For decades, density gradient centrifugation has been the cornerstone of red blood cell (RBC) senescence research. However, there has been persistent controversy over the suitability of this technique for isolating senescent RBCs. From a methodological standpoint, it is likely that many of the commonly used media fail to fulfill essential requirements of providing both isopycnic equilibrium sedimentation (ie, separation based on density) and a physiologic environment for RBCs. From an interpretive standpoint, it is unclear what components of the total RBC population are represented by the densest RBCs. Although many of the densest RBCs may be pathologic and destined for rapid clearance, it is not clear if the same population is senescent.

To avoid this controversy, investigators have developed novel approaches to the study of RBC senescence, such as the hypertransfusion and biotinylation methodologies. Overall, results from these approaches have cast increasing doubt on the presumed positive correlation between RBC density and senescence, at least in rodents and rabbits. These approaches have also provided invaluable information on some of the biochemical processes that occur during normal RBC maturation and senescence. However, these studies remain somewhat suspect in their relevance to human RBC senescence, because they have to date used animals with relatively high random RBC loss and short RBC life spans (~60 days).

The biotinylation strategy for collecting unequivocally senescent RBCs relies on the ability of immobilized avidin to retrieve biotinylated erythrocytes after circulating in vivo. An important feature of the method is that theoretically, it avoids the controversial use of density gradient centrifugation to collect presumably old RBCs. Furthermore, the dog is an appropriate model for human RBC senescence because it has a low degree of random RBC loss and a similarly long RBC life span (~110 days). Two dogs had 97% to 100% of their circulating RBCs biotinylated by infusion of N-hydroxysuccinimido biotin (Clontech, Palo Alto, CA; Calbiochem, La Jolla, CA) dissolved in dimethyl sulfoxide. At postbiotinylation days 104 and 107 for one dog and day 110 for the other dog, biotinylated RBCs were isolated by magnetic cell sorting and analyzed for the presence of autologous IgG using 125I-labeled sheep-antidog IgG (SAD IgG). On all 3 days, there were at least three times more SAD IgG molecules per RBC on senescent biotinylated RBCs than on control (unfractionated) RBCs (day 104: 11,677 ± 3,399; day 107: 6,710 ± 2,115; day 110: 6,042 ± 1,838 molecules of SAD IgG per senescent v control RBC). Furthermore, it is unlikely that an immune response to the conjugated biotin had been elicited, because fresh in vitro biotinylated RBCs that were incubated in autologous plasma (taken after exposure to circulating biotinylated RBCs for 113 days) and then exposed to the SAD IgG showed no increase in antibody binding over control (nonbiotinylated) RBCs (1,431 ± 1,378 cpm/106 biotinylated v control RBCs; P > .20). These results suggest that senescence of canine biotinylated RBCs is characterized by binding of autologous IgG and that antibiotin antibodies do not contribute to this process.

Senescence of Canine Biotinylated Erythrocytes: Increased Autologous Immunoglobulin Binding Occurs on Erythrocytes Aged In Vivo for 104 to 110 Days

By John A. Christian, Alan H. Rebar, G. Daniel Boon, and Philip S. Low

We have evaluated senescence related changes in canine red blood cells (RBCs) using the biotinylation system, where RBCs are labeled in vivo with biotin at the beginning of their life span, and retrieved from circulation on immobilized avidin at the end of their life span. This approach avoids the controversial use of density gradient centrifugation to collect presumably old RBCs. Furthermore, the dog is an appropriate model for human RBC senescence because it has a low degree of random RBC loss and a similarly long RBC life span (~110 days). Two dogs had 97% to 100% of their circulating RBCs biotinylated by infusion of N-hydroxysuccinimido biotin (Clontech, Palo Alto, CA; Calbiochem, La Jolla, CA) dissolved in dimethyl sulfoxide. At postbiotinylation days 104 and 107 for one dog and day 110 for the other dog, biotinylated RBCs were isolated by magnetic cell sorting and analyzed for the presence of autologous IgG using 125I-labeled sheep-antidog IgG (SAD IgG). On all 3 days, there were at least three times more SAD IgG molecules per RBC on senescent biotinylated RBCs than on control (unfractionated) RBCs (day 104: 11,677 ± 3,399; day 107: 6,710 ± 2,115; day 110: 6,042 ± 1,838 molecules of SAD IgG per senescent v control RBC). Furthermore, it is unlikely that an immune response to the conjugated biotin had been elicited, because fresh in vitro biotinylated RBCs that were incubated in autologous plasma (taken after exposure to circulating biotinylated RBCs for 113 days) and then exposed to the SAD IgG showed no increase in antibody binding over control (nonbiotinylated) RBCs (1,431 ± 1,378 cpm/106 biotinylated v control RBCs; P > .20). These results suggest that senescence of canine biotinylated RBCs is characterized by binding of autologous IgG and that antibiotin antibodies do not contribute to this process.

