Comparison of Transferrin Receptor-Mediated Endocytosis and Drug-Induced Endocytosis in Human Neonatal and Adult RBCs

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Neonatal RBCs can undergo receptor-mediated endocytosis; normal adult RBCs cannot. Previously, we showed that drug-induced endocytosis, which can occur in adult RBCs exposed to amphipathic cations like primaquine, is greatly enhanced in all density-defined fractions of neonatal RBCs. To investigate the similarities and differences between receptor-mediated endocytosis and drug-induced endocytosis, we characterized transferrin receptor-mediated endocytosis in neonatal RBCs and compared it with drug-induced endocytosis. Primaquine drug-induced endocytosis is dependent on RBC ATP levels, is invariably preceded by stomatocytosis, and is inhibited by vanadate. In contrast, receptor-mediated endocytosis of transferrin is not preceded by stomatocytosis, is not nearly so dependent on ATP levels as is drug-induced endocytosis, and is not inhibited by vanadate. Furthermore, receptor-mediated endocytosis is quantitatively blocked by preincubation of neonatal RBCs with sodium cyanide, whereas cyanide does not inhibit drug-induced endocytosis in either adult or neonatal RBCs. Morphologic observation of the neonatal RBCs established the fact that only puckered RBCs that exhibited brilliant cresyl blue staining reticulum were capable of undergoing receptor-mediated endocytosis of transferrin. These characteristics identify them as motile R-1 reticulocytes. Reticulocytes in normal adult RBCs were incapable of exhibiting this phenomenon. Thus, receptor-mediated endocytosis, a property of motile reticulocytes in neonatal RBCs, differs from drug-induced endocytosis in its energy requirements, response to inhibitors, and morphologic concomitants.

MATERIALS AND METHODS

Experimental Procedures

Materials. Iron-free human transferrin and Pronase were purchased from Calbiochem (San Diego). Na125I (10 μCi/mL), and 57Cyanocobalamin (10 μCi/mL) were purchased from Amersham (Arlington Heights, IL). 55FeCl3 (1 mCi/mL) was obtained from New England Nuclear (Boston), sodium vanadate from Fisher Scientific (Pittsburgh) and sodium cyanide from J.T. Baker Chemical (Phillipsburg, NJ). Primaquine, fluorescein isothiocyanate (FITC), antimycin A, and oligomycin were obtained from Sigma Chemical (St Louis). Enzyme beads were obtained from BioRad, (Richmond, CA). Silicon oil was obtained from Dow Corning (New Bedford, MA). All materials used were of the highest reagent grade available.

RBCs. Heparinized human cord blood was obtained from placental vessels immediately upon delivery. Freshly drawn heparinized venous blood was obtained from normal adult volunteers and patients with reticulocytosis. All blood samples were drawn according to a protocol approved by the Stanford Medical Committee for the protection of Human Subjects in Research. The blood was centrifuged, plasma and the buffy coat were removed, and the RBCs were washed three times with ice-cold Hanks' balanced salt solution (HBSS), pH 7.4. RBCs were kept on ice for no longer than two hours before use. Reticulocyte counts were performed on washed RBC samples stained with brilliant cresyl blue. The proportion of reticulocytes ranged from 3% to 5% in the cord blood (n = 3), <2% in adult blood.

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normal adult blood (n = 3), and >18% in selected patients with extreme reticulocytosis (n = 2).

Labeling of transferrin. Human transferrin (Tf) was saturated with iron and labeled when required with 59Fe (59Fe-Tf). The amount of iron bound by the Tf was estimated from the ratio of A465 nm: A280 nm (A is the absorption or optical density), which was 0.046 at full saturation.6 Diferric Tf in 0.15 mol/L NaCl/0.02 mol/L Tris-Cl, pH 7.4, was also radiolabeled with 125I (125I-Tf) using immobilized enzymebound lactoperoxidase-glucose oxidase (BioRad) according to the manufacturer’s instructions. After incubation, the sample was passed over a Sepharose column equilibrated with 0.15 mol/L NaCl/0.02 mol/L Tris-Cl, pH 7.4. The specific activity of 125I-Tf was 6 x 10^8 cpm/μmol transferrin and that of 59Fe-Tf was 4 x 10^8 cpm/μmol Tf. Diferric transferrin was also fluorescently labeled with FITC using established procedures48 in which diferric Tf in 100 mmol/L sodium borate solution, pH 9, was conjugated with FITC (FITC-Tf) dissolved in 3 mmol/L borate solution at room temperature for three hours. Unbound FITC was removed by dialysis against phosphate-buffered saline (PBS). The ratio of fluorochrome to protein (A495 nm: A280 nm) was 8:1. Albumin was also fluorescently labeled with FITC as described above.

