Membrane Skeletal Alterations During In Vivo Mouse Red Cell Aging

Increase in the Band 4.1a:4.1b Ratio

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Abstract

We have examined membrane protein profiles for alterations during red blood cell aging. To obtain populations of in vivo-aged red cells, we maintained mice in a state of continuous erythropoietic suppression for up to 8 wk using serial hypertransfusion. The circulating $t_{1/2}$ of red cells from mice which had been erythropoietically suppressed for 8 wk was < 1 d compared with a $t_{1/2}$ of 15 d for red cells from normal animals. The most obvious alteration in membrane proteins was an increase in the ratio of the membrane skeletal components 4.1a:4.1b from 0.3 for the normal red cell population to > 1 for these old cells. The 4.1a:4.1b ratio thus appears to be a useful index of red cell age. Analyses of the density profile of cells aged in the hypertransfused mice disclosed that these old cells had a density range similar to that of controls, suggesting that cell density does not increase significantly with red cell age in the mouse.

Introduction

Erythrocytes circulate for a finite time, after which they are removed by cells of the reticuloendothelial system. A variety of cellular changes have been reported in "old" red cells (for a recent review, see ref. 1), including: (a) metabolic depletion and decreased enzymatic activities, (b) oxidative damage, (c) alterations in cell-surface carbohydrates and glycoproteins, (d) increased immunoglobin binding, (e) decreased cellular deformability, and (f) increased cell density. However, the relevance of these changes to old red cell clearance remains unclear. We have focused on changes that occur in the red cell membrane and its attendant membrane skeleton as cells age because a "signal" for clearance is most likely manifest in the cell membrane, as this is the structure through which the cell communicates directly with the external milieu, and age-associated decreases in cell deformability, which decrease the cell's ability to negotiate the narrow passages of the microcirculation, probably reflect alterations in membrane skeletal components (2).

To identify biochemical changes during in vivo red cell aging and to critically evaluate the role of these changes in senescent cell clearance, it was necessary to obtain homogeneous populations of old cells. The notion that red cells become more dense as they age in circulation forms the basis for the most widely used method of preparing old cells, namely, separation by density centrifugation (1). However, there are conflicting reports concerning the utility of density techniques for generating truly senescent cells, and the relationship of cell age to density has been questioned (for a comprehensive discussion of this controversy, see ref. 1). An alternative approach for production of homogeneous populations of old red cells was used by Ganzoni et al. (3), who suppressed erythropoiesis in rats for nearly one red cell life-span by serial red cell hypertransfusion. We have used this hypertransfusion protocol of Ganzoni, but have substituted mice for rats because erythropoietic suppression by hypertransfusion is more complete in mice (4, 5), and gastric ulceration is not a complication (6). Our results demonstrate changes in key red-cell membrane skeletal components as cells age in the circulation.

Methods

Mice. C$_{57}$Bl$_6$ retired breeder mice (6–11 mo) were obtained from the Jackson Laboratory, Bar Harbor, ME.

Blood samples for estimating packed cell volumes (PCV)[1] and reticulocytes. PCVs were determined by a micromethod, and reticulocyte percentages were determined from blood smears stained with new methylene blue followed by Wright-Giemsa (to allow easier quantitation). Mice were warmed under an examination lamp to induce vasodilation. Blood was obtained by puncturing a tail vein with a 27-gauge needle.

Packed red cells for hypertransfusion. Blood was collected from ether-anesthetized mice into heparin-rinsed syringes with 21-gauge needles via cardiac puncture, red cells were pelleted by centrifugation and the plasma and buffy coat were discarded. Packed red cells were injected intraperitoneally with a 21-gauge needle for initial and early serial hypertransfusions. For later hypertransfusions, packed red cells were injected intravenously into a tail vein with a 25-gauge needle. Blood from five to six donor mice was required for the initial hypertransfusion of each mouse.

Preparation of aged red cell populations. Aged red cells were prepared by a modification of the procedure of Ganzoni et al. (3) as illustrated in Fig. 1. Red cells for the initial hypertransfusion were obtained from other normal retired breeder mice of the same strain.

Erythropoiesis was inhibited for up to 56 d by using, at ~ 2-wk intervals, half of the hypertransfused group as donors to maintain elevated PCVs in the remaining hypertransfused mice. Thus, the red cells of both donor and recipient mice had the same (progressively older) mean red cell age. At the sixth week, mice were either hypertransfused again or given daily actinomycin D (80–100 μg/kg) to maintain erythroid suppression (7). Erythropoietic inhibition was confirmed by examining blood smears for the presence of reticulocytes at the time of each serial hypertransfusion.

The bottom portion of Fig. 1 presents the theoretical age distribution of circulating red cells after various periods of erythropoietic inhibition. In normal animals, there is a continuum of ages from young to old (mean age, 30 d). When erythropoiesis is inhibited, no new cells enter

