Reticulocytes I. Isolation and In Vitro Maturation of Synchronized Populations

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Studies of reticulocyte maturation have been limited by the inability to obtain pure populations of age-synchronized reticulocytes and by the absence of well-defined methods for the maturation of reticulocytes in vitro. Many of these problems were overcome using temporary suppression of erythropoiesis with thiamphenicol and phlebotomy resulting in a highly reproducible reticulocyte response. Percoll density gradient separation of cells yielding essentially pure populations of age-synchronized reticulocytes, and liquid culture techniques where cell lysis is minimal. The system allows reproducible study of well-defined cohorts of reticulocytes as they mature into erythrocytes. During in vitro maturation we serially monitored changes in reticulocyte count, glucose consumption, $^{125\text{I}}$-transferrin binding, fluorescein (FITC)-labeled transferrin binding, the activities of four erythrocyte enzymes (glucose-6-phosphate dehydrogenase, pyruvate kinase, phosphofructokinase, and lactate dehydrogenase) and the appearance of cells on scanning electron microscopy. These variables changed at different rates suggesting that multiple mechanisms underlie these maturational events. Transferrin binding and reticulocyte count decreased most rapidly and reached values near zero after three to four days in culture. The four enzyme activities decreased much more slowly, and only two reached pretreatment values after seven days in culture. In contrast to the findings of others, scanning electron microscopy suggested that cells do not assume the normal biconcave shape in this system. The methods described make it feasible to study the process of reticulocyte maturation in vitro. The data presented represent a first step in the study of the coordination and interrelationships of various maturational processes.

METHODS

Thiamphenicol treatment and bleeding. Male and female Long-Evans outbred rats, 6 to 8 months of age, were used for all experiments. Unless specified, all reagents were obtained from Sigma Chemical Company (St Louis). Thiamphenicol (1.1 g/100 g bleeding on erythroid precursor cells in hematopoietic tissues of mice has been studied extensively.$^{10,11}$ Using rats we have characterized the response in peripheral blood rather than in hematopoietic tissues. While the resultant reticulocytes are clearly "stress" reticulocytes and may not be entirely normal, the ability to define how long these cells have been in the circulation allows accurate definition of their properties. Reticulocytes were isolated in pure populations by a one-step discontinuous Percoll gradient and incubated in liquid culture medium for up to seven days. Cell lysis was low, indicating that the population of cells studied over time did not change due to loss of subpopulations of cells.

As a first step in applying these methods to the study of reticulocyte maturation, we measured the loss of new methylene blue staining, transferrin binding, glucose consumption, the activities of four "age-related" enzymes and the appearance of the reticulocytes on scanning electron microscopy as they matured into red cells.

METHODS

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body weight) was placed in sterile dialysis bags. About 5 mL of sterile water was added, forming a suspension. The bags were implanted subcutaneously into the neck of each rat. After four days the bags were removed. They always contained some undissolved drug at this time. On days 3, 5, and 7, 3 mL of blood was removed from each male rat and 2 mL from each female rat by cardiac puncture under ether anesthesia. On other days, 20 to 50 µL of blood were taken by clipping the end of the tail. The data presented are for reticulocytes during in vitro maturation from 11 rats. Baseline data were obtained 1 week before thiamphenicol treatment.

Reticulocyte harvest. All procedures used sterile solutions and were carried out in a tissue culture hood. On day 11, 136 hours after the dialysis bags were removed, animals were killed and blood was drawn from ether anesthetized rats. Sterile isotonic Percoll solutions of densities 1.096 and 1.058 g/mL were made by diluting Percoll to a final concentration of 10 mmol/L triethanolamine, 117 mmol/L NaCl, 5 mmol/L glucose, and 1.5 mg/mL bovine serum albumin (BSA). These solutions were between 295 and 310 mOsm. Five milliliters of Percoll solution (density, 1.096 g/mL) was added to sterile, 15 mL conical centrifuge tubes. Two milliliters of Percoll solution (density, 1.058 g/mL) was layered over this. Two to 4 mL of whole blood were layered on top of each tube. Tubes were centrifuged at 250 g for 30 minutes in an IEC refrigerated centrifuge with swing-out tube holders (IEC, Boston).

After centrifugation, reticulocytes and some white cells were at the interface of the two Percoll layers. Plasma and white cells at the top were aspirated and the reticulocyte layer transferred to 50 mL syringe barrels to a settled volume of at least 3 mL and equilibrated with PBS. Reticulocytes were added and washed through with at least 40 mL of PBS.

