Different Resistance of Mammalian Red Blood Cells to Hemolysis by Bile Salts

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To evaluate why hemolysis of red blood cells (RBC) by bile acids varies in different mammalian species, we determined the mean corpuscular volume (MCV), lipid content and the concentrations of the conjugates of deoxycholate and of NaCl inducing 50% hemolysis of RBC from healthy humans, pigs, horses, cows, sheep and jaundiced humans. A volume of 0.05 mL of washed RBC at 1% hematocrit, which has the same lipid content but different phospholipid composition and number of erythrocytes (owing to the variable MCV), was incubated in taurodeoxycholate (TDC) solution (0–5 mM) to determine the TDC concentration inducing 50% hemolysis (TDC_{50}). The TDC_{50} was highest in RBC of sheep and decreased within the series sheep > pig > cow > horse > healthy human > jaundiced human, which have generally increasing MCV. The osmotic resistance followed an inverse order, with jaundiced human > healthy human > horse > cow > pig > sheep. Although we found no correlation between the TDC_{50} and phospholipid composition of the erythrocytes tested, the extent of bile salt-induced hemolysis seemed to depend on both the MCV and the number of erythrocytes in the incubation medium.
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Red blood cells (RBC) from different mammalian species have a remarkably similar lipid content per milliliter of packed cells (1), but their phospholipid composition (1–3), mean corpuscular volume (MCV) (2) and osmotic resistance (4) vary widely. Sphingomyelin (SM) and phosphatidylcholine (PC) are preferentially located in the outer leaflet of the RBC membrane and represent about 45–60% of total phospholipids (PL) (5); their proportions vary in RBC from different animal species (1). Furthermore, the lipid composition of RBC membranes has been considered an important factor influencing the susceptibility to damage from bile salts. Coleman *et al.* (6) have shown that cow and sheep RBC, whose PC content is very low and is replaced by SM, are more resistant to glycocholate-induced hemolysis than human erythrocytes.

In this study we evaluated whether criteria, besides RBC membrane composition, were correlated with bile salt-induced hemolysis of RBC from different species. Variations in the MCV of mammalian erythrocytes may contribute to the different hemolytic effects observed with detergent bile salts.

MATERIALS AND METHODS

Taurodeoxycholate (TDC) and glycodeoxycholate (GDC) (Na^+ salt, grade A) were purchased from Calbiochem (Milan, Italy) and were more than 96% pure as judged by thin-layer chromatography (TLC). Blood from healthy human subjects and from humans with obstructive jaundice, which have large RBC (7), was drawn into heparinized vacutainer tubes. Blood from pigs, cows, sheep and horses was obtained from local slaughterhouses. Cell indices (MCV and mean cell hemoglobin) were evaluated in an S-plus Coulter Counter Analyzer (Coulter, Hialeah, FL). Blood samples were centrifuged and the plasma buffy coat was removed. Then the RBC were washed three times with 15 mM *tris*(hydroxymethyl)aminomethane buffer (pH 7.4) containing 145 mM NaCl and 5 mM glucose. The lipid content of RBC was determined after extracting 1 mL of packed RBC according to Rose and Oklander (8); PL (9) and cholesterol (Biochemia kit no. 676535, Boehringer Mannheim, Mannheim, Germany) were determined on the chloroform extracts. The PL classes contained in RBC membranes were separated by two-dimensional TLC on silica gel G plates (Merck, Darmstadt, Germany), using chloroform/methanol/aqueous ammonia (65:35:5, by vol) as the first solvent and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, by vol) as the second (10). The fractions were located with iodine vapor and scraped off the silica gel plates; PL were measured after digestion with perchloric acid (9). Fractions were identified by comparison with standards (Supelco, Milan, Italy).