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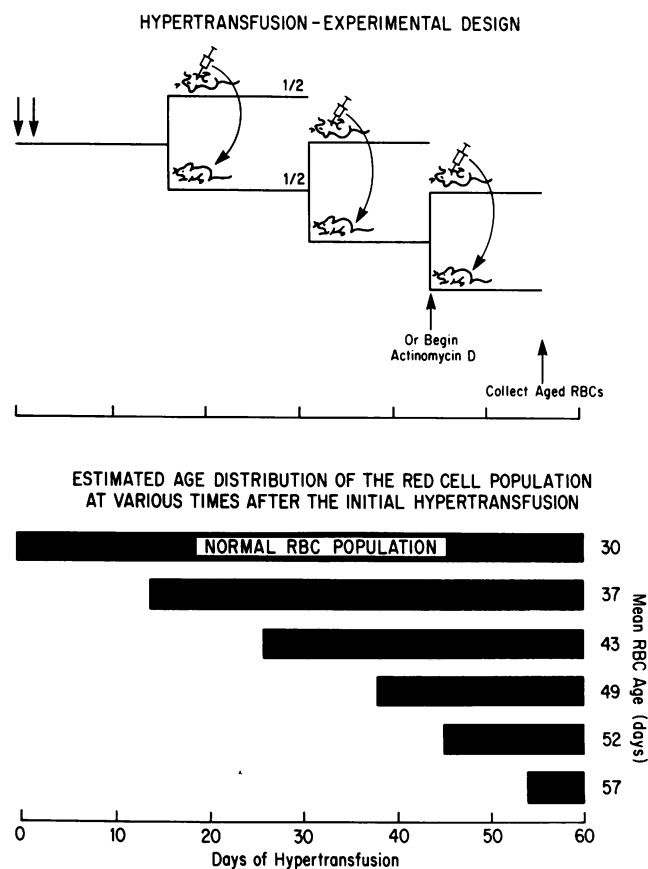*Received for publication 27 March 1986.*

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1. *Abbreviations used in this paper:* BSS, balanced salt solution; DTT, dithiothreitol; PCV, packed cell volume; PMSF, phenylmethanesulfonyl fluoride.

HYPERTRANSFUSION - EXPERIMENTAL DESIGN

ESTIMATED AGE DISTRIBUTION OF THE RED CELL POPULATION
AT VARIOUS TIMES AFTER THE INITIAL HYPERTRANSFUSION

Figure 1. Diagram illustrating the protocol for generating populations of in vivo-aged mouse red cells via the continuous suppression of erythropoiesis by serial hypertransfusion. Note that at ~ 6 wk, the erythropoietic blockade can be maintained either by another hypertransfusion, administration of daily, low-dose Actinomycin D, or by sublethal, whole-body irradiation (see Methods).

the circulation, therefore, the existing cells become progressively older. For instance, 14 d after the first hypertransfusion, the age distribution is 14–60 d with a mean cell age of 37 d. After 54 d of erythroid inhibition, the age distribution of cells is 54–60 d with a mean cell age of 57 d. The net effect is that the average age of the cells increases and the age range decreases. Eventually the entire red cell population becomes old and exhibits a very narrow age distribution.

Hypertransfusion schedule. Before hypertransfusion, recipient mice were screened and those having PCV values less than 40% were discarded. Most of the initial hypertransfusions were given in two approximately equal volumes 1 d apart, but a few were given as one injection. The volume of packed red cells was adjusted for body weight to attain a PCV on day 2 of 70–75% (8). For example, mice weighing 25–27 g were given a total of 2.0 cm³ of packed red cells. PCVs of the hypertransfused mice were determined 1–4 d after the initial hypertransfusion.

At ~ 2-wk intervals, the PCVs of the hypertransfused mice were determined; blood was collected from the half of the hypertransfused group with the lowest, but still elevated PCVs and examined for the presence of reticulocytes. Packed cells then were prepared and injected into the remaining hypertransfused mice. The volume of packed red cells injected at each serial hypertransfusion ranged from 0.6 to 0.7 cm³. PCVs were determined 2–5 d after each serial hypertransfusion. A total of 50 mice was required to obtain 1 ml of packed old red cells produced by 56 d of erythroid suppression.

Survival of old red cells. The survival of red cells was evaluated after labeling with ^{51}Cr. Mice were maintained polycythemic for 44 d by serial hypertransfusion. Erythropoiesis was inhibited an additional 12 d by

daily subcutaneous or intraperitoneal injection of 80–100 µg/kg actinomycin D so that erythropoiesis was suppressed for a total of 56 d. Because of concern about possible increased fragility of old red cells, the labeling with ^{51}Cr was done in vivo. Hypertransfused and normal mice were given 1 mCi of $Na_2{}^{51}CrO_4$ in saline intravenously 4 or 5 d before red cell collection. On the day of blood collection, a 10-µl sample of blood was obtained from a tail vein and placed on top of 400 µl of Percoll density gradient media containing 9 parts stock Percoll density gradient media (Pharmacia Fine Chemicals, Piscataway, NJ), 1 part 10× Hanks' balanced salt solution (BSS) without calcium or magnesium, and 10 parts CATCH media (9). The red cells were separated by centrifugation at 600 g for 10 min at room temperature and the supernatant (containing white cells, platelets, and plasma) and the red cell pellet were counted for radioactivity. In normal mice, 90% of the radioactivity was in the red cell fraction; in 56-d erythroid-suppressed mice, 99% was in the red cell fraction. Blood was then collected from anesthetized mice via cardiac puncture into preservative-free heparin-rinsed syringes. The old red cell-enriched blood and the blood from normal mice were quickly transfused into the tail veins (with a 27-gauge needle) of five recipient female $C_{57}Bl_6$ mice weighing 15–17 g. Each mouse received 0.1 cm³ blood containing either 182,430 cpm (blood from 56-d erythroid-suppressed mice) or 173,010 cpm (blood from normal mice). 10-µl blood samples were collected from puncture of a tail vein with a 27-gauge needle at 1, 24, 48, 72, and 96 h for determination of radioactivity. Additional samples were collected at 7 and 15 d from the group that received normal cells. The proportion of the injected radioactivity circulating at 1 h was calculated by assuming a blood volume of 6% of body weight. The rate of disappearance of radioactivity from the circulation was calculated by expressing the radioactivity in the daily samples as a percentage of the 1-h values. The radioactivity values of the daily samples were corrected for radioactive decay and blood loss, assuming a blood loss of 15 µl per d.

