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Erythrocyte homeostasis: Antibody-mediated recognition of the senescent state by macrophages

(aging/hypertransfusion/phagocytosis/autologous antibodies)

J. A. SINGER*, L. K. JENNINGS*†, C. W. JACKSON‡, M. E. DOCKTER*§, M. MORRISON*, AND W. S. WALKER¶

Departments of *Biochemistry, ‡Hematology-Oncology, and ¶Immunology, St. Jude Children's Research Hospital, Memphis, TN 38101

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ABSTRACT **We tested the hypothesis that the accumulation of bound autologous antibody on a "senescent epitope" identifies aged erythrocytes for phagocytic removal by macrophages. Erythrocytes were collected from mice maintained on a hypertransfusion protocol designed to yield cells of defined age. The mouse erythrocytes were assayed for the presence of bound antibody by measuring their susceptibility to ingestion by macrophages from mouse peritoneal exudates and by flow cytofluorometry. Both assays disclosed that only the oldest mouse erythrocytes bore detectable levels of antibody. Flow cytofluorometric analysis revealed that the frequency distribution of IgG isotypes bound to the cells reflected their levels in normal serum. Finally, treatment with trypsin abolished the ability of the macrophages to ingest erythrocytes aged *in vivo*. These findings support the hypothesis that antibody mediates the clearance of senescent mouse erythrocytes from the circulation and demonstrate that the presence of a trypsin-sensitive recognition structure on macrophages is an essential requirement in this homeostatic process.**

Senescent erythrocytes are selectively recognized and removed from the circulation by macrophages located primarily in the spleen and liver (1, 2). Although various mechanisms have been proposed to account for this selectivity (3), attention has focused on whether an age-dependent accumulation of autoantibody on a "senescent epitope" (3–6) targets aged erythrocytes for removal by the reticuloendothelial system.

As part of our continuing study of erythrocyte homeostasis (7), we adapted the Ganzoni hypertransfusion procedure for *in vivo* aging of rat erythrocytes (8) to the mouse and showed that this method yields populations of uniformly aged mouse erythrocytes. In this report we show that the susceptibility of mouse erythrocytes to phagocytic uptake by mouse peritoneal exudate macrophages (PEM) is a function of cell age and correlates positively with an increase in proportion of IgG-bearing mouse erythrocytes. By the end of their normal life span (≈60 days), mouse erythrocytes bore detectable amounts of autologous antibody, which appeared to mediate the phagocytic removal of the aged cell via a trypsin-sensitive receptor on the macrophage.

MATERIALS AND METHODS

Mice. C57BL/6 female mice weighing ≈20 g each (The Jackson Laboratory) were used as a source of macrophages. Retired breeder females of the same strain and from The Jackson Laboratory were used in the hypertransfusion protocol for the preparation of old erythrocytes.

Macrophages. PEM were obtained 3–4 days after the animals had been injected intraperitoneally with 1.0 ml of Brewer's thioglycollate broth (Difco). The cells were washed and resuspended in RPMI 1640 containing 10 mM Hepes (pH 7.2) and 0.07% bovine serum albumin (assay medium).

Isolation and Radiolabeling of Erythrocytes. Mouse erythrocytes were collected in heparin and acid citrate/dextrose (National Institutes of Health Formula A) and radiolabeled with ^{51}Cr as described (9). ^{51}Cr-labeled mouse erythrocytes were washed three times in RPMI 1640/10 mM Hepes, pH 7.2, and resuspended in assay medium.

Antisera. Rabbit antisera to mouse IgG and the IgG isotypes were obtained from Litton Bionetics; fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG was purchased from Sigma; and purified rabbit anti-mouse IgG and anti-human IgA (α-chain specific) antisera were from Cooper Biomedical (Malvern, PA).

Flow Cytofluorometric (FCM) Analysis. Samples (150 μl) of mouse blood in heparin and acid citrate/dextrose were centrifuged at 800 rpm for 10 min. The buffy coat and plasma were removed, and the mouse erythrocytes were washed three times in saline. The cells were resuspended in 1.0 ml of phosphate-buffered saline (PBS, 150 mM NaCl, 6 mM sodium phosphate, pH 7.4) and then further diluted 1:5 in PBS. A 500-μl aliquot of the diluted suspension was mixed with a saturating concentration (15 μl) of rabbit anti-mouse IgG antibody (15 μg of protein), and the mixture was incubated at room temperature for 1 hr. The cells were then washed twice in PBS, resuspended to 500 μl, and 10 μl (50 μg, a saturating amount) of FITC-conjugated goat anti-rabbit IgG was added. After 30 min at room temperature, the cells were washed twice with PBS and resuspended in PBS containing 0.5% bovine serum albumin. The suspensions of mouse erythrocytes were analyzed by established procedures (10) with a Coulter EPICS V flow cytometer, using the 488-nm excitation line of the argon laser operated at 800 mW. A 510-nm bandpass filter and green photomultiplier tube combination were used to detect the positive fluorescein signal, and the accumulated data from each assay were stored in a Coulter Multiparameter Data Analysis and Digital Storage (MDADS) system. Typically, 100,000 cells were analyzed in each assay. The results were processed by the IMMUNO program of the Coulter MDADS system that calculates the percentage of cells that display positive fluorescence over the background level of a control cell sample.