The hemolytic effect of TDC and GDC was evaluated as follows: Washed RBC at 1% hematocrit were incubated in TDC and GDC solutions (0–5 mM) in 15 mM of the abovementioned TRIS buffer, pH 7.4, for 45 min, at a final volume of 5 mL. Afterwards, the samples were centrifuged at 5,000 rpm for 4 min (model 153, Microfuge; Beckman Instruments, Fullerton, CA), and the extent of hemolysis was evaluated by comparing absorbance at 546 nm of 100-fold serial dilutions of the supernatant with that of RBC totally lysed in distilled water.

Osmotic fragility of the different RBC was evaluated according to Parpart *et al.* (11). The mean cell fragility was taken as the NaCl concentration (g/dL) at which 50% of the RBC were hemolyzed (12).

RESULTS

Table 1 reports the cell parameters of the RBC tested. The MCV increased along the order sheep, cow, horse, pig and human; the largest RBC came from jaundiced humans (MCV 115 μm^3). Cholesterol and phospholipid content (mg/mL) of the packed RBC was relatively constant in all species, as previously reported by Nelson (1). Even though the lipid content per RBC was greater in humans than in pigs, horses, cows and sheep, the lipid content of RBC from cholestatic humans was even higher (Table 1).

The same volume of packed RBC (0.05 mL) from the various species tested in the hemolysis experiments contained different numbers of cells, even though the total lipid content and the cholesterol to PL molar ratio were roughly the same (Table 2).

Figure 1 shows the hemolysis induced by increasing concentrations of TDC. RBC from sheep, pig and cow were

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Abbreviations: GDC, glycodeoxycholate; MCV, mean corpuscular volume; PL, phospholipid; PS, phosphatidylserine; RBC, red blood cells; SM, sphingomyelin; TDC, taurodeoxycholate; TDC_{50}, TDC concentration inducing 50% hemolysis; TLC, thin-layer chromatography.

TABLE 1

Parameters of Erythrocytes from Different Animal Species[a]

	MCV[b] (μm^3)	S.A.[c] (μm^2)[g]	MCH[d] (pg)	Ch[e] (mg/mL)	PL[f] (mg/mL)	Ch/PL molar ratio	Lipids (g/cell) ($\times 10^{-13}$)[h]
Healthy humans (n = 5)	87 ± 3	142	30 ± 1	1.21 ± 0.3	2.69 ± 0.4	0.91	3.3 ± 0.3
Obstructive jaundice (n = 3)	115 ± 2	160	33 ± 2	1.34 ± 0.2	2.88 ± 0.5	0.94	4.6 ± 0.2
Cow (n = 4)	49 ± 2	90	14 ± 1	1.38 ± 0.3	2.86 ± 0.4	0.97	1.9 ± 0.4
Pig (n = 4)	66 ± 2	95	17 ± 1	1.37 ± 0.3	2.82 ± 0.5	0.98	2.6 ± 0.3
Horse (n = 3)	54 ± 4	83	18 ± 1	1.38 ± 0.2	2.85 ± 0.6	0.98	2.2 ± 0.1
Sheep (n = 3)	25 ± 3	70	12 ± 1	1.39 ± 0.2	2.84 ± 0.4	0.99	1.6 ± 0.1

[a]The values indicate means ± SD.
[b]MCV, mean corpuscular volume.
[c]S.A., surface area.
[d]MCH, mean cell hemoglobin.
[e]Ch, cholesterol; mg/mL of packed red blood cells.
[f]PL, phospholipid; mg/mL of packed red blood cells.
[g]Values were taken from References 12, 13 and 15–17.
[h]Grams of lipids per red blood cell ($\times 10^{-13}$).

much more resistant to TDC than healthy human erythrocytes; the latter cells were less prone to TDC-induced hemolysis than the larger ones that were obtained from jaundiced humans. Horse RBC showed an intermediate behavior. Because the GDC concentrations causing 50% hemolysis overlapped the values reported for TDC, these have not been reported.