Analysis of red cell density profiles in Stractan gradients. The density profiles of red cells from two mice whose erythropoiesis had been suppressed for 56 d were compared to those of red cells from two untreated mice in eight-step, discontinuous Stractan gradients (density range of 1.0741–1.1056 g/ml in 0.0045-g/ml increments). The red cells were washed three times and resuspended to a hematocrit of 40% before 250 µl of each of the four cell suspensions were layered on top of the gradients and centrifuged 30 min at 25,000 rpm in an ultracentrifuge. The density profiles were photographically recorded.

Density fractionation of red cells in Percoll gradients for analysis of bands 4.1a/4.1b ratio. Usually 0.6 ml of mouse blood was mixed with 10 ml of 90% Percoll density medium (Pharmacia Fine Chemicals) diluted in CATCH medium (9). The Percoll was made isotonic with 10× Hanks' BSS before dilution. Density fractionation was accomplished by centrifugation at 30,000 g for 30 min in an angle head rotor.

Enzyme activities. Red cell glutamic-oxalacetic transaminase and glucose-6-phosphate dehydrogenase activities were measured with kits obtained from Sigma Chemical Co. (St. Louis, MO), and the units of activity were expressed per gram of hemoglobin (determined by a cyanomethemoglobin method) (10). Activities of red cells from erythroid-suppressed mice are given as a percentage of normal red cell activity determined in parallel.

Preparation of mouse erythrocytes for analysis of membrane proteins. Mouse blood was drawn into heparin supplemented with a red cell preservative, acid citrate-dextrose (ACD; 150 µl/850 µl blood), and maintained at 4°C. Blood was processed within 1 d of drawing. White cell contamination was removed by filtration at 23°C over columns packed with a mixture (1:1 by weight) of microcrystalline cellulose (Sigmacell, type 50, Sigma Chemical Co.) and α-cellulose (11). The bed volume was generally at least twice that of blood. Initial experiments that did not employ this filtration frequently showed proteolytic degradation of red cell membrane proteins. Cells, flushed from the column with saline (0.15 M NaCl), were diluted with cold saline and centrifuged. The cells were washed with saline four additional times. The last wash was in a 1.5-ml microcentrifuge tube in order to more accurately measure packed cell volumes.

Preparation of red cell membranes. Stroma was prepared by lysis and

repeated washing (five to six times) with 50 vol of 7 mM cold phosphate buffer, pH 7.4, containing 1 mM EDTA plus 0.2 mM phenyl-methanesulfonyl fluoride (PMSF) (lysis buffer).

Preparation of membrane skeletons. Membrane skeletons were prepared from equivalent volumes of packed stroma derived from normal (control) or in vivo aged red cells by extraction with 6 volumes of cold 1% vol/vol Triton X-100 in 7 mM phosphate buffer, pH 7.4, containing 1 mM EDTA and 1 mM dithiothreitol (DTT), and centrifugation. After removal of the supernatant, the Triton-insoluble residue was washed once with cold lysis buffer and resuspended to seven times the original stroma volume with lysis buffer. Samples were immediately boiled with gel sample buffer.

Gel electrophoresis. Membrane proteins were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Membranes were boiled for 3 min with gel sample buffer, and the solubilized proteins separated on 6–15% or 7.5–15% gradient slab gels using the discontinuous buffer system of Laemmli (12, 13). Gels were stained with Coomassie Blue R-250 (13). Stained bands were quantitated by densitometry.

Preparation of antibodies to band 4.1. Red cell stroma were stripped of the bulk of peripheral proteins, except band 4.1, by extraction with 10 volumes of cold NaOH, pH 11–11.5, and centrifugation. After one to two additional washes, the packed pellet was resuspended with cold NaOH, pH 12, or 0.1 N NaOH to release band 4.1 and centrifuged (14, 15). Final purification of band 4.1 was achieved by preparative SDS slab gel electrophoresis (12–20% acrylamide gradient) using the discontinuous buffer system of Laemmli (12, 13). The bands were visualized by lightly staining with Coomassie Blue and destaining. Bands 4.1a + 4.1b were excised from the gel, homogenized with an equal volume of Freund's complete adjuvant and injected intramuscularly into the rear flanks of female New Zealand rabbits (200–400 μg/rabbit). Rabbits were boosted approximately every 2 mo with bands 4.1 in Freund's incomplete adjuvant. In some boosts, Coomassie-stained gel slices, rather than eluted proteins, were homogenized in Freund's adjuvant.

Affinity purification of anti-band 4.1 antibodies. Antibodies to bands 4.1a + 4.1b were affinity/purified by elution from nitrocellulose strips following immunoblotting (see below). The 4.1 bands of skeletal residues were visualized on the blots by Amido-Schwartz staining, cut out, and reacted with the 4.1 antiserum as described below. Antibodies specific for the 4.1 bands were eluted from the nitrocellulose strips by incubation at 23°C for 1.5 h with 4 M KSCN and dialyzed at 4°C vs. cold phosphate-buffered saline (PBS) containing 0.01% NaN₃. The specificity of the antibodies for band 4.1 was confirmed by comparing immunoblots of cells or stroma from normal human donors and from a patient with hereditary elliptocytosis whose red cells are devoid of band 4.1 (15, 16). The affinity-purified antibodies reacted with band 4.1a and b from samples of normal cells or stroma. However, no reaction was observed with samples devoid of band 4.1.