In vitro maturation of reticulocytes. Cell suspensions were washed once in PBS and once in Alpha-minimum essential medium (MEM) augmented with 25 mmol/L HEPES, 20 mg/dL glucose, 5% newborn calf serum (Whittaker MA Bioproducts, Walkersville, MD), 100 U/mL penicillin, and 0.1 mg/mL streptomycin, pH 7.5. Cells were counted in a Coulter Model Fx cell counter (Coulter Electronics, Inc, Hialeah, FL) and suspended at 2 x 10^7 cells/mL in 175 cm² culture flasks. An aliquot of cells was used for zero-time determinations. Flasks were placed in a 37°C incubator and capped because adequate pH control could not be achieved in the presence of 5% CO₂. The pH was kept between 7.3 and 7.5 by periodic additions of 1 N NaOH. A standard curve of pH change per microliter NaOH per milliliter medium was constructed and used for pH adjustments. At no time did pH fall outside the 7.3 to 7.5 range. Samples were taken at 24-hour intervals for four days when the remaining incubation medium was centrifuged, the supernatant aspirated and replaced with fresh Alpha-MEM.

Determination of cell lysis. Cell lysis was estimated by measuring the percentage of total lactate dehydrogenase activity (LDH) released into the medium during in vitro incubation of cells. Consumption of glucose. Glucose consumption was determined by measuring the decrease in glucose in the medium from zero-time through four days at 24-hour intervals. Samples of medium were added to ice-cold 0.6 N perchloric acid and frozen at −70°C for later assay of glucose concentration.

Preparation of cell aliquots for assay. At 24-hour intervals for four days and then after seven days, samples of cells were removed from the tissue culture flasks. The pH was immediately measured. Cells were centrifuged at 1,625 g for ten minutes and resuspended in fresh serum-free medium for 30 minutes at 37°C to remove bound
transferrin. After a second centrifugation, cells were suspended at approximately $1 \times 10^8$ cells/mL in PBS and the cell count determined. These cell suspensions were used for all subsequent determinations.

**Reticulocyte count and white cell contamination.** One milliliter of cell suspension was centrifuged, the supernatant aspirated, and an equal volume of normal rat plasma was added. At zero-time, Wright’s stained smears were used to determine white cell contamination. Samples containing white cells were discarded. An equal volume of new methylene blue stain was added to the cells, mixed, incubated for 20 minutes, and smeared for reticulocyte counts. At least 500 cells were counted.

**Transferrin binding.** Binding of $^{125}$I-labeled human diferric transferrin (Tf) was measured according to Ward and Kaplan. A 0.1 mL aliquot of cell suspension was added to a volume of 0.9 mL containing $5.9 \times 10^{-4}$ mol/L $^{125}$I-labeled Tf, 2 mg/mL BSA in serum-free alpha-MEM. Background assays contained $1.25 \times 10^{-3}$ mol/L cold transferrin. Cells were incubated for one hour at 37°C, placed on ice, washed three times with ice-cold PBS, lysed with 1 mL 1% SDS, and counted in a Beckman gamma counter. Assays were performed in duplicate. Scatchard plot analysis indicated that 91% of the receptors were occupied at the concentration of Tf used. Results were expressed as molecules Tf bound per cell.

Fluorescin isothiocyanate (FITC)-labeled diferric rat Tf (Pel-freeze Biologicals, Rogers, AR) replaced $^{125}$I-labeled human Tf in the same assay system. After three washes with PBS, cells suspended in PBS were immediately analyzed in a Coulter EPICS C Flow Cytometer. Ten thousand cells were counted and plotted as number of cells vs log green fluorescence. Cells with fluorescence greater than that of channel 17 were analyzed. A measure of the mean fluorescence of a population of cells, relative fluorescence units (RFU) were calculated using the formula:

$$RFU = 2 - \frac{(255-P)^2}{8}$$

P is the peak channel of each fluorescence distribution.

**Scanning electron microscopy.** One milliliter of $1 \times 10^7$ cells/mL in PBS was applied to a glass cover slip (22 x 22 mm). After five minutes, cover slips were gently placed in Karnovsky’s fixative for five minutes, washed twice for 30 seconds in 0.1 mol/L sodium phosphate, pH 7.4, dried in a series of 35%, 50%, 70%, 90%, 95%,

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![Fig 2](https://via.placeholder.com/150)