At a constant volume of incubated RBC, the TDC concentration causing 50% hemolysis (TDC_{50}) increased in the order sheep > pig > cow > horse > healthy human > jaundiced human (Table 2). The plot of TDC_{50} *vs.* erythrocyte MCV gave a strong negative correlation, which reached statistical significance ($r = -0.83$)($P < 0.02$) (Fig. 2).

At TDC_{50}, the number of bile salt molecules per RBC in the incubation tubes was lowest for horse and highest for pig (Table 2); no correlation was found between this ratio and TDC_{50} ($r = 0.5$; P = not significant).

TABLE 2

Parameters for TDC-Induced Hemolysis[a]

	Number of RBC in the incubation tube ($\times 10^6$)	Lipid content in the incubation tube ($g \times 10^{-7}$)	TDC_{50}[b] (mM)	TDC molecules[c] / RBC number ($\times 10^8$)
Healthy humans (n = 5)	574 ± 42	1894 ± 107	1.38 ± 0.2	71.4 ± 5.4
Obstructive jaundice (n = 3)	435 ± 40	2001 ± 124	1.24 ± 0.2	84.2 ± 6.2
Cow (n = 4)	1064 ± 82	2022 ± 138	2.43 ± 0.3	68.6 ± 4.7
Pig (n = 4)	806 ± 71	2096 ± 99	3.00 ± 0.4	110.8 ± 7.2
Horse (n = 3)	960 ± 68	2112 ± 107	1.89 ± 0.2	58.8 ± 5.0
Sheep (n = 3)	1280 ± 98	2048 ± 111	3.72 ± 0.4	86.0 ± 6.4

[a]The values indicate means ± SD. The final volume of incubation medium was 5 mL (1% Ht).
[b]TDC_{50}: Taurodeoxycholate (TDC) concentration inducing 50% hemolysis.
[c]Number of TDC molecules per erythrocyte in the incubation medium at TDC_{50}. RBC, red blood cells.

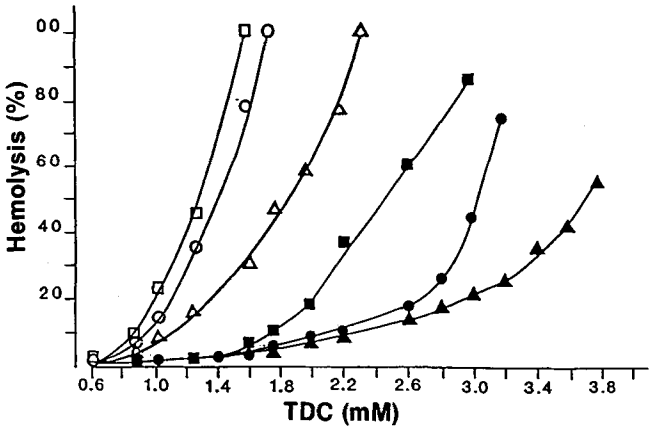

FIG. 1. Hemolysis of red blood cells from healthy human (○), cholestatic patient (□), cow (■), sheep (▲), pig (●) and horse (△), induced by increasing concentrations of taurodeoxycholate (TDC). The data shown are the means of three experiments. The final volume of the incubation medium was 5 mL (1% Ht).

TABLE 3

NaCl Concentrations Inducing 50% Hemolysis[a]

	NaCl (g %)	MCV (μm^3)
Healthy humans (n = 5)	0.44 ± 0.05	87 ± 3
Obstructive jaundice (n = 3)	0.41 ± 0.06	115 ± 2
Cow (n = 4)	0.57 ± 0.08	49 ± 2
Pig (n = 4)	0.62 ± 0.10	66 ± 2
Horse (n = 3)	0.52 ± 0.08	54 ± 4
Sheep (n = 3)	0.63 ± 0.07	25 ± 3

[a]The values indicate means ± SD. MCV, mean corpuscular volume.

Hemolytic behavior also seems to be correlated with the physical characteristics of the erythrocytes studied. The osmotic resistance of RBC from different mammalian species decreased with MCV (Table 3). The correlations between the NaCl concentration inducing 50% hemolysis and both MCV ($r = -0.86$) ($P < 0.001$) and TDC_{50} ($r = 0.95$, $P < 0.003$) were statistically significant.