Immunoblotting. Membrane proteins separated on SDS polyacrylamide gels were electrophoretically transferred to nitrocellulose paper at 23°C using the Hoefer Transphor apparatus as described by Towbin et al. (17), but substituting ethanol for methanol in the blotting buffer. After 1.5 h at 0.5 A, the nitrocellulose was removed, rinsed for 30 min with PBS, and treated with immune stain buffer containing 10 mM Tris base, 150 mM NaCl, 0.02% NaN₃, and 2% casein to block nonspecific protein binding to the nitrocellulose. The nitrocellulose was then incubated overnight at 23°C with specific rabbit antibody diluted in immune stain buffer. Bound antibody was detected by incubating with ¹²⁵I-labeled protein A (1 × 10⁶ cpm/ml for 1 h) followed by autoradiography.

Results

Red cell survival. If cells produced by this regimen of continuous erythropoietic suppression are truly old, their survival time in vivo should be greatly decreased. After 52 d of continuous erythropoietic inhibition, red cells in suppressed animals were labeled in vivo by intravenous injection of ⁵¹Cr. 4 d later, the labeled cells were collected and transfused into normal recipients for

survival studies. Cells from normal animals were similarly labeled 4 d before collection and injected into normal recipients. As seen in Fig. 2, the circulating $t_{1/2}$ of red cells collected from mice after 56 d of erythropoietic suppression is ~ 1 d, compared with a $t_{1/2}$ for control cells of 15 d. Thus, the half-life of these old cells was only 7% that of control cells. This marked decrease in survival time confirmed that these old cells were truly senescent. As no reticulocytes were seen in blood samples obtained at each hypertransfusion, the cells in the suppressed animals must be at least 51–56 d old (up to 5 d may be required for the complete cessation of erythropoiesis; (18), very close to the estimated 60-d life span of circulating mouse erythrocytes (19).

Further evidence of increased red cell age after hypertransfusion was provided by measuring red cell enzyme activities. Glutamic-oxalacetic transaminase activity (GOT) and glucose-6-phosphate dehydrogenase (G6PD) activity were depressed to 62±3% (SD) (two mice) and 58±14% (two mice) of the activity of normal cells, respectively.

Membrane alterations. Having established the validity of this serial hypertransfusion regimen for generating populations of very old mouse red cells, we asked the question, Do changes occur in the red cell membrane as the cells age in vivo? Membranes isolated from normal red cells and from in vivo aged red cells obtained after 55 d of hypertransfusion, were solubilized and analyzed by SDS gel electrophoresis. As seen in Fig. 3, the most striking change in the Coomassie Blue-stained profiles occurred in bands 4.1a and 4.1b. In control stroma, band 4.1b predominates and comprises ~ 70% of the total band 4.1 (4.1a + 4.1b) when analyzed by quantitative densitometry. Following 55 d of in vivo red cell aging, the staining intensity of bands 4.1a and 4.1b is roughly equivalent. The ratios of (4.1a + 4.1b): band 3 and (4.1a + 4.1b):band 4.2 remain relatively constant, suggesting a decrease in the content of membrane-associated band 4.1b and an equivalent increase in 4.1a. To confirm that these bands are, in fact, the mouse red cell homologues of human erythrocyte bands 4.1a and b, we reacted normal and in vivo-aged mouse red cell stroma with affinity-purified antibodies directed against bands 4.1a + b isolated from human red cell membranes. These components of mouse membranes reacted with the anti-4.1 antibodies, showing changes in intensity of the 4.1a and b bands similar to those observed in the Coomassie Blue-stained gels.

Kinetics of the change in the 4.1a:4.1b ratio. A major advantage of the serial hypertransfusion protocol for generating populations of old cells is the potential for kinetic evaluation of
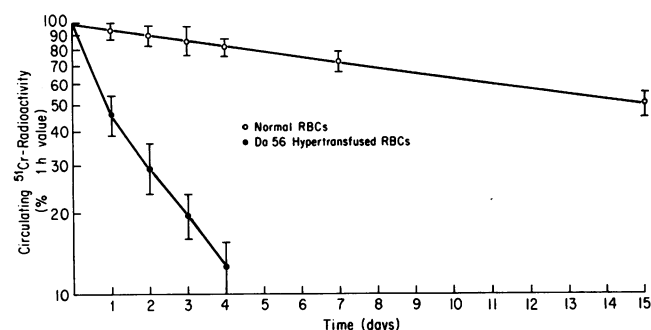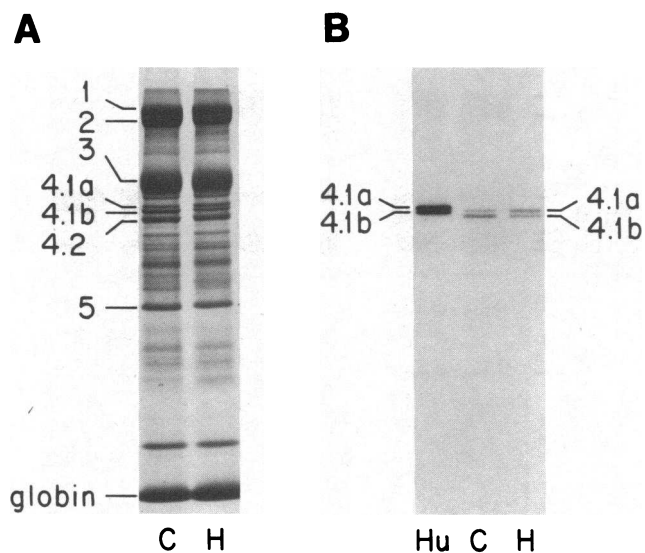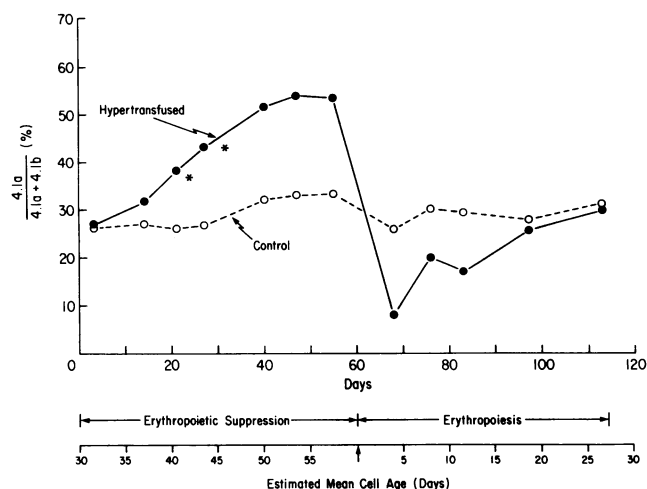

