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Reticulocytes Have a Higher Resistance to Complement Lysis than Erythrocytes

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Old RBC have been shown to express less decay-accelerating factor (CD55), CD59 and complement receptor 1 (CR1, CD35) than young RBC [1–5]. All 3 proteins are known to regulate complement and CD55 and CD59 are essential for the protection of RBCs against autologous lysis by the continuous complement activation that takes place in plasma in the vicinity of RBCs [6–8]. Paroxysmal nocturnal haemoglobinuria, in which CD55 and CD59 are lacking because of a deficient synthesis of their GPI anchor, is the best illustration of their protective role [9]. Whether CR1 protects the RBC itself is uncertain, although when transfected to CHO cells, CR1 has been shown to reduce complement-mediated lysis [10]. Reticulocytes express approximately 3-fold more of all 3-complement regulators when compared to erythrocytes [2, 4, 5, 11] (here we define erythrocytes as non-reticulocytes, and RBC as the total population). To our knowledge, whether these differences would be of biological significance has not been tested. We therefore set out to measure reticulocytes before and after haemolytic assays performed with 4 combinations of ABO incompatibility, using different concentrations of serum and incubation times, as well as 2 temperatures (25 and 37°C).

We defined RBCs as the population of erythrocytes + reticulocytes. We used EDTA blood from group A or B healthy donors (n = 4). The blood was centrifuged for 10 min at 4°C and 500 *g* and the plasma removed. The cells underwent 4% dextran sedimentation for 30 min on ice in order to remove the white blood cells. The RBCs were then washed 3 times with cold 0.9% NaCl. They were counted and adjusted to the concentration of 7×10^{10} cells/ml.

The sera used as sources of antibodies and complement were from Rh-positive donors with blood group O. The sera were collected and used on the same day.

Four samples of RBCs from normal donors with blood groups A and B were exposed to various concentrations of different sera from blood group O donors in a simple haemolytic assay comparable to the standard CH50 assay. Three parameters were varied: the serum concentration, the time of incubation, and the temperature. The percentage reticulocytes was determined before and after haemolysis, and the percentage of cells remaining after haemolysis was measured. Briefly, 10^8 RBC were exposed to different concentrations of serum. NaCl was added as negative control. Total lysis was achieved by adding dis-

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Table 1. Relationship between percentage reticulocytes and percentage lysis at 37°C

	Lysis, %					
	0	24.5–26.3	55.2–57.1	67.4–71.4	85–89	91.9–96.1
Reticulocytes, %	0.8–1.3	1.0–1.6	1.5–2.5	2.1–3.5	2.4–4.5	2.7–6.5
Increase in reticulocytes, %	0	23.1–27.3	75–100	92.3–172.7	172.7–281.8	237.5–400

Haemolysis was measured after 45 min incubation at 37°C. The percentage of reticulocytes in healthy volunteers varies from 0.8 to 1.3. This percentage increased proportionally to the percentage lysis. When 91.9–96.1% of RBC were lysed, the percentage increase of reticulocytes ranged from 237.5–400, which is 3.375–5 times the starting percentage of reticulocytes ($p < 0.01$).

tilled water to the RBC. The RBC suspensions were incubated under constant agitation at either 37°C for 25–45 min or at 25°C for 65–125 min. The samples were then centrifuged for 10 min at 4°C and 500 *g* and the RBC lysis (haemoglobin) in the supernatant determined by spectrophotometer at 554 nm, using a Spectramax 190 photometer.

The reticulocyte counts were determined using the ADVIA 120T Hematology System (Bayer) in the 10^8 RBC population before exposure to different concentrations of serum and after lysis.

This index of resistance (iR) was calculated according to the following formula. It corresponds to the resistance of reticulocytes to lysis, with 1 being complete resistance (i.e. correlates inversely with the lysis of reticulocytes).

$$\delta = \alpha \, X \, \frac{100\%}{100\% - \gamma}$$

$$iR = \frac{\varepsilon}{\delta}$$

where γ = measured lysis (in %) of the RBC population, α = measured reticulocytes (in %) of the RBC before lysis, ε = measured reticulocytes (in %) of the RBC after lysis, and δ = theoretical maximal amount of reticulocytes (in %) of the RBC after lysis.