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MATERIALS AND METHODS

Dogs were healthy, mature male beagles or beagle/basset hound crosses obtained from a commercial random source vendor. They were housed indoors and maintained according to Purdue University Animal Care and Use Committee regulations. Dogs were tested for blood and intestinal parasites and received appropriate therapy for intestinal parasitism. All dogs were free from heartworm microfilaria. Results from complete blood counts, reticulocyte counts, and routine clinical chemistry panels were within acceptable limits.

N-hydroxysuccinimido (NHS)-biotin was from Clontech (Palo Alto, CA) or Calbiochem (La Jolla, CA). Dimethylacetamide and bovine serum albumin (BSA) were obtained from Sigma (St Louis, MO). Dimethyl sulfoxide (DMSO) (90%, medical grade) was from Syntex (West Des Moines, IA). For purification of biotinylated RBCs by magnetic cell sorting (MACS), avidin fluorescein isothiocyanate (FITC), steel-wool columns (MACS columns) and biotinylated magnetic microbeads (MACS microbeads) were purchased from Becton Dickinson (San Jose, CA). Agents for the biocinchoninic acid (BCA) protein assay and lodoantigen were obtained from Amersham (Arlington Heights, IL) and desalting columns (PD-10) were from Pharmacia (Piscataway, NJ). All other chemicals were of reagent grade or better and were purchased from major suppliers.

Phosphate-buffered saline (PBS) contained 125 mmol/L sodium chloride, 20 mmol/L sodium phosphate with 5 mmol/L EDTA and 0.01% sodium azide, pH 7.4. PBS with glucose (PBS-G) consisted of PBS with 5 mmol/L glucose. BSA solution contained 1% BSA in PBS-G.

In vitro biotinylation of canine RBCs. Canine RBCs were biotinylated in vitro using a modified form of Suzuki and Dale's method. Heparinized whole blood was washed three times in PBS, removing the buffy coat, and resuspended at 10% hematocrit in PBS-G. NHS-biotin (0.55 mg/10 mL RBCs or 161.1 µmol/L final) dissolved in dimethylacetamide (~5 mg NHS-biotin/0.250 mL dimethylacetamide) was added and the mixture was incubated 1 hour at 37°C. Biotinylated RBCs were then washed three times in PBS.

In vivo biotinylation of canine RBCs. Two dogs (Ro2, Ri5) had 97% to 100% of circulating RBCs biotinylated by intravenous (IV) transfusion of NHS-biotin dissolved in DMSO (modified from Dale and Norenberg as described by Christian). NHS-biotin was dissolved in medical grade 90% DMSO (solubility is approximately 200 mg/mL) and administered very slowly through an indwelling (IV) catheter. Dog Ro2 received 20 mg NHS-biotin per kilogram body weight over approximately 60 minutes. Dog Ri5 received 50 mg NHS-biotin per kilogram body weight total, in two divided doses separated by about 4 hours. Pretreatment with atropine sulfate (0.05 mg/kg) was used to counteract cholinergic-like side effects (eg, bradycardia) of DMSO.

To produce a larger population of biotinylated RBCs for collection at the end of the RBC life span, biotinylation was performed during reticulocytosis in response to an iatrogenic blood-loss anemia. The dogs were bled 3 to 4 times over a 3- to 5-day interval to reduce hematocrits to approximately 25% (normal = 37% to 55%) and biotinylation was performed 8 to 11 days after the first bleeding.

Isolation of in vivo aged biotinylated RBCs by magnetic cell sorting. Detailed procedures for isolating in vivo aged biotinylated RBCs by MACS have been described elsewhere. Heparinized whole blood was briefly washed three times in cold PBS-G, removing the buffy coat and plasma proteins. The percentage of biotinylated RBCs in circulation was determined by incubating an aliquot of RBCs (2% hematocrit) with avidin-FITC and analyzing for fluorescent cells by flow cytometry. Then, the biotinylated cells in washed, unfractionated samples were labeled for sorting by incubating with avidin-FITC in PBS-G for 10 minutes on ice (0.025 mg avidin-FITC per 0.020 mL packed biotinylated RBCs in 2.5 mL aliquots). The cells were washed twice in PBS-G followed by labeling with biotinylated MACS magnetic microbeads (0.0045 mL microbeads per 0.020 mL packed biotinylated RBCs in 2.5-mL aliquots for 5 minutes on ice). The avidin-FITC served as a bridge connecting the biotinylated cells to the biotinylated MACS microbeads and provided a fluorescent marker that facilitated rapid assessment of sorting efficiency.