Figure 2. ⁵¹Cr-survival of old red cells collected 56 d after the initiation of red cell hypertransfusion (*closed circles*) compared to survival of the normal red cell population (*open circles*). Values represent the mean±1 SD for five mice in each group.

A

1
2
3
4.1a
4.1b
4.2
5
globin —

C   H

B

4.1a
4.1b

Hu   C   H

4.1a
4.1b

Figure 3. (*A*) Comparison of the Coomassie Blue staining profiles of normal mouse stroma (*C*), and stroma isolated from mouse red cells aged for 55 d in a hypertransfusion experiment (*H*). Stroma samples were dissolved in gel sample buffer and electrophoresed on an SDS slab gel with the discontinuous Laemmli buffer system. (*B*) Immunoblot of mouse red cell membranes using anti-human-band 4.1 antibodies. The blots were reacted with affinity-purified antibodies directed against bands 4.1a + b isolated from human red cell stroma. Bound antibodies were detected with ^{125}I-Protein A followed by autoradiography. *Hu:* human membrane skeletal residue; *C:* stroma isolated from normal mouse red cells; *H:* stroma isolated from in vivo-aged mouse red cells following 56 d of continuous erythroid inhibition.

Figure 4. Increase in the 4.1a:b ratio as mouse cells progressively age during a hypertransfusion protocol. Erythropoiesis was inhibited by three serial hypertransfusions at ~ 2-wk intervals. At 6 wk, ~ 2 wk after the last transfusion, erythropoietic suppression was maintained by daily injections of Actinomycin D until day 60, at which time the erythropoietic block was released and erythropoiesis allowed to resume. Blood was collected at various times during the protocol. Blood from normal mice was routinely drawn and analyzed at each time point. Washed stroma were solubilized in gel sample buffer and electrophoresed on 7.5–15% acrylamide gradient gels using the Laemmli buffer system. Proteins were visualized by Coomassie Blue staining and quantitated by densitometry. * Points of blood collection from animals that had been suppressed by administration of low-dose Actinomycin D alone, without any hypertransfusion.

changes related to in vivo cell aging. We have evaluated the change in the 4.1a:b ratio as a function of time, or mean cell age, during a hypertransfusion regimen (Fig. 4). At each time point, samples from both erythroid-suppressed mice and controls were drawn and the 4.1a:b ratio evaluated by quantitative densitometry of Coomassie Blue-stained gels. In membranes from control cells (mean cell age, 30 d), band 4.1a accounted for 30% of the total band 4.1a. As cells became progressively older (Fig. 1), there was a progressive increase in the 4.1a:b ratio. This change appears to be a sensitive index of red cell age and can be detected as early as 2 wk after the initiation of the erythropoietic blockade, when the estimated mean age of the circulating red cell population is only 37–38 d. After 55 d of continuous suppression, when the mean cell age of the remaining red cell population is ~ 57.5 d, band 4.1a accounts for ~ 54% of the total band 4.1. In fact, the increase in the 4.1a:b ratio appears to plateau around day 47 of the protocol, or slightly before the end of the estimated 60-d red cell life span.

Since the altered 4.1a:b ratios might result from the prolonged elevation of hematocrits, rather than from the aging process, we analyzed the contents of bands 4.1a and b in stroma from mice in which erythropoiesis was suppressed for up to 32 d by daily injections of low-dose Actinomycin-D (80–100 μg/kg; see ref. 7). As seen in Fig. 4, suppression for 23 and 32 d resulted in a progressive increase in the 4.1a:b ratio, and the values observed are in close agreement with those seen during erythropoietic suppression by serial hypertransfusion for similar lengths of time. These observations provide further evidence that the increase in the 4.1a:b ratio seen with the red blood cell hypertransfusion

regimen is a result of in vivo red cell aging and not an effect of elevated PCV.