Measurement of Endocytosis

Drug-Induced Endocytosis

Morphologic observations. Neonatal RBCs were incubated with 3 mmol/L primaquine in HBSS, pH 7.4, at 37°C for 15 to 60 minutes as previously described.13 Drug-treated RBCs were washed three times with 0.15 mol/L NaCl, 20 mmol/L sodium phosphate, pH 7.4 (PBS) postincubation, and 20 μL was fixed in 200 μL 2% glutaraldehyde in PBS, pH 7.4. Drug-induced shape change, endocytosis, and vacuole formation were evaluated and photographed with phase contrast and Nomarski microscopy. In some experiments, FITC-labeled albumin was used as a fluid-phase marker that would be engulfed during drug-induced vacuole formation, thus allowing us to track the now fluorescent vacuoles by fluorescence microscopy.

Semiquantitative binding assay. The exoface of neonatal RBCs was labeled with 59Co-Vitamin B12 binding protein.11 The RBCs were then incubated with primaquine as above. 59Co-Vitamin B12 not trapped in endocytic vacuoles was removed from the RBC surface by trypsin digestion and subsequent washings.13 The amount of 59Co remaining after trypsinization and washings reflects the degree of endocytic vacuole formation. The extent of endocytosis produced by primaquine was always confirmed by phase-contrast microscopy.

Receptor-Mediated Endocytosis

Morphologic observations. Endocytosis in neonatal RBCs was induced by incubating 2 to 4 x 10^8 RBCs in 1 to 2 mL HBSS containing 0.2 to 0.5 mmol/L FITC-Tf at 37°C for 30 minutes. The RBCs were then washed three times with HBSS, pelleting the RBCs by centrifugation at 2,000 g for three minutes each time. RBCs were then fixed in 1% to 2% glutaraldehyde in PBS, and their shape and appearance were observed both by phase-contrast and fluorescence microscopy.

Binding assay. RBC suspensions containing 2 to 4 x 10^8 RBCs in a volume of 1 to 3 mL HBSS were incubated either with 59Fe-Tf or 125I-Tf at a concentration of 0.095 to 2 mmol/L. After the desired incubation periods at either 4º or 37°C, duplicate 200-μL aliquots of cell suspension were removed and layered on 0.3 mL Dow silicone oil cushion in 1-mL Eppendorf microcentrifuge tubes. The cells were aspirated from the incubation medium by centrifugation at 11,000 x g for 30 seconds. The supernatant and the oil were carefully aspirated, and the total cell-associated radioactivity was determined by y-well counting. The extent of internalization of ligand was determined by incubating the cells with the radiolaabeled ligand as described above, after which the RBCs were washed three times with ice-cold PBS and then incubated with 0.2% Pronase in HBSS for 30 minutes at 4°C. The RBCs were separated from the incubation medium by centrifuging duplicate 200-μL aliquots through a cushion of silicone oil. The level of radioactivity in the pellet was measured by y-well counting.

Nonspecific binding and uptake were determined with a 200- to 200-fold excess of unlabeled ligand and ranged from 20% to 58% of the total binding. All specific binding data were corrected accordingly. The cellular uptake of 125I-Tf or 59Fe-Tf was calculated from the observed counts, measured specific activity of the radiolaabeled ligands, and the number of RBCs in the sample.