The RBC of the species studied had variable amounts of choline-containing PL (Table 4), as reported by Nelson (1,3). In particular, SM accounted for more than 50% of total PL in cow and sheep RBC, whereas PC content was near zero in these species. In contrast, SM proportions are similar in human, pig and horse RBC, even though the latter two have smaller MCV and a higher resistance to TDC than human RBC. The correlation between SM content of RBC membranes (expressed as percent of total PL) and the TDC_{50} was not statistically significant (Fig. 3).

DISCUSSION

Previous studies have indicated that RBC containing SM are more resistant to bile salt-induced hemolysis (6). Several hematological parameters, such as MCV, surface area and surface-to-volume ratio differentiate erythrocytes from different mammalian species (12–17); surface area is negatively correlated with the volume of the RBC (18). Hypotonic stress reduces the surface-to-volume ratio, and hemolysis occurs after the cells have become spherical and have reached a critical volume. Osmotic resistance is strongly correlated to the surface-to-volume ratio of the RBC (7,18), and it increases with MCV (4,12) (Table 3). An increment of both the surface area and the surface-to-volume ratio, as occurs in cholesterol-enriched RBC of cholestatic humans, enhances osmotic resistance (7) (Table 3); these erythrocytes are resistant to hemolysis from hypotonic stress but are susceptible to the action of deoxycholic acid conjugates. By contrast, erythrocytes with

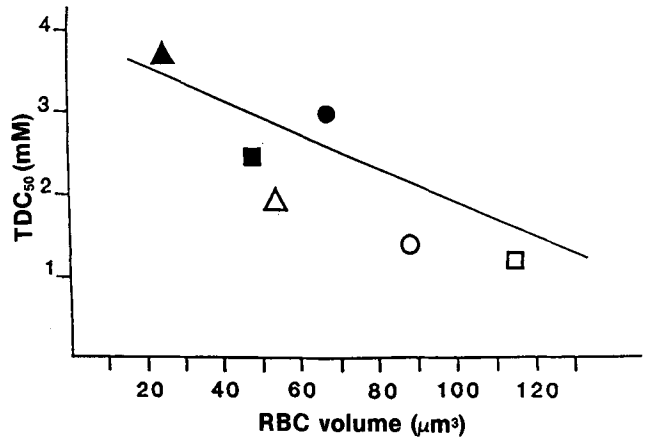

FIG. 2. Correlation between taurodeoxycholate (TDC) concentrations able to induce 50% hemolysis (TDC_{50}) and mean corpuscular volume of the red blood cells (RBC) tested (μm^3) ($r = -0.83$, $P < 0.02$). Symbols indicate RBC from healthy human (○), cholestatic human (□), cow (■), sheep (▲), pig (●) and horse (△).

TABLE 4

Phospholipid Composition of Red Blood Cells from Various Animal Species[a]

	(% of total phospholipids)						
	LPC	PI	PS	SM	PC	PE	Other
Healthy humans	1.6	1.8	12.9	25.7	39.5	26.2	3.1
Obstructive jaundice	1.3	1.8	12.6	23.4	32.4	26.1	2.2
Cow	—[b]	2.1	15.9	50.8	1.5	26.8	2.7
Pig	0.9	2.7	16.1	20.1	30.3	28.2	1.6
Horse	1.2	1.2	16.2	20.1	34.3	25.0	1.9
Sheep	—[b]	1.8	14.2	56.2	1.6	24.4	1.6

[a]The data were taken from three healthy humans, two humans with obstructive jaundice and three animals each for cow, pig, horse and sheep. LPC, lysophosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine.
[b]—, Not detected.

