Myosin Content and Distribution in Human Neonatal Erythrocytes Are Different From Adult Erythrocytes

By Francine C. Colin and Stanley L. Schrier

Neonatal erythrocytes (N-RBC) are different from adult erythrocytes (A-RBC). N-RBC are larger, less deformable, and undergo enhanced spontaneous and drug-induced endocytosis. The reticulocyte population of N-RBC is also different, consisting primarily of the youngest (R1) reticulocytes that are motile and capable of receptor-mediated endocytosis. Processes such as motility could require a contractile system. Myosin, a contractile protein, was identified in both A-RBC and N-RBC. We proposed to compare myosin content and distribution in A-RBC and N-RBC by immunofluorescence and enzyme-linked immunosorbent assay (ELISA) using a monospecific polyclonal rabbit antimyosin. There was bright immunofluorescence on 44% of N-RBC with some heterogeneity contrasting with a barely detectable fluorescence on A-RBC. ELISA measurements showed that A-RBC had 4,315 myosin copies/RBC, whereas N-RBC had 10,855 copies/RBC (or 2.5 times as much). ELISA measurements of white ghosts showed that A-ghosts contained 1,250 copies of myosin/RBC (29% of total) whereas N-ghosts contained 3.4 times as much at 4,320 copies/RBC (39% of total). Therefore, N-RBC not only have more myosin, but the amount that is membrane-associated is disproportionately increased. It is proposed that such differences in amount and distribution of myosin could account for some of the unusual properties of neonatal RBC indicated.

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MATERIALS AND METHODS

Materials

Swine serum was purchased from Gibco Laboratories (Grand Island, NY). Fluorescein-isothiocyanate (FITC) swine antirabbit antibody, α cellulose and crystalline cellulose (Sigma-cellulose-50), alcian blue, alkaline phosphatase, and Sigma 104-105 phosphatase substrate were purchased from Sigma Chemical Co (St Louis, MO). Methanol and acetone were obtained from Merck, Em Science (Gibbstown, NJ).

Methods

Blood samples. N-RBC were obtained from placental vessels just after normal full-term vaginal delivery. A-RBC were obtained by venipuncture from healthy adult volunteers and was anticoagulated with heparin. All samples were drawn according to a protocol approved by the Stanford Medical Committee for the Protection of Human Subjects in Research. RBC were extensively washed and column-filtered through cellulose as previously described to remove platelets and white blood cells (WBC). Ghosts were prepared as described, using a ratio of 40 vol of 5 mmol/L phosphate lysis buffer pH 8.0 to 1 vol of RBC and performing three such washes.

Purification of platelet myosin and preparation of antimyosin antibody. Human platelet myosin was purified exactly as previously described. Polyclonal antibodies to this human platelet myosin were raised in rabbits using the human platelet myosin extract, and the antiserum was then affinity-purified against a column of the purified antigen. Myosin and antibodies were stored at 4°C. The antibody specificity was checked by immunoblotting. Western blotting on washed sonicated RBC and on ghosts was performed as described (Fig 1), except that 8% polyacrylamide gels were used, 5% bovine serum albumin (BSA) was used as a blocking agent, and no swine serum was used (Fig 1). The antibody used in this study is different than the one we used in our initial investigation and was diluted in BSA before use.

Immunofluorescence. A 1/1,000 dilution of washed RBC was fixed in 0.2% glutaraldehyde in phosphate-buffered saline (PBS) pH 7.4 for 60 minutes at 4°C and then washed once with 5 mmol/L.
NEONATAL RBC MYOSIN

Fig 1. Western blotting of washed sonicated RBC and of ghosts from neonatal and adult RBC was performed using our antiplatelet myosin antibody. Equal numbers of RBC and equal amounts of membrane protein were applied to the gels and run in parallel pairs. The RBC and ghosts were run at different times; therefore, the length of the gels are not comparable. To detect myosin in RBC, the gels have to be heavily overloaded and this probably account for the appearance of nonspecific bands in the midportion of the gels. The immunoreactive myosin band in the ghosts appears just below ankyrin as visualized in the amido black-stained nitrocellulose transfer papers (not shown), and thus has a molecular weight of 200 Kd, consistent with myosin heavy chain.

ELISA. The method of double-antibody ELISA was performed exactly as described. Platelets and WBC were removed by cellulose filtration. Then wells were coated with the antiplatelet antibody (10 μg protein/well, 4-hour incubation at 37°C). Standards of purified platelet myosin or sonicated N- and A-RBC and ghosts in incremental amounts were added for a 1-hour incubation at 37°C. The plates were then incubated with alkaline phosphatase-conjugated affinity-purified IgG antihuman platelet myosin for 2 hours at 37°C. The reaction was quantified with phosphatase substrate and the readings were performed at 405 nm. Between each step washes were performed in PBS-0.05% tween 20-0.1% BSA.