Metabolic Studies

ATP depletion. Neonatal RBCs were metabolically depleted by incubating them in PBS, pH 7.4 (hematocrit 35% to 40%) with gentle agitation, in horizontally aligned tubes for 24 hours at 37°C, in presence of 200 U/mL penicillin and streptomycin, respectively. ATP was measured by the coupled hexokinase G6PD assay,1 at the beginning and at the end of the depletion procedure, at which point ATP levels were routinely <0.1 μmol/mL packed RBCs. 125I-Tf binding and uptake were measured in ATP-depleted neonatal RBCs by incubating them with 125I-Tf in PBS for 20 minutes at 37°C. RBCs from the same placental blood samples, maintained in HBSS at 4°C overnight, were used as the reference point and always had an ATP content of >0.6 μmol/mL of packed RBCs.

Effect of Metabolic Inhibitors. Drug-induced endocytosis was measured in the presence of sodium cyanide and sodium vanadate. 125I-Tf binding and receptor-mediated endocytosis were measured in the presence of sodium fluoride, sodium cyanide, sodium azide, sodium vanadate, and the antibiotics antimycin A and oligomycin. Sodium fluoride, sodium cyanide, sodium azide, and sodium vanadate were dissolved in HBSS, and the pH was adjusted to 7.4. Antimycin and oligomycin were dissolved in dimethylsulfoxide (DMSO). Neonatal RBCs (hematocrit 15% to 20%) were preincubated with these drugs in HBSS for 60 minutes at 37°C. Drug-induced endocytosis and receptor-mediated endocytosis of Tf were measured as described above. Control samples for the studies using antibiotic inhibitors contained an equivalent amount of DMSO. The effect of these agents on RBC shape change before and after incubation was monitored by phase-contrast microscopy. None of the drugs induced any shape change in neonatal RBCs at the concentrations used.

RESULTS

Morphologic Observation of Drug-Induced Endocytosis and Receptor-Mediated Endocytosis in Neonatal RBCs

To compare and characterize the two forms of membrane internalization in neonatal RBCs, these processes were observed morphologically during endocytic vacuole formation. Primaquine was used to produce drug-induced endocytosis in neonatal RBCs with the fluid phase-marker FITC-albumin added. Receptor-mediated endocytosis of Tf was induced by incubating neonatal RBCs with FITC-Tf. After these exposures, neonatal RBCs were studied by Nomarski and fluorescence microscopy, specifically evaluating shape change and the extent of endocytic vacuole formation. Primaquine caused the expected discocyte-stomatocyte transformation and produced large endocytic vacuoles as previously reported.14 More than 95% of the primaquine-treated
neonatal RBCs exhibited fluorescent vacuoles since FITC-albumin was trapped during the process of drug-induced endocytosis. Drug-induced vacuoles formed at or around the lip of the stomatocytic cup. These vacuoles did not exhibit Brownian motion, and when ghosts were prepared by hypotonic lysis these vacuoles sedimented with the plasma membranes. Furthermore, these drug-induced fluorescent vacuoles could not be dislodged from the RBC membrane by sonication and recentrifugation (not shown). In contrast, neonatal RBCs showing FITC-TF endocytosis were discocytes, and only 3% to 5% exhibited fluorescent vacuoles (endosomes) (Fig 1A and B). Occasionally, more than three endosomes per RBC were observed. The endosomes moved freely within the RBC cytosol, exhibited Brownian motion, and could be isolated by disrupting the RBC. With differential gradient centrifugation, a homogeneous fraction of the endosomes could be obtained (manuscript in preparation). That only 3% to 5% of the neonatal RBC population exhibited endosome formation raised the possibility that the cells undergoing receptor-mediated endocytosis were reticulocytes. We investigated this possibility as follows: FITC-Tf was used to induce endosome formation in the neonatal RBCs. As expected, ~3% to 5% neonatal RBCs exhibited brilliant fluorescent endosomes under ultraviolet light. The supravital stain, brilliant cresyl blue, was then added directly to the slides and, under white light, the cells containing the fluorescent endosomes invariably exhibited blue staining reticulum. These RBCs also had the puckered discocyte shape described for motile R, reticulocytes in the literature.16 Conversely, when a puckered reticulocyte was identified under white light, it contained the fluorescent endosome when observed by fluorescent microscopy. For comparison, normal adult RBCs and RBCs from adult patients with reticulocytosis were studied as described above. In normal adults (n = 3), <2% of the RBCs were reticulocytes, but none of these cells exhibited any fluorescent vacuoles; the same was true for patients with mild reticulocytosis of 3% to 7%. In contrast, patients with extensive reticulocytosis of 18% to 20% (n = 2), 8% to 10% of these RBCs exhibited both reticulum staining and fluorescent endosomes. This finding indicated that in the case of extreme reticulocytosis about one-half of the adult patients' reticulocytes engaged in receptor-mediated endocytosis of Tf.

