HS (n = 3)	100	99	95 $\pm$ 3	84 $\pm$ 7	42 $\pm$ 6	29 $\pm$ 1

Values are % transmission of the cell-free supernatant following 30 min incubation in buffer/water.
HS erythrocytes were from patients LF, FLF and FG.

Table 4. Anion ($^{32}PO_4^{3-}$) efflux from pre-loaded control and HS erythrocytes.

	6 min	12 min	32 min	66 min	66 min/DIDS
Control (n = 2)	9/9	18/20	36/38	60/64	0.3/0.7
HS (n = 3)	9 $\pm$ 1	17 $\pm$ 2	35 $\pm$ 1	54 $\pm$ 4	1.6 $\pm$ 0

Values are % ^{32}P in the cell-free supernatant compared to the total intracellular amount of ^{32}P at time zero. HS erythrocytes were from patients LF, FLF and FG.

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Exovesicles isolated following incubation of control and HS erythrocytes with C12-zwittergent (263 μM, 30 min) are shown in the insets of (*c*) and (*d*), respectively. (*e*) control and (*f*) HS erythrocytes treated with the stomatocytogenic amphiphile chlorpromazine (200 μM, 30 min). Control erythrocytes incubated (*g*) with and (*h*) without glucose (24 h, 37°C). (*i*) exovesicles released from control erythrocytes at treatment (*h*). HS erythrocytes incubated (*j*) with and (*k*) without glucose (24 h, 37°C). (*l*) exovesicles released from HS erythrocytes at treatment (*k*). (*a*)–(*h*), (*j*) and (*k*) are similarly enlarged, see bar in (*a*). (*c*) inset, (*d*) inset, (*i*) and (*l*) are similarly enlarged, see bar in (*c*) inset.

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