For sorting, the RBCs were diluted to 1% hematocrit in BSA, applied to the separation column containing plastic-coated steel wool, and placed in the magnetic field of the permanent magnet where labeled RBCs adhered to the steel wool and nonlabeled RBCs were eluted. The column was then removed from the magnet and biotinylated RBCs were flushed from the column. The sorting procedure was then repeated using only the positive fraction. The purity of biotinylated (fluorescent) RBCs in various fractions was assayed by flow cytometry.

Iodination of SAD Ig. Standard procedures were used to radiolabel SAD IgG with 125I using iodobeads. (0.5 mCi) was added to a plastic 1.5-mL centrifuge tube containing five washed iodobeads in 0.5 mL PBS. After 5 minutes, 5.5 mg of antoglobulin in 0.5 mL PBS was added and incubated for 30 minutes at room temperature. The fluid was removed and unreacted free 125I was separated from the protein bound form by passage through a desalting column. Protein concentration was determined by the bicinchoninic acid method and radioactivity was measured in a Packard Cobra Gamma counter (Packard Instrument Co, Meriden, CT).

125Iodine SAD IgG binding assay: Detection of autologous IgG on senescent biotinylated RBCs. For each sample (RBCs collected at days 104, 107, and 110) four presorted aliquots, four negative aliquots, and (depending on the number of cells available for analysis) one or two positive aliquots of RBCs isolated by MACS were examined. In each case, 0.060 mL of RBCs at a 50% hematocrit in BSA was mixed with 0.050 mg (~0.020 mL) of 125I-SAD IgG or nonspecific control IgG. The mixture was incubated 90 minutes at 37°C and the RBCs were washed five times in BSA solution. Radioactivity (cpm) was assayed, volumes were measured and manual RBC counts (three replicates per aliquot) were performed. The number of SAD IgG molecules per RBC were calculated from these data. Because data from days 107 and 110 included only one positive aliquot, the results were described but were not subjected to statistical analysis. Data on day 104 was statistically analyzed using Student's t-test. A P value of <.05 was considered statistically significant.

Assay for autologous antibiotorin antibodies. Plasma from dog Ri5 was withdrawn 113 days postbiotinylation and frozen. Blood from the same dog was withdrawn 120 days postbiotinylation, when circulating biotin-positive RBCs were negligible. This blood was used to prepare unmodified (control) and freshly biotinylated RBCs for use in evaluating the existence of antibiotorin IgG in the plasma. Three aliquots of each RBC sample (0.040 mL, 50% hematocrit) were incubated with autologous plasma (0.060 mL) for 60 minutes at 37°C. The RBCs were washed three times in PBS-G and resuspended in 0.050 mL of BSA solution. The RBC suspensions were then incubated with 125I-SAD IgG (0.050 mg) for 60 minutes at 37°C followed by three washes and resuspension with 0.125 mL BSA-G. The radioactivity (cpm) was determined, volumes were measured and manual RBC counts (three replicates per aliquot) were performed.
INCREASED IgG ON CANINE SENESCENT RBCs

Data were statistically analyzed using Student’s t-test. A P value of <.05 was considered statistically significant.

RESULTS

Biotinylation, survival and magnetic sorting of canine RBCs. For both dogs, prebiotinylation phlebotomies resulted in a greater than fourfold increase in the number of reticulocytes per microliter of blood at peak production (dog Ro2: 35,000 v 149,000 and dog Ri5: 48,000 v 220,000 reticulocytes/µL blood at baseline v peak production). Hematocrits returned to normal values in approximately 30 days.

Infusion of NHS-biotin labeled 97% to 100% of circulating RBCs. The percentage of biotinylated RBCs in circulation at the end of the study was 2.2% (dog Ri5, day 104), 1.8% (dog Ri5, day 107), and 3.7% (dog Ro2, day 110). These values are consistent with normal RBC life spans for these dogs based on previous 14C cyanate labeling studies (data not shown).

The purity of biotinylated RBCs in final isolates was 90% (day 104), 81% (day 107), and 83% (day 110). Negative fractions from magnetic sorting contained 1% or fewer biotinylated RBCs.

Detection of autologous IgG on in vivo aged canine RBCs. 125I-labeled SAD IgG was used to detect autologous IgG on senescent canine biotinylated RBCs from two dogs. Results of assays at a total of three time points are shown in Fig 1 (dog Ri5, day 104), Fig 2 (dog Ri5, day 107), and Fig 3 (dog Ro2, day 110). In each figure, values for presorted and negative fractions are the mean of four replicates (±1 SD). For each day’s analysis, there was no evidence of a statistical difference in IgG binding between presorted RBCs and the negative fraction of sorted cells (P > .20 for each day). The positive fraction in Fig 1 is the mean of two aliquots (±1 SD). However, as explained previously, only one aliquot of positive cells was available on days 107 and 110. Therefore, SD lines are not included for the positive fraction in Figs 2 and 3.