Transferrin Binding and Uptake in Neonatal RBCs

$^{125}$I-TF binding to the neonatal RBCs began almost instantaneously, both at 0 and 37°C. At 0°C, the total Tp uptake reached a stable maximum after ~5 minutes (Fig 2). In contrast, at 37°C, total Tp binding and uptake was rapid during the first 10 minutes of incubation and then increased slowly over the next 15 minutes (Fig 2). The amount of Tp internalized at 37°C was determined by treating the RBCs with pronase to remove Tp from the external RBC surface. After 30-minute incubation at 37°C, 61% of the total cell-bound Tp in neonatal RBCs was pronase resistant as a result of being internalized by the endocytic process. Adult RBCs did not exhibit any difference in Tp uptake when incubated at 0 and 37°C (Fig 2). After pronase treatment, no radioactivity was associated with the adult RBCs, indicating that $^{125}$I-Tp had not been internalized (data not shown).

We examined the effect of varying the concentration of Tp on its binding and uptake by the neonatal RBCs. Neonatal RBCs were incubated with either $^{59}$Fe-TF or $^{125}$I-Tp in HBSS at concentrations of 0.1 to 10 μmol/L for 20 minutes at 37°C and for 90 minutes at 0°C. The $^{125}$I-Tp binding isotherm at 0°C, corrected for nonspecific binding and plotted as a Scatchard analysis, yields an apparent $k_d$ of ~2.24 x 10$^{-9}$ mmol/L and a receptor number of ~1.01 x 10$^5$/neonatal reticulocyte (data not shown). At 37°C, total Tp binding increased rapidly up to a concentration of ~2 μmol/L (Fig 3A) and then continued to increase slowly as the concentration of Tp was further raised. The rate of Tp uptake was 2.2 x 10$^5$ molecules of Tp/neonatal reticulocyte/min. The rate of iron accumulation was linear with time, when the cells were

![Fig 1.](image-url)
incubated with 5.0 μmol/L ⁵⁹Fe-TF at 37°C for 25 minutes (Fig 3B). Unlike neonatal RBCs, adult RBCs incubated with ¹²⁵I-TF under similar conditions exhibited neither endocytosis nor Tf uptake (Fig 2).

**Effect of ATP Depletion on Transferrin Binding and Uptake in Neonatal RBCs**

The effect of ATP depletion on Tf binding and uptake in the neonatal RBCs was examined (Fig 4). Control neonatal RBCs exhibited an initial rapid binding and uptake of Tf during the first 2 minutes of incubation at 37°C, followed by a slower uptake for the next 6 minutes and then a plateau as full saturation of Tf receptors was achieved. In ATP-depleted neonatal RBCs (Fig 4), Tf uptake was slower after the initial binding as compared with the control. Tf binding and uptake never reached control levels in ATP-depleted RBCs even when the incubation was prolonged.

**Effect of Metabolic Inhibitors on Drug-Induced Endocytosis and Receptor-Mediated Endocytosis in Neonatal RBCs**

The effect of sodium fluoride (6 mmol/L) on Tf binding and uptake in neonatal RBCs was similar to the effect of ATP depletion on this process; ie, there was an initial lag in Tf binding and uptake in fluoride-treated cells (data not shown). After 20-minute incubation, the amount of Tf taken up by the fluoride-treated cells was 30% less than that in the control RBCs at the steady state (data not shown).