If this increase in the 4.1a:b ratio during erythropoietic suppression is, in fact, related to red cell aging, then populations of very *young* red cells should have 4.1a:b ratios lower than those of control mice. Removal of the erythropoietic block after 60 d of continuous suppression, or about one red cell life span, allows the resumption of erythropoiesis with the production of reticulocytes and young red cells. Analysis of stroma isolated from red cells obtained 8 d after the resumption of erythropoiesis, where the estimated mean cell age is ~ 4 d, disclosed that band 4.1a accounted for only 8% of the total band 4.1. Continued sampling of the red cell population disclosed that the 4.1a:b ratio returned to normal as the age distribution of the cells gradually returned to normal.

Thus, the ratio of membrane-associated band 4.1a:b appears to be a reflection of the in vivo red cell age. Very young mouse red cells (0–8 d old) have 4.1a:b ratios of ≤ 0.1, while populations of old red cells (mean cell age ~ 57 d) have 4.1a:b ratios slightly > 1.

Band 4.1b is converted to 4.1a as cells age. There are at least two plausible explanations for this change in the 4.1a:b ratio. One is that the membrane-associated 4.1 bands are in equilibrium with cytosolic bands 4.1, and that as red cells age, band 4.1b on the membrane is gradually replaced by cytosolic band 4.1a. A second explanation is that band 4.1b is converted to 4.1a as cells age, resulting in a precursor-product relationship for band 4.1b and 4.1a. Support for a precursor-product relationship comes from analyses of total cellular bands 4.1a and b by electrophoresis of whole cells on SDS gels as seen in Fig. 5.
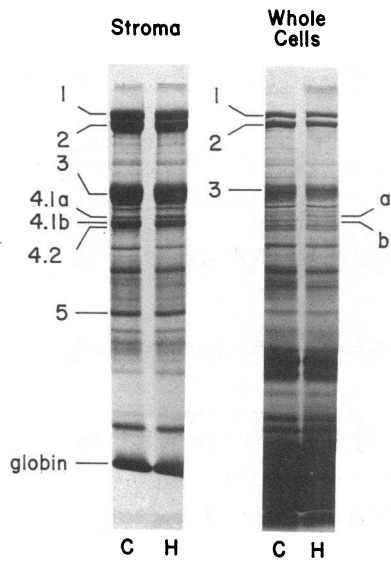
Figure 5. Comparison of bands 4.1a,b in whole red cells isolated from control mice (*C*) and from mice which were hypertransfused for 44 d (*H*). Whole cell and stroma samples were boiled in gel sample buffer and electrophoresed on a 6–15% gradient gel. Proteins were visualized by staining with Coomassie Blue.

In this figure, whole control cells are compared with cells obtained after 44 d of hypertransfusion when the change in the 4.1a:b ratio has nearly reached a plateau (see Fig. 4). These old cells have a mean cell age of $\sim$ 52 d, compared with 30 d for the control cells. Examination of the 4.1 region of these whole cell samples disclosed changes similar to those observed in stroma. Densitometric measurements of control cell profiles indicated that band 4.1a accounts for 30% of the total band 4.1a + b. In the profile of the in vivo aged whole cells, band 4.1a constitutes 53% of the total band 4.1. Because the total cellular complement of band 4.1 was analyzed, these data suggest that during red cell aging there is, in fact, a conversion of band 4.1b to 4.1a. This implies that the components are identical peptides undergoing posttranslational modifications. Peptide mapping studies have indicated that bands 4.1a and 4.1b are, in fact, sequence-related polypeptides (20, 21).

Triton extraction of old cell membranes. What effect, if any, does this change in the 4.1 bands have on the composition or structural integrity of the membrane skeleton? We have compared Triton X-100 extraction of stroma isolated from control red cells and from cells aged in vivo for 55 d (Fig. 6). This conversion of band 4.1b to 4.1a does not appear to have any striking effect on the extractability of the other key skeletal components, spectrin (bands 1 + 2) and actin (band 5), indicating that no gross dissolution of the skeletal core has occurred. Skeletons derived from these old cells do have an enhanced content of globin chains. Membranes from both control and in vivo aged mouse red cells retain significant amounts of globin under the conditions of lysis and washing used in these experiments (7 mM phosphate buffer, pH 7.4 containing 1 mM EDTA and 0.2 mM PMSF). However, the membrane-associated globin in control cells is mainly Triton-soluble, whereas the membrane-associated globin of in vivo aged cells is mainly Triton insoluble.

Density distribution of in vivo aged cells. We have previously shown that little difference exists in the density distribution of red cells from control and hypertransfused mice when cells are centrifuged on self-forming Percoll gradients (22). Here, we have analyzed cell density profiles using equilibrium density gradient centrifugation on discontinuous Stractan gradients. As seen in Fig. 7, gradient profiles of red cells from normal animals were compared with profiles from animals in which erythropoiesis
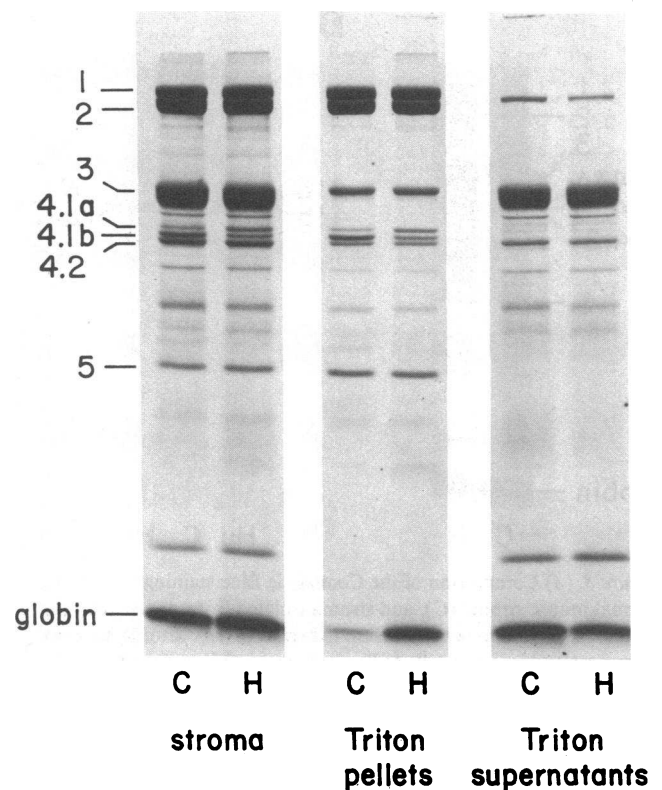