(A) Percentage of cells with reticulocyte staining during incubation. After isolation of reticulocytes, new methylene blue stained cells represented 96% of the population. After 24 hours in culture the percentage decreased rapidly, reaching baseline values after three to four days. A log-linear regression of reticulocyte count with time points at 1, 2, and 3 days indicated that the half-life for these reticulocytes was 12 hours ($r = 0.99$). (B) Cell lysis during in vitro maturation. The greatest lysis of cells occurred in the first 24 hours when mean lysis was 6.8% ± 13.9%. After 24 hours, cell lysis showed a very small, steady increase in the next three days. The large error bars are the result of considerable variability in the lysis seen at 24 hours, which is carried over to other time points. For seven of the 11 samples, lysis was <2% at 24 hours. (C) Reticulocyte binding of $^{125}$I-labeled human Tf. Initially, an average of $133 \times 10^3$ Tf molecules bound per cell. This fall rapidly in culture. A log-linear regression of Tf binding over time indicated a half-life of 16.5 hours ($r = 0.96$) for the Tf receptor in this system. (D) Reticulocyte binding of FITC-labeled rat Tf over time for samples from eight rats. Data are expressed as mean ± SD of RFU. The data confirm that Tf binding decreased rapidly during maturation. The half-life was 18.3 hours ($r = 0.96$).
100%, and 100% ethanol solutions for 15 seconds each, two solutions of Freon 113 for 30 seconds each, allowed to air dry, and placed in a dessicator. Cells were gold sputter coated with a Hummer III of Freon 113 for 30 seconds each, allowed to air dry, and placed in a (Technics, Alexandria, VA) and observed at 15 kV in a JOEL JSM-35 (Japan Electron Optics and Lenses, Ltd, Tokyo) scanning electron microscope.

Enzyme assay. One milliliter of cell suspension was added to 0.5 mL 3X dilution buffer containing 1 mg/mL saponin and mixed well. A 0.1 mL aliquot of this hemolysate was added to cuvettes containing reaction mixtures warmed to 37°C for the enzymes glucose-6-phosphate dehydrogenase (G6PD), pyruvate kinase (PK), and phosphofructokinase (PFK) according to Beutler.12 The LDH assay was done with 0.025 mL of hemolysate.13 Activities were expressed as IU/10^6 cells and were carried out in duplicate.

RESULTS

Peripheral blood response to thiamphenicol treatment and bleeding. The peripheral blood response to thiamphenicol treatment and phlebotomy is shown in Fig 1. Thiamphenicol treatment leads to total suppression of erythropoiesis followed by a reticulocyte response when the drug is removed. Increasing the length of exposure to the drug by one to two days does not appear to alter the length of time for recovery. In 43 animals, the severity of the anemia was compared with the number of reticulocytes per milliliter of peripheral blood 136 hours after the removal of dialysis bags. Mature RBC per milliliter x reticulocytes per milliliter whole blood was plotted. The correlation coefficient (r) was equal to +0.04, indicating no relationship between the severity of anemia and the reticulocyte response. The range was 21.6 to 52.8 x 10^6 mature RBC/mL, which represents reductions from the baseline of 27% to 70%, respectively. These data suggest that all animals were sufficiently anemic to produce a maximal reticulocyte response.

Reticulocyte yield, reticulocyte count, and cell lysis during in vitro maturation. The recovery of reticulocytes from peripheral blood after Percoll separation was 36.2% ± 12.8% for the 11 rats used in these studies. The mean reticulocyte count of these samples was 94.5% ± 4.8% and fell during in vitro maturation as shown in Fig 2A. After a small decrease in the first 24 hours stained cells declined dramatically to baseline by day 3 and 4. The degree of cell lysis observed during the period of culture is shown in Fig 2B. Not shown is the finding that mature cells lyse more readily than reticulocytes.

Decline in transferrin binding during in vitro maturation. Data on reticulocyte binding of 125I-labeled human Tf and FITC-labeled rat Tf are shown in Figs 2 C and D, respectively. Both measures indicate a rapid decline in binding consistent with loss of Tf receptors during maturation.

Reticulocyte staining and morphology during in vitro maturation. Figure 3 shows photographs of reticulocytes from one rat over the period of culture as seen in new methylene blue stained smears (Fig 3, A through E) and on scanning electron microscopy (Fig 3, F through J). Photographs indicate that, with maturation, staining diminishes, cells become smaller, and bizarre forms are sometimes seen.

Glucose consumption. The glucose consumption for each 24-hour time interval was calculated for each of six samples over four days in culture. The average glucose consumption for the first 24 hours was 2.4 ± 0.4 mmol/L/b/10^12 cells. From 24 to 96 hours the consumption was constant at 1.0 ± 0.6 mmol/L/b/10^12 cells.

DISCUSSION

The system developed was well-suited for studies of reticulocyte maturation in vitro. By bleeding the animals while exposed to thiamphenicol, we assume that many erythropoietic stimuli were increased so that when the suppression was released, a predictable and reproducible reticulocyte response ensued. The linear rate of increase in blood reticulocytes after 96 hours suggests that reticulocytes were produced at maximal levels after this time. The response varies little from animal to animal (Fig 1).

Circulating reticulocytes can be harvested at any time after they appear; thus their age can be specified within narrow limits. The 0 to 40 hour age range used here yielded large numbers of reticulocytes. Smaller numbers of younger, more homogeneous cells can be easily isolated at earlier times.