The effect of sodium cyanide, a potent inhibitor of oxidative phosphorylation, on drug-induced endocytosis and Tf binding and internalization in neonatal RBCs was then examined. Cyanide did not inhibit drug-induced endocytosis in either adult or neonatal RBCs. The amount of ⁵⁷Co-Vitamin B₁₂ internalized was similar in neonatal RBCs with or without the addition of cyanide (Table 1). Similarly, cyanide did not block the discocyte-stomatocyte transformation associated with primaquine drug-induced endocytosis (data not shown). In contrast, sodium cyanide caused a marked inhibition of Tf binding and uptake in neonatal RBCs (Fig 4). The early phase of binding of Tf to cyanide-treated cells was markedly reduced (~50% of the controls), and this inhibition persisted even after 20-minute incubation. In contrast, the control cells exhibited saturation kinetics of Tf uptake (Fig 4). The inhibitory effect of cyanide was confirmed morphologically by incubating cyanide-treated cells with FITC-Tf and observing them by fluorescence microscopy. Cyanide-treated cells exhibited fluorescent rims as a result of FITC-Tf binding to its receptors on the RBC surface. The degree of rim fluorescence observed in cyanide-treated cells was approximately one-half of that which occurred in the controls (data not shown), suggesting that cyanide treatment had diminished the number of Tf receptors on the cell surface. Fluorescent-internalized endosomes were not detected in cyanide-treated RBCs but were clearly visible in control cells.

Unlike cyanide, the other mitochondrial inhibitors (antimycin A, oligomycin, and sodium azide) caused only a slight but reproducible reduction in Tf uptake by the neonatal RBCs at the concentrations used (Table 2). Sodium vanadate, a known inhibitor of phosphohydro-lases and ATPases inhibits drug-induced endocytosis and the associated spherostomatocytic shape change in adult RBCs. We examined the effect of vanadate on drug-induced endocytosis and Tf binding and uptake in neonatal RBCs (data not shown). As in adult RBCs, vanadate effectively blocked the discocyte-stomatocyte-transforming effect of primaquine in neonatal RBCs. In parallel, the amount of drug-induced endocytosis was reduced 76% (Table 1). These observations confirmed the previous observations of the role of vanadate in blocking drug-induced endocytosis in RBCs. In contrast, vanadate did not inhibit Tf binding or uptake in RBCs (Fig 5) even when the vanadate concentration was increased 25-fold over that necessary to block drug-induced endocytosis.
Fig 3. (A) Concentration dependence of $^{125}$I-transferrin and Fe-Tf uptake and endocytosis by neonatal reticulocytes. RBCs were incubated for 20 minutes at 37°C with $^{125}$I-Tf or $^{59}$Fe-Tf at the concentrations shown. RBC-associated radioactivity was determined as described in the Materials and Methods section. Experimental points are the mean of two separate experiments. (B) $^{59}$Fe accumulation from Fe-Tf by neonatal reticulocytes as a function of time. Amount of $^{59}$Fe accumulated by the neonatal reticulocytes at each time point was determined as described in the Materials and Methods section ($n = 5$).

DISCUSSION

Receptor-mediated endocytosis has been proposed to occur in neonatal RBCs and not in adult RBCs. We therefore investigated the binding and uptake of the physiologic ligand transferrin and compared it with drug-induced endocytosis in neonatal RBCs in an attempt to identify similarities and differences in the underlying mechanisms of these two processes. As expected, primaquine-induced endocytosis involved an antecedent stomatocytic shape change in neonatal RBCs similar to that reported in adult RBCs. Drug-induced vacuoles occurred in 95% of the RBCs and formed at the lip of the stoma and inside the stomatocytic cup of neonatal RBCs. In contrast, receptor-mediated endocytosis of transferrin did not involve a stomatocytic shape change in neonatal RBCs that appeared to be discocytes (Fig 1A B). Furthermore, fluorescence microscopy showed that most of the drug-induced vacuoles seemed to be attached to the membrane, whereas the transferrin endosomes were free in the cytosol. Therefore, these two processes differ morphologically in at least two ways: In contrast to drug-induced endocytosis, receptor-mediated endocytosis requires no antecedent shape change; transferrin receptor-mediated endocytic vacuoles are free in the cytosol, whereas drug-induced vacuoles are membrane bound.