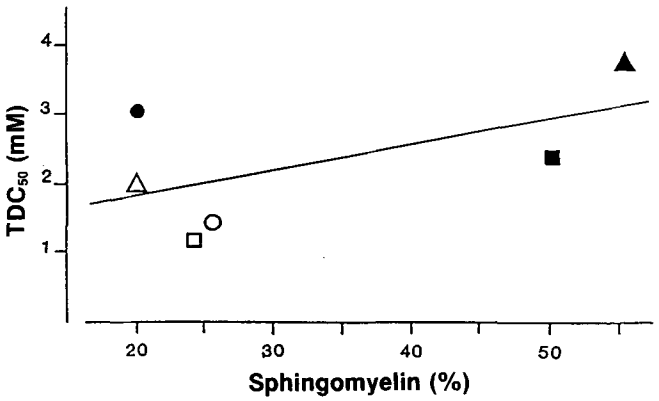

FIG. 3. Correlation between taurodeoxycholate (TDC) concentration inducing 50% hemolysis (TDC_{50}) and the percentage of sphingomyelin in red blood cell (RBC) membranes (r = 0.60; P = N.S.). Symbols indicate RBC from healthy human (○), cholestatic patient (□), cow (■), sheep (▲), pig (●) and horse (△).

small surface-to-volume ratio, such as those from sheep and cow, are osmotically fragile but resistant to bile salts (19). Erythrocytes with small MCV would lyse at lower bile salt concentrations if cell swelling alone were responsible for breaking down the cell membrane. Instead, the smaller cells are more resistant to the detergent effect of tauroconjugates of deoxycholic acid (Table 2) (Fig. 2), even if the number of TDC molecules per erythrocyte required to obtain 50% hemolysis does not vary accordingly (Table 2).

Few data are available on the mechanisms underlying hemolysis induced by bile salts; at appropriate concentrations bile salts solubilize RBC membrane constituents (20), but like other anionic drugs, sublytic concentrations of bile salts actually protect against hypotonic stress (21). Hypotonic hemolysis and hemolysis induced by bile salts thus occur *via* different mechanisms.

Bile salt hemolysis is traditionally evaluated by incubating packed RBC with serial concentrations of bile salts (6). In our hemolysis experiments, 0.05 mL of packed RBC contained a variable number of cells but the same amount of lipids (Table 2); therefore the volume of packed erythrocytes used in the hemolysis experiments contained more cells when these had a small MCV, as for sheep, pig and cow.

As the concentration of TDC required to determine 50% hemolysis was inversely correlated with the volume of the erythrocytes tested (r = −0.83; see Fig. 2), it seems that some physical characteristics of the single erythrocyte play a role in determining the extent of hemolysis in the presence of TDC.

The variable resistance to damage by bile salts has been attributed to the peculiar PL composition of RBC from some animal species (2,6). In fact, SM largely replaces phosphatidylcholine in cow and sheep RBC (1) (Table 4). SM-rich membranes are typically less fluid and are lysed more slowly by bile salts than are more fluid membranes (19). Moreover, SM reduces the permeability to water (22) and phosphates (23). The influx of glycerol, phosphate and urea decreases when the ratio of poly- to monounsaturated fatty acids in the membrane decreases (4,5), as is the case in pig and sheep RBC (23,24).

The correlation between TDC-induced hemolysis and SM percentage in RBC membranes was not statistically significant (Fig. 3). Schubert and Schmidt (25) demonstrated *in vitro* that SM does not stabilize membranes against bile salt damage, as suggested by Lowe and Coleman (19) for erythrocytes. The latter authors incubated RBC at a concentration of 2% (by vol), so that the number of cells changed widely for the different animal species tested. Support for the importance of additional factors besides lipid composition comes from the hemolytic behavior of RBC from pig and horse; the erythrocyte PL composition in these species compares well with that in humans, but pig and horse RBC are smaller and have a TDC-resistance similar to those rich in SM.

In conclusion, several factors, including membrane characteristics and PL composition (6), influence the resistance of RBC to bile salts. In this study we have shown that both the MCV and the number of erythrocytes present strongly influence the extent of bile salt-induced hemolysis.