Isolation of essentially pure populations of reticulocytes from peripheral blood has not been reported previously. Our success may be due to the fact that the mature red cells present at the time of harvest were relatively “old.” Figure 1 indicates that mature cells present at harvest had been mature cells for at least six days and any decrease in cell density associated with reticulocyte maturation should be completed. In addition, rat reticulocytes released in response to stress of anemia are apparently larger than those produced under similar conditions in humans.15 On the other hand, the discontinuous Percoll gradient method is also useful for enrichment of normal rat reticulocytes; peripheral blood of normal rats containing 3% to 5% reticulocytes has been enriched to 81% reticulocytes (data not shown).

It is interesting that the reticulocyte yield was only 36%. The remaining reticulocytes remain with the mature RBCs at the bottom of the gradient. Whether the isolated reticulocytes represent the entire reticulocyte population in terms of age or whether they represent a further subpopulation is unclear and depends on the distribution of densities of reticulocytes released into the circulation at one time. We may say only that the blood reticulocytes as well as the isolated reticulocytes have been in the circulation 0 to 40 hours.

In vitro maturation has the advantage that the same cohort of cells may be followed for a number of days. However, because of high cell lysis seen using other methods, this is the first method that ensures that the population examined over time is the same. Where the same exact cohort of cells must be followed, only samples with very low lysis may be used.

Based on the decrease in reticulocyte count, the in vitro maturation time for these prematurely released reticulocytes is 48 to 72 hours (Fig 2A), which is well within the range...
Fig 3. Photographs of new methylene blue stained smears from one animal at the time of harvest (A) and at 24 hour intervals for four days in culture (B through E, respectively). At zero-time reticulocytes = 98%. Original magnification x1,570. (F through J) Scanning electron micrographs of samples corresponding to those in A through E (original magnification x 1,780). The zero-time cells (F) resemble those of Bessis and are clearly polylobular. During in vitro maturation (G through J) the cells become smaller and progressively more abnormal in shape. Small membrane projections are seen that appear to be “budding off.” By four days (J), bizarre forms are seen frequently.

expected for in vivo maturation. Thus the in vitro system supplies the components necessary for normal loss of ribosomal RNA. Similarly, while the initial number of Tf receptors is somewhat higher than previously reported for stress reticulocytes, the fall to baseline in four days is as expected since mature red cells have no Tf receptors.

The increases in reticulocyte enzyme activities over baseline were very similar for LDH (295%), PFK (279%), and G6PD (275%) and considerably higher for PK (492%). The finding that LDH is age-related in these cells is somewhat surprising since it is not age-related in humans. LDH is, however, elevated in sheep reticulocytes. The decline in the activity of enzymes reveals a very different picture from either new methylene blue staining or Tf binding; the enzymes decline very slowly. Although this may result from abnormal in vitro conditions, the fact that glucose consumption remains constant between two and four days of incubation indicates that these cells remain metabolically active.
during this period. If this is so, the decline in enzyme activities may truly reflect in vivo degradation mechanisms. The fact that by day 7 all enzymes approach or reach pretreatment activities supports the results of Beutler and Hartman and Suzuki and Dale, which suggest that many red cell enzyme activities remain constant after the reticulocyte stage for most of the cell’s lifespan.

These reticulocytes do become smaller in vitro (Fig 4). Whether the mechanism of this size reduction is the same as that which normally occurs in vivo is unclear. Scanning EM suggests that small pieces of membrane are lost, which may cause the formation of spherocytes and echinocytes rather than normal biconcave disks. The data contrast with that of Gronowitz et al who conclude that “all aspects of normal reticulocyte maturation occur in vitro, independent of the spleen, including the assumption of the mature biconcave disk shape.” This conclusion was based on finding more biconcave cells on scanning EM at 6 hours than at zero-time. In contrast to their conclusions, they state that 60% of the cells were spherical or spherocytocinocytic at 48 hours when maturation is complete. Their initial reticulocyte count was 26% and declined to 4% during a 48-hour incubation after which hemolysis was 21%. It seems difficult to be sure which cells were being followed. We conclude that the question of whether reticulocytes can mature into cells with normal morphology in vitro is unclear.

In summary, we have developed an in vitro system that comes closer to an ideal method than previous methods because the reticulocyte response is very reproducible from animal to animal, the age range of cells studied is defined, the cells are essentially pure reticulocytes, and cell lysis is very low. We have used this system to define maturational changes in reticulocyte count, transferrin binding, and enzyme activity. These variables decline at rates that are consistent with those thought to occur in vivo. Morphological data, however, indicate that these reticulocytes do not assume a normal biconcave shape in this in vitro system and once again raise the question of whether normal morphological maturation is possible under in vitro conditions. Regardless of whether all maturational processes occur normally or not, the system has great potential for further understanding of these processes and what is required for their successful execution.

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