The mean number of SAD IgG per RBC on presorted and
negative versus positive fractions was 3,399 and 3,167 versus 11,677 on day 104; 2,115 and 2,255 versus 6,710 on day 107; and 1,838 and 1,791 versus 6,042 on day 110. Compared with respective controls (presorted and negative fractions), positive fractions of in vivo aged biotinylated RBCs bound more than three times as many SAD IgG per cell. On day 104 (Fig 1), this difference was statistically significant ($P = .0044$). Although the single aliquot of senescent RBCs on days 107 and 110 preclude statistical analysis, these values are very compatible with day 104 results in that the positive fractions are consistently greater than 3 SDs above the mean of their respective control population, a range that should include 99% of all control samples (assuming a normal distribution).

Because negative fractions had been depleted of biotinylated (and thus old) RBCs, they might have been expected to show considerably less anti-IgG binding than presorted fractions. However, differences in the amount of biotinylated RBCs in whole blood (presorted) versus negative fractions were 3% or less, and so the differences in anti-IgG binding might be expected to be negligible.

Detection of autologous antibiotin antibodies on senescent canine RBCs. Demonstrating increased autologous antibody binding to senescent biotinylated RBCs provides no information on the antigen that is recognized. Of concern is the possibility that the biotin on the membrane proteins might have been antigenic and induced an immune response during the ~110 days in circulation. Although a significant antibiotin response seems unlikely considering the observed normal survivability of the biotinylated cells (data not shown), we still looked for evidence of antibiotin antibodies in the serum of a dog whose blood contained biotinylated RBCs for the previous 113 days. For this purpose, the above serum was incubated with nonbiotinylated and freshly biotinylated cells from the same dog, and the levels of autologous IgG binding were compared (Fig 4).

The mean of three replicates for control and biotinylated RBCs are strikingly similar ($1,378$ and $1,431$ cpm/10$^6$ RBCs, respectively), exhibiting less than a 4% difference between them ($P > .20$). Thus, there is no evidence for antibiotin antibodies arising in dog RIs's serum and contributing to the increased IgG binding observed on the 104- and 107-day-old cells (Figs 1 and 2). This finding is consistent with observations reported for in vivo aged biotinylated RBCs in the rabbit.28

**DISCUSSION**

We provide evidence that canine senescent RBCs do indeed bind more autologous IgG than younger cells and that this increased binding is not likely a result of labeling RBCs with biotin. Because the results were obtained using unquestionably senescent RBCs from an animal species that, like humans, has low random RBC loss and long RBC survival, it seems reasonable to suggest that human RBC senescence may be characterized by enhanced autologous antibody binding. Such binding on dense, presumably old, human RBCs has been documented repeatedly.19,22,26 Furthermore, several studies suggest that increased IgG binding mediates phagocytic removal of dense/old human RBCs19,30,31 and senescent RBCs from hypertransfused mice.23 Extrapolation from such studies suggests that increased IgG on canine senescent RBCs are likely to be important in facilitating removal of old RBCs. Even so, the primary function of bound IgG in senescent RBC removal may be to initiate complement fixation rather than serve as an independent opsonin.18,30

Our findings contrast with the lack of antibody binding described when the biotinylation system was used to isolate senescent rabbit RBCs.24 However, they are similar to findings based on analysis of erythrocytes from hypertransfused mice,23 another unambiguous method for collecting old RBCs. It is very possible that these differences reflect species variations in the mechanisms governing RBC turnover. However, a reason for immunologic removal of senescent RBCs in some species but not in others is not immediately obvious. Comparative physiology of the rabbit and the dog might suggest that nonimmunologic removal of senescent rabbit RBCs is associated with the rabbit's higher random RBC loss and shorter RBC life span.11 However, this association is not supported in the mouse, which has random RBC loss and a short RBC life span similar to the rabbit.12

It is unfortunate that low numbers of available cells allowed examination of only one positive fraction each on days 107 and 110. However, given the degree of increase in IgG binding over control samples and the agreement of this increase with the statistically significant difference between positive and control fractions on day 104, it is unlikely that the antibody counts on positive fractions for days 107 and 110 represent a random fluctuation of their control counts. Nonetheless, this difference cannot be verified statistically and thus requires cautious interpretation.

Finding increased quantities of IgG on old RBCs does not clarify the nature of membrane changes responsible for IgG binding. Since numerous sites and mechanisms for IgG binding to old RBCs have been suggested,29,31,33,34 further
experiments are required to identify the senescence-associated antigenic sites. Previously, we have presented considerable evidence that the senescence antigen resides on the membrane protein band 3 and that band 3 clustering is required for its expression. The biotinylation system in the dog provides an excellent methodology to test this and other hypotheses.

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