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REFERENCES

1. Nelson, G.J. (1967) *Biochim. Biophys. Acta 144*, 221-232.
2. Smith, J.E., Mohandas, N., and Shohet, S.B. (1979) *Am. J. Physiol. 5*, H725-730.
3. Nelson, G.J. (1972) in *Blood Lipids and Lipoprotein: Quantitation, Composition and Metabolism* (Nelson, G.J., ed.) pp. 317-386, Wiley-Interscience, New York.
4. Coldman, M.F., Gent, M., and Good, W. (1970) *Comp. Biochem. Physiol. 34*, 759-772.
5. Sheetz, M.P., and Singer, S.J. (1974) *Proc. Natl. Acad. Sci. USA 71*, 4457-4461.
6. Coleman, R., Lowe, P.J., and Billingtone, D. (1980) *Biochim. Biophys. Acta 599*, 294-300.
7. Cooper, R.A., Diloy-Puray, M., Lando, P., and Greenberg, M.S. (1972) *J. Clin. Invest. 51*, 3182-3192.
8. Rose, H.G., and Oklander, M. (1965) *J. Lipid Res. 6*, 428-431.
9. Bartlett, G.R. (1959) *J. Biol. Chem. 124*, 466-471.
10. Nelson, G.J. (1972) in *Blood Lipids and Lipoprotein: Quantitation, Composition and Metabolism* (Nelson, G.J., ed.) pp. 25-73, Wiley-Interscience, New York.
11. Parpart, A.K., Lorenz, P.B., Parpart, E.R., Gregg, J.R., and Chase, A.M. (1947) *J. Clin. Invest. 26*, 636-649.
12. Good, W. (1971) in *Experiments in Physiology and Biochemistry*, (Kertut, G.A., ed.) Vol. 4, pp. 163-181, Academic Press, London.
13. Wintrobe, M.M. (1974) *Clinical Hematology*, pp. 1805-1814, Lea & Febiger, Philadelphia.
14. Brooks, D.L., Tillman, P.C., and Niemi, S.M. (1984) in *Laboratory Animal Medicine* (Fox, J.G., Cohen B.J., and Loew, F.M., eds.) pp. 282-283, Academic Press, London.
15. Smith, J.E. (1983) in *Hematology* (Williams, W.J., Beutler, E., Erslev, A.J., and Lichtman M.A., eds.) 3rd edn., pp. 117-125, McGraw-Hill, New York.
16. Hoffman, J.F. (1986) in *Physiology of Membrane Disorders* (Andreoli, T.E., Hoffman, J.F., Fanestil, D.D., and Schultz, S.G., eds.) 2nd edn., pp. 221-234, Plenum Publishing Corporation, New York.
17. Romano, L. (1989) *Cell. Biol. Int. Rep. 13*, 851-855.
18. Bowdler, A.J., Dougherty, R.M., and Bowdler N.C. (1981) *Gerontology 27*, 224-231.

19. Lowe, P.J., and Coleman, R. (1981) *Biochim. Biophys. Acta 640*, 55–65.
20. Richards, M.H., and Gardner, C.R. (1978) *Biochim. Biophys. Acta 543*, 508–522.
21. Isomaa, B., Hägerstrand, H., Paatero, G., and Engblom, A.C. (1986) *Biochim. Biophys. Acta 860*, 510–524.
22. Barenholz, Y. (1986) in *Physiology of Membrane Fluidity* (Shinitzky, M., ed.) pp. 131–173, CRC Press, Boca Raton.
23. Gruber, W., and Deuticke, B. (1973) *J. Membrane Biol. 13*, 19–36.
24. Deuticke, B., and Gruber, W. (1970) *Biochim. Biophys. Acta 211*, 369–372.
25. Schubert, R., and Schmidt, K.H. (1988) *Biochemistry 27*, 8787–8794.

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