Further morphologic studies showed that the neonatal RBCs containing the endosomes were invariably reticulocytes. Reticulocytes that were puckered exhibited more brilliant cresyl blue staining of RNA and are described in literature as motile R-1 reticulocytes engaged in receptor-mediated endocytosis. These are the earliest reticulocytes that have just lost their nucleus; they retain their motility,
however, and because they are actively synthesizing hemoglobin they have a requirement for iron.

Reticulocytes in normal adult RBCs were incapable of undergoing receptor-mediated endocytosis of TF. The reticulocytes of patients with a mild degree of reticulocytosis (3% to 7%) were similarly incapable of undergoing receptor-mediated endocytosis. However, ~50% of reticulocytes in patients with extreme reticulocytosis were able to undergo receptor-mediated endocytosis of TF. These RBCs were similar in their puckered morphology to the neonatal RBCs that exhibited TF endocytosis. Therefore, probably only R-1 reticulocytes are capable of receptor-mediated endocytosis of TF.

As in other cell systems, receptor-mediated endocytosis of TF in neonatal RBCs is a temperature-dependent phenomenon requiring metabolic energy. Neonate RBCs exhibited a twofold increase in TF binding and uptake at 37°C over that observed at 0°C (Fig 2). This probably is owing to the rapid recycling of TF receptors which is optimal in the physiologic temperature range. In contrast, after the initial binding of TF to its receptor at 0°C, there is no further uptake because endocytosis is an energy-requiring process and because receptor cycling is inhibited at the low temperature. Therefore, the amount of TF bound to neonatal RBCs at 0°C is a reflection of the number of receptors that are then present on the cell surface. The recycling of transferrin receptor complex was morphologically observed by incubating the neonatal RBCs with internalized FITC-TF at 37°C. The incubated RBCs were repeatedly washed, and within 30 to 40 minutes virtually all fluorescence was lost as the transferrin–transferrin receptor complex was presumably recycled back to the cell surface (data not shown).

We then characterized and compared the metabolic requirements of receptor-mediated and drug-induced endocytosis. Primaquine drug-induced endocytosis in adult and neonate RBCs is absolutely dependent on ATP levels. Similarly, uptake of TF-bound iron, used in hemoglobin synthesis is dependent on a supply of metabolic energy. Endocytosis and receptor turnover are also dependent on ATP stores and functional metabolic pathways of the cells under study. As expected, ATP-replete RBCs exhibited an initial rapid binding and uptake of TF, which plateaued at the steady state. However, in both ATP-

Table 1. Effect of Sodium Cyanide (10 mmol/L) and Sodium Vanadate (100 μmol/L) on Drug-Induced Endocytosis in Neonatal RBCs

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total 125I-TF Uptake (Mean±SD)</th>
<th>Packed RBCs (cpm/mL, mean of two experiments)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>Control NaCN Vanadate</td>
</tr>
<tr>
<td>Primaquine endocytosis</td>
<td>4,580</td>
<td>4,630</td>
</tr>
<tr>
<td>Sodium azide (6 mmol/L)</td>
<td>85</td>
<td></td>
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</tbody>
</table>

The Neonatal RBCs were preincubated with cyanide and vanadate in HBSS for 60 minutes at 37°C before 3 mmol/L primaquine was added. Drug-induced endocytosis was measured as described in the Materials and Methods section.

![Fig 4. Effect of cyanide addition and ATP depletion on total binding and uptake of 125I-TF by neonatal reticulocytes. RBC-associated radioactivity was measured as described in the Materials and Methods section (n=3).](image-url)
depleted (Fig 4) and fluoride-treated neonatal RBCs (data not shown), initial binding was reduced and uptake of T₉ was slower and continued to be so as steady state was reached. The fraction of transferrin internalized in ATP-depleted neonatal RBCs at the steady state was one-half of that in the control. This was the fraction resistant to pronase treatment (data not shown). ATP-depleted neonatal RBCs probably have a diminished number of surface receptors initially, because the transferrin receptors are trapped inside neonatal RBCs as a consequence of a slower rate of recycling owing to a lack of metabolic energy. This results in a decrease in transferrin uptake.