Figure 6. Comparison of membrane skeletons (Triton pellets) of stroma from control (normal) mouse red cells (*C*) and from red cells following 55 d of hypertransfusion (*H*). Stroma were extracted with cold 1% Triton X-100 and centrifuged. Samples from equivalent amounts of stroma were analyzed on 7.5–15% acrylamide gradient SDS gels using the Laemmli buffer system. Proteins were visualized by Coomassie Blue staining.

had been suppressed for 56 d. Although the red cells from erythroid-suppressed mice show a slight shift in more dense regions, the gradient profiles of age-enriched and control cells are most notable for their similarities rather than their differences. Thus, even after 56 d of continuous erythropoietic suppression, the old cells have a broad distribution of densities that is similar to cells from control (normal) animals.

4.1a:b ratio in density-fractionated cells. We have also evaluated the 4.1a:b ratio of the most dense cells isolated from density
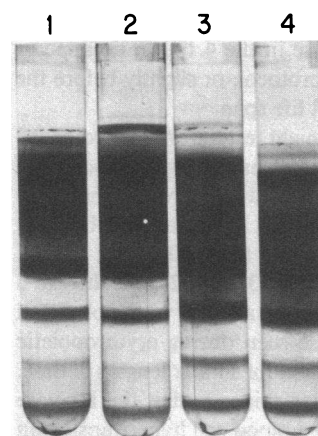

Figure 7. Discontinuous Stractan density gradient profiles of red cells from control mice (*lanes 1* and *2*) and from mice which were hypertransfused for 56 d (*lanes 3* and *4*). Each tube contains cells isolated from a different mouse.

gradients (Fig. 8). In membranes isolated from normal mouse red cells, band 4.1a comprises 29.7±3.3% (SD) ($n = 14$) of the total band 4.1. As described earlier, this value increases to 50–55% in in vivo aged red cells. In the densest cells isolated from the gradients, band 4.1a accounted for 30.1±3.5% (SD) ($n = 4$) of the total 4.1. It must be noted that the dense cells used in these analyses represent very small percentages (1.7–3.7%) of the total cells on the gradients. Thus, stroma isolated from the most dense cells showed no apparent change in the 4.1a:b ratio, an indicator that changes very early after the initiation of erythropoietic suppression by hypertransfusion. By contrast, a decreased 4.1a:b ratio of 17.9±5.4% (SD) ($n = 4$) was observed in membranes isolated from the least dense cells (0.9–6.0% of the total cells on the gradient).

Discussion

Our results demonstrate that continuous inhibition of erythropoiesis by serial hypertransfusion, an approach first employed by Ganzoni et al. in rats (3), is an effective technique for generating homogeneous populations of very old in vivo aged mouse red cells. Although the volume of blood obtained from a single mouse is less than that obtained from a rat, we chose mice for these experiments because the suppression of erythropoiesis by hypertransfusion is more complete in mice (4, 5) and gastric ulceration is not a complication (6). The erythropoietic blockade in mice can be routinely maintained for at least 8 wk. Thus, the cells remaining in circulation after 8 wk of suppression must be at least 52–56 d old (Fig. 1), which is very close to the end of

Figure 8. Comparison of the Coomassie Blue staining profiles of stroma isolated from unfractionated, control mouse red cells (*WB*) and of stroma isolated from the most dense cells (1.6% of the cells on the gradient) from a Percoll gradient (*D*). Stroma samples were boiled in gel sample buffer and electrophoresed on a 6–15% SDS gel.

the estimated 60-d survival time of mouse red cells (19). The extremely short survival time of cells collected from animals after 8 wk of continuous erythroid suppression ($t_{1/2}$ of 1 d compared with $t_{1/2}$ of 15 d for controls) confirmed that these red cells are indeed very old. The decrease in GOT and G6PD enzyme activities to ~ 60% of control values is also consistent with the conclusion that these are older cells. However, recent results obtained by Beutler and Hartman (23) suggest that the decrease in activity of many human red cell enzymes occurs during maturation of the reticulocyte to the mature red cell and may not decline significantly during in vivo aging of the mature red cell (24).