Because the high concentration of hemoglobin and other compounds in sonically lysed RBC could interfere with the detection of myosin by the antiplatelet myosin, myosin standard curves were also performed with the addition of the actual amounts of sonically disrupted adult and neonatal RBC used in the assays. In parallel plates, the myosin content was independently determined in A-RBC and N-RBC. The myosin curves in the presence of the lysate were still linear with a recovery of 64% using adult RBC and 70% using neonatal RBC.

RESULTS

To detect the presence and analyze the distribution of myosin in the population of N-RBC compared with A-RBC, immunofluorescence staining of these two types of RBC was performed on four sets of A- and N-RBC. The immunofluorescence results obtained on permeabilized N-RBC (Fig 2) showed that in this experiment about 44% of N-RBC showed a bright fluorescent rim. A-RBC (Fig 2) showed, at best, a marginal and dim fluorescence.

To be certain that nonspecific binding or variations in permeabilization did not account for these findings, these
A-RBC and N-RBC were, in parallel, incubated with preimmune rabbit serum and also with our affinity column-purified rabbit polyclonal antispectrin antibody. There was uniform permeabilization and minimal nonspecific staining (Fig 3).

To more precisely determine the amount of myosin that was membrane-associated compared with the whole RBC content in neonates and adults, ELISA was performed on ghosts and sonically disrupted RBC. Before any measurement, platelets and WBCs were removed. ELISA measurements performed on four sets of adult and neonatal RBC allowed us to determine that A-RBC contained 2,760 myosin copies per cell, 800 copies (29%) of which are bound to the membrane. N-RBC contain 7,600 myosin copies per cell, of which 2,960 (39%) are membrane-associated. If one corrects these actual values for the recoveries observed for myosin standards added to sonically lysed RBC, the values are shown in Fig 4 with 4,315 myosin copies per A-RBC, of which 1,250 are in A-ghosts, while N-RBC contained 10,855 copies per RBC, of which 4,230 are in N-ghosts. Therefore, there are 2.5 times more myosin in N-RBC than in A-RBC and 3.4 times more myosin in N-ghosts than in A-ghosts.

**DISCUSSION**

In 1985, Fowler et al\(^7\) and Wong et al\(^8\) identified and purified RBC myosin. Fowler et al\(^7\) found it both in cytoplasm\(^9\) and in the membrane, with about one third present in the membrane. It was possible that some of the differences between N-RBC and A-RBC noted previously could be caused in part by differences in myosin content and distribution. Matovcik et al\(^5\) previously showed that N-ghosts contained more myosin than A-ghosts, but the Western blotting methods used were not quantitative.

The extensive cross-reactivity between platelet and RBC myosin has been well documented.\(^10,11\) Therefore, we prepared a monospecific polyclonal antiplatelet myosin antibody against highly purified human platelet myosin, extracted as previously described.\(^14\)

Immunofluorescence showed that approximately half (44%) of N-RBC showed bright staining, while only a dim fluorescence could be seen in A-RBC (Fig 2). The glutaraldehyde fixation used can cause a degree of heterogeneity in immunoreactivity, as seen with the antispectrin immunofluorescence in Fig 3. However, we propose that this modest variation does not account for the differences seen in antimyosin reactivity in neonatal versus adult RBC in Fig 2. Neonatal blood contains only 3% to 5% reticulocytes, much less than 44% of N-RBC, which were quite positive for myosin by immunofluorescence. Therefore, the myosin increase seen in N-RBC is not the consequence of a small subpopulation of unusual RBC containing very large amounts of myosin.

Our ELISA data were in general agreement with that Fowler et al\(^7\), who identified 6,000 myosin copies per adult RBC, and with Wong et al\(^8\), who reported 2,400. We detected 4,315 copies per adult RBC and showed that there...
were 2.5 times more myosin in N-RBC (10,855 copies) than in A-RBC. A-ghosts contained 1,250 myosin copies on a per-cell basis or 29% of the total cellular myosin, while N-ghosts contained 4,230 copies on a per-cell basis or 39% of N-RBC myosin. Therefore, there are 3.4 times more membrane-associated myosin copies in N-RBC compared with A-RBC, partly because the membrane-associated fraction in N-RBC is disproportionately increased. Immuno-fluorescence is probably dependent on levels of myosin between 4,315 and 10,855 copies per RBC, explaining why immunofluorescence was not readily detectable in A-RBC.

The ELISA and immunofluorescence data are supported by myosin Western blotting of RBC and ghosts (Fig 1), showing increased immunoreactivity of myosin heavy chains when equal numbers of RBC and equal amounts of ghost protein are analyzed.

Myosin has been shown to be involved in cell motility and shape alteration through attachment to the plasma membrane in human intestinal epithelial cells or platelets. The increased amount of myosin in N-RBC and especially in the membrane-associated fraction could account for some of the mechanical differences noted previously, particularly if myosin binds to protein 4.1 in vivo as it does in vitro.

REFERENCES

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