The percentage reticulocytes increased with increasing lysis of total RBCs for all 4 samples of RBCs tested (table 1, fig. 1a), and this was observed at the various time intervals during the incubation (not shown) and at both temperatures tested(table 2, fig. 1b). These data indicated clearly that reticulocytes were more resistant to lysis than erythrocytes. The resistance of reticulocytes was significantly higher at 25°C than at 37°C ($p < 0.023$).

To directly compare the resistance to lysis of reticulocytes versus erythrocytes, we calculated the iR of reticulocytes according to the formula above (fig. 2). The iR of reticulocytes illustrated the major difference between reticulocytes and erythrocytes: when 70% of the erythrocytes were lysed less than 20% of the reticulocytes were lysed. At the other end, when the lysis of erythrocytes was limited (up to 20%), there was very low lysis of reticulocytes (fig. 2a). These differences were even more evident when the assays were performed at 25°C (fig. 2b).

The reticulocytes were strikingly more resistant to complement-mediated lysis than were erythrocytes. This should come as no surprise considering the significantly higher surface concentration of the 3 complement regulators on reticulocytes, which are 1–3 days old, versus erythrocytes, which have a half-life of 60 days. Whereas it is known that reticulocytes lose some membrane proteins by the release of vesicles when transforming into erythrocytes, the loss of the 3 complement regulators continues thereafter, as evidenced by the measures comparing young versus old erythrocytes. Although the precise mechanisms of these losses are not yet fully understood, much evidence suggests that this is due to ongoing vesiculation (ectocytosis) at the surface of the RBC, possibly related to attacks by different agents [12–14]. In addition, all 3 regulators are lost similarly and are found on vesicles (ectosomes) released by in vitro aged erythrocytes [3]. Iida et al. [13] have elegantly shown that successful fixation of the C5–C9 complex does not lead invariably to lysis, but that this complex can be removed by active formation and release of ectosomes, which include the C5–C9 complex. In our assay it was not possible to define whether the resistance of reticulocytes was due to inhibition of C5–C9 deposition on the cells by the high concentration of complement regulators or to a higher capability of the cells to get rid of the C5–C9 by ectocytosis. The even higher resistance of reticulocytes at 25°C could have resulted from a reduced complement activation at 25°C (table 2, fig. 1b) than at 37°C