In addition to generating homogeneous populations of truly old red cells, a second advantage of erythroid suppression as a means to produce aged red cells is that one can follow biochemical changes as a function of mean cell age by collecting blood at various times during the regimen. As seen in Fig. 4, kinetic evaluation of the change in the 4.1a:b ratio shows that this event can be detected very early during the hypertransfusion protocol and, in fact, appears to plateau 2 wk before the end of the 60-d red cell life span. Since it is likely that any "signal" for clearance produced by senescent red cells would be manifest very late in a cell's life, the change in the 4.1 bands is unlikely to be directly involved in senescent cell recognition. However, the conversion of band 4.1b to 4.1a could be an early participant in a cascade of events leading to the generation of a purported signal for clearance. The association of band 4.1 with transmembrane components in the human erythrocyte membrane (15, 25–27) provides an avenue for transmission of intracellular changes to the cell surface. While many biochemical changes might be expected to occur during cell aging, the kinetics of appearance of these changes may vary widely. In contrast to the kinetics of the conversion of band 4.1b to 4.1a, we found that the appearance of Triton-insoluble globin, which appears to be associated with the skeleton (see Fig. 6), is a late event during the hypertransfusion regimen whose onset occurs at about the same time that the change in the 4.1 bands has reached a maximum (28). Other changes in the protein profiles of the membranes of these in vivo aged cells can also be detected (see Figs. 3 and 6). However, the identity of the proteins is unknown and the kinetics of appearance of these changes has not been examined.

The 4.1a:b ratio appears to be a useful indicator of mouse red cell age (Fig. 4). Animals that were rebounding from 60 d of continuous erythroid suppression and had a red cell population with a very young mean age (~ 4 d as indicated in Fig. 4) thus have a very low 4.1a:b ratio of 0.1 or less. In contrast, very old cells generated by the inhibition of erythropoiesis for 6–8 wk have 4.1a:b ratios of slightly > 1. It thus appears that the 4.1a:b ratio is a useful index for evaluating whether a population of mouse red cells is enriched with old cells. However, the fact that the 4.1a:b ratio appears to plateau in the mouse hypertransfusion system ~ 2 wk before the end of one red cell life span suggests that this parameter alone is not a sufficient criterion for demonstrating that the cell population is on the very threshold of being cleared from circulation.

Similar changes in the 4.1a:b ratio also appear to occur in human red cells. Blood samples with elevated reticulocyte counts have low 4.1a:b ratios (29). Ravindranath and Johnson (30), as well as we (unpublished observations), have detected elevated 4.1a:b ratios in red cell membranes from patients with transient erythroblastopenia of childhood (TEC) in whom a transient inhibition of erythropoiesis has resulted in a population of cir-
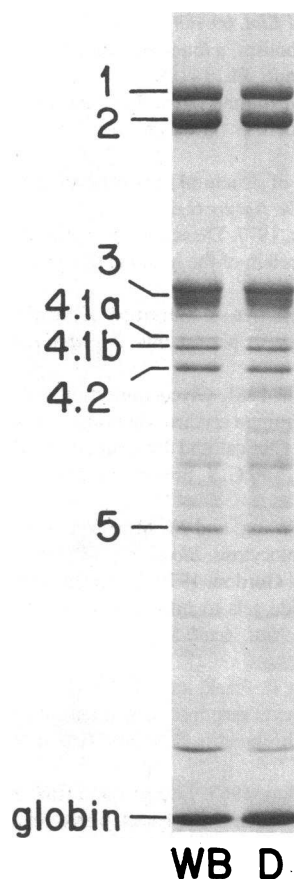
culating erythrocytes with an increased mean cell age. Obviously, hypertransfusion experiments in humans cannot be performed, so populations of senescent human erythrocytes must be isolated by physical/chemical methods. The 4.1a:b ratio may be useful in assessing the efficacy of such methods for their ability to enrich for older human red cells.

Bands 4.1a and 4.1b appear to be sequence-related proteins (20, 21). However, the nature of the difference in the two components has not been elucidated. Whether or not the two proteins represent the products of two different but closely related genes or are the result of posttranslational processing has not been clearly shown. The finding of altered ratios of the two components in reticulocyte-enriched blood (29) would appear to favor the latter interpretation. Our demonstration that band 4.1b is converted to 4.1a as cells age in vivo suggests that these are identical proteins that undergo posttranslational modification during in vivo aging while intercalated in the membrane skeleton. The nature of the processing event remains unknown. However, several posttranslational events that could cause changes in apparent molecular weight on SDS gels have been reported for band 4.1, including phosphorylation (20, 31), glycosylation (32, 33), acylation (34), and methylation (35). In addition, although ubiquitination of red cell membrane proteins has not been reported, ubiquitin is present in red cells (36), and the addition of peptides via the formation of isopeptide bands might also occur. Although proteolytic processing results in fragments of lower molecular weight, in theory one could observe products of higher apparent molecular weight if the release of small peptides alters SDS binding to the parent molecule causing a decrease in electrophoretic mobility.

While the density distribution of red cells showed a small shift toward higher densities as the period of erythroid inhibition increased, one is struck by how little change occurs during 8 wk of continuous erythropoietic inhibition. Even after 56 d, the in vivo-aged cells exhibit a broad range of densities similar to that of normal blood. This finding is inconsistent with current dogma that there is a correlation between red cell age and density. Luthra et al. (37) found an imperfect correlation between age and density of human red cells which had been labeled in vivo with radioactive iron. The failure to detect any significant elevation of the 4.1a:b ratio among the most dense cells isolated from normal blood also suggests that the densest mouse cells isolated from density gradients do not represent homogeneous populations of the oldest cells. In addition, Morrison et al. (22) have found that the densest fractions may, in fact, contain the youngest mouse red cells. These data do not support the notion that cell age and density are well correlated in mice.

We have thus shown that the technique of continuous erythroid suppression via serial hypertransfusion is a useful technique for generating homogeneous populations of senescent mouse red cells. The use of this approach also enables one to study the kinetics of various biochemical changes as a function of mean cell age. One can then begin to evaluate whether biochemical changes in membrane components are related to physiological parameters such as decreased cellular deformability or senescent cell clearance.

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