Thus, although drug-induced endocytosis and receptor-mediated endocytosis both require ATP, there are profound quantitative differences. Treatment with fluoride or ATP depletion to 0.1 μmol/mL reduced drug-induced endocytosis by ~90%. However, this level of residual ATP in neonatal RBCs was sufficient for receptor recycling, although at a slower rate. Another and not mutually exclusive interpretation is that energy production in neonatal RBCs undergoing receptor-mediated endocytosis can also occur through ongoing oxidative phosphorylation in the mitochondria persistent in the R-1 reticulocytes. Indeed, sodium cyanide, a potent inhibitor of oxidative phosphorylation, inhibited receptor-mediated endocytosis in neonatal RBCs. In cyanide-treated RBCs, the initial binding of T₉ was ~50% less than that of the controls (Fig 4), possibly as a result of a diminished number of surface receptors. However, T₉ uptake by the process of receptor-mediated endocytosis was completely inhibited. Cyanide could exert this effect by reducing the ATP content of the neonatal RBCs by inhibiting both oxidative phosphorylation and glycolysis. Cyanide certainly inhibits electron transport and blocks ATP synthesis by the oxidative phosphorylation route. If as a result of this inhibition NAD⁺ availability, the critical step in glycolysis involving the conversion of glyceraldehyde-3-phosphate to 1,3 diphosphoglycerate would also be blocked.

We previously showed that vanadate effectively inhibits drug-induced endocytosis and the associated stomatocytic shape change and speculated that vanadate was inhibiting some ATP-requiring phosphohydrolytic step. Vanadate completely blocked the discocyte-stomatocyte transformation and drug-induced endocytosis in primaquine-treated neonatal RBCs (Table 1). However, vanadate did not inhibit T₉ receptor-mediated endocytosis of neonatal RBCs (Fig 5). Therefore, if ATP is required in receptor-mediated endocytosis, it is not used by an ATPase or by a phosphohydrolytic reaction inhibitable by vanadate.

Thus, normal neonatal RBCs are capable of two forms of endocytosis, drug-induced endocytosis and receptor-mediated endocytosis, whereas normal adult RBCs can only exhibit drug-induced endocytosis. The two forms of endocytosis, superficially similar, are actually very different. Primaquine drug-induced endocytosis observed in >95% of neonatal RBCs (and ~50% to 75% of adult RBCs) is preceded by an obligatory stomatocytic shape change. The vacuoles formed do not exhibit Brownian motion and are attached to the membrane. ATP is required, and endocytosis is blocked by vanadate but not by sodium cyanide. In contrast, receptor-mediated endocytosis of T₉, which occurs only in R-1 reticulocytes in normal neonatal RBC populations, is not associated with an antecedent shape change. The vacuoles that are formed exhibit Brownian motion, are freely movable in the cytosol, and can be easily separated from the RBC membrane. ATP depletion to ~0.1 μmol ATP/mL RBCs and sodium fluoride treatment produce modest inhibition, but sodium cyanide is a powerful inhibitor. In contrast, sodium vanadate has no effect on receptor-mediated endocytosis.

Several conclusions can be derived. Two seemingly similar endocytic processes are actually very different. Therefore, studies on RBC endocytosis must clearly delineate which process is being studied. Which, if either, of these processes causes accumulation of spontaneous endocytic vacuoles in neonatal RBCs that leads to formation of the "pocked" neonatal RBCs is still not clear. Furthermore, receptor-mediated endocytosis is not a unique property of neonatal RBCs. Rather it is property of the R-1 reticulocytes which, from this study, appear to be most of the reticulocytes in cord blood. In adults, R-1 reticulocytes are uncommon and can only be demonstrated under unusual circumstances in which the reticulocyte count reaches levels of ~20%. Why the kind of reticulocytes in cord blood is different from that in adult blood is a subject for further study.

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