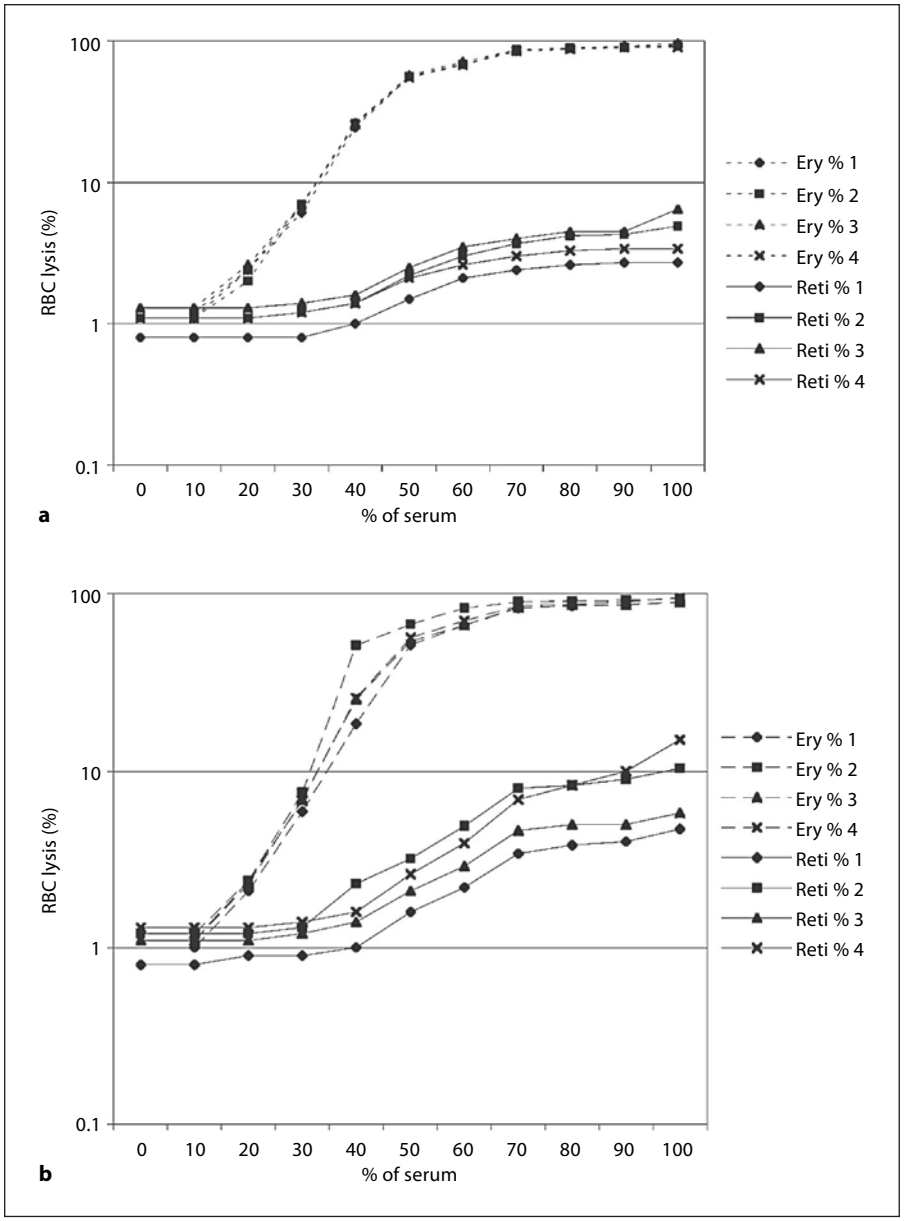

Fig. 1. Percentage of RBC lysis and percentage of reticulocytes after lysis. The figure represents percentage RBC lysis along with the percentage of serum. Each serum concentration corresponds the percentage of erythrocytes and of reticulocytes lysed. The RBC lysis was measured **a** after 45 min incubation at 37°C and **b** after 125 min incubation at 25°C. Reti, Ery 1 and 2 are from the A blood group, Reti, Ery 3, 4 are from the B blood group.

Table 2. Relationship between percentage reticulocytes and percentage lysis at 25°C

	Lysis, %					
	0	18.6–26.0	51.7–56.9	67.0–71.3	83.9–89.0	90.1–95.4
Reticulocytes, %	0.8–1.3	1.0–1.6	1.6–2.6	2.2–3.9	3.4–8.3	4.7–15.1
Increase in reticulocytes, %	0	23.1–27.3	90.9–118.2	163.9–200	318.2–538.5	427–1,061

Haemolysis was measured after 125 min incubation at 25°C. The percentage of reticulocytes increased proportionally to the percentage lysis. In the conditions where 90.1–95.4% of RBC were lysed, the percentage increase in reticulocytes ranged from 427–1,061 which represents 5.27–11.61 times the starting percentage of reticulocytes ($p < 0.0113$).

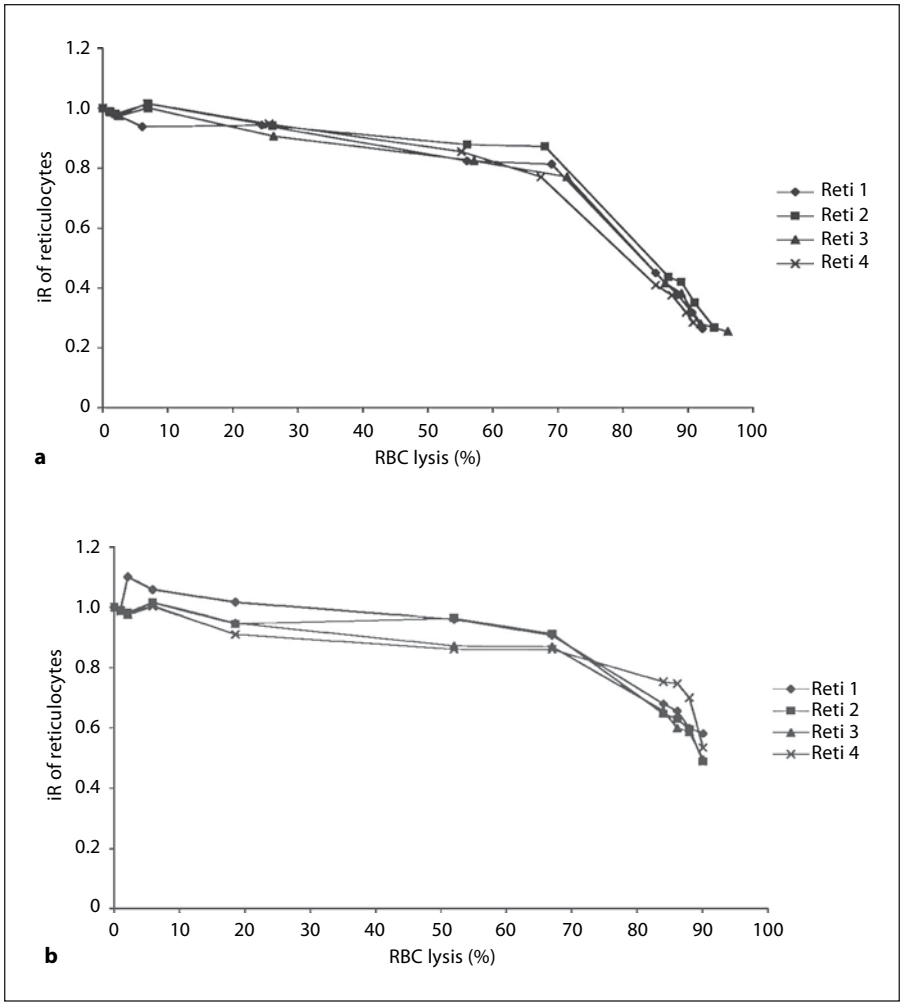

Fig. 2. iR of reticulocytes compared to the lysis of RBC. The iR is a measure of the resistance of reticulocytes for different amounts of RBC lysis, with 1 describing complete resistance of reticulocytes and lower values a partial resistance. **a** Temperature 37°C. In all conditions inducing <20% lysis of the total RBCs, the iR of reticulocytes was 1 (corresponding to 100% resistance, i.e. no changes in the absolute number of reticulocytes). In conditions inducing around 60% RBC lysis, the iR of reticulocytes was of 0.8 (corresponding to 80% resistance) only 20% of reticulocytes were lysed. When approximately 95% of RBC were lysed, the iR of reticulocytes was 0.3 (corresponding to 30% resistance) 70% of reticulocytes were lysed. **b** Temperature 25°C. The differences in the resistance to lysis were even more evident at 25°C. In all conditions inducing <20% lysis of the total RBCs, the iR reticulocytes was 1 (resistance of reticulocytes was 100%). In conditions inducing around 60% RBC lysis, iR of reticulocytes was of 0.85 (corresponding to 85% resistance) only 15% of reticulocytes were lysed. When approximately 95% of RBC were lysed, the iR of reticulocytes was of 0.5 (resistance of reticulocytes was of 50%) only 50% of reticulocytes were lysed. Reti 1 and 2 are from the A blood group, Reti 3 and 4 are from the B blood group.

(table 1, fig. 1a). Thus, the specific mechanism(s) responsible for the increased resistance of reticulocytes remains to be defined.

In conclusion, the intrinsic property of reticulocytes to resist lysis distinguishes them from the general population of erythrocytes, and may also explain why reticulocytes continue to be released in the blood stream at high rates in haemolytic anaemia without being harmed in the bone marrow before release. Finally, in haemolytic anaemia, the lysis most probably occurs under limiting conditions, resembling the experiments performed with low serum concentration or at 25°C. It is tempting to speculate that the 'reticulocytosis' observed under these circumstances (i.e. haemolytic anaemia) is not only due to enhanced production, which evidently occurs, but also to the intrinsic resistance of reticulocytes, which do not lyse under limiting conditions.

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