Phagocytosis Assay. The phagocytic uptake of ^{51}Cr-labeled mouse erythrocytes was measured (7, 9). Briefly, PEM and erythrocytes were mixed in a final volume of 40 μl in 12 × 75-mm plastic tubes and incubated at 37°C in a humidified atmosphere of 7% CO_2/93% air. At the end of the incubation period, 2.0 ml of medium was added to the tubes to stop the

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Abbreviations: PEM, mouse peritoneal exudate macrophages; FCM, flow cytofluorometric; FITC, fluorescein isothiocyanate.

†Present address:Department of Medicine/Nematology-Oncology, University of Tennessee, Memphis, TN 38163.
§Present address: Department of Medicine/Infectious Diseases, University of Tennessee, Memphis, TN 38163.

reaction. The contents were mixed and centrifuged. The supernatant fluids were removed, and the radioactivity in 1.0-ml aliquots was determined. The cell pellets—consisting of PEM, ingested target cells, and target cells that had not been ingested—were resuspended in 2.0 ml of water to lyse the noningested erythrocytes. After the assay tubes were centrifuged, the water-supernatant fluids were harvested, and the amount of radioactivity in 1.0-ml aliquots was determined. The cell pellets, containing PEM and the ingested mouse erythrocytes, were resuspended in 1.0 ml of water, and the amount of radioactivity was determined.

Ingestion of target cells was expressed as the ratio of the radioactivity of the cell pellets after the water lysis step compared with the total radioactivity in the culture.

Preparation of Age-Defined Mouse Erythrocytes. The hypertransfusion method of Ganzoni *et al.* (8) was adapted to mice (7, 9, 11). Briefly, blood was collected by cardiac puncture, and the plasma and buffy coat were removed. Sufficient erythrocytes were injected into another group of mice to produce a packed cell volume of 65–75%. Packed cell volumes and reticulocyte levels were determined in all hypertransfused mice at regular intervals to ensure maintenance of the polycythemic state. Every 2 weeks, one-half of the hypertransfused mice served as erythrocyte donors for the remaining mice. This procedure, repeated for 6 weeks, suppressed erythropoiesis for up to 8 weeks, roughly the life span of mouse erythrocytes (2). Erythrocytes collected from mice at increasing intervals of hypertransfusion showed progressive changes in a cytoskeletal protein that may be an indicator of erythrocyte age (11) and markedly increase susceptibility to phagocytic uptake both *in vitro* and *in vivo* (7).

RESULTS

Preferential Phagocytosis of Aged Mouse Erythrocyte by PEM. We have shown that the hypertransfusion procedure of Ganzoni *et al.* (8), when adapted to the mouse, yields populations of uniformly aged mouse erythrocytes, judged from several characteristics, including an enhanced susceptibility to phagocytic uptake both *in vitro* and *in vivo* (7). Fig. 1 illustrates this enhanced susceptibility by comparing the phagocytic uptake of erythrocytes from mice that had been hypertransfused for 56 days (mean erythrocyte age of 58 days) with the uptake of erythrocytes isolated from normal mice (mean erythrocyte age of 30 days). First, PEM ingested about 4-fold more erythrocytes from hypertransfused mice than from normal animals. Second, the proportions of cells ingested did not increase as the numbers of macrophages were increased, indicating that only a subpopulation of erythrocytes in either preparation was susceptible to phagocytic removal. To ensure that ingestion of this subpopulation was complete, PEM to mouse erythrocyte ratios of 2.5 or greater were used in all experiments.

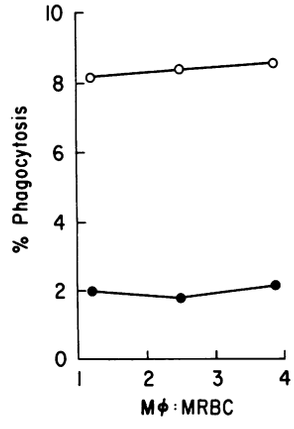

FIG. 1. Phagocytosis of ^{51}Cr-labeled erythrocytes from normal (●) and 56-day hypertransfused (○) mice. Erythrocytes (MRBC, 4 × 10^5) were mixed with the indicated numbers of peritoneal exudate macrophages (Mϕ), and the proportions of target cells ingested were determined 100 min later.

To establish that the enhanced phagocytic susceptibility of erythrocytes from hypertransfused mice was related to age, we mixed mouse erythrocytes obtained at different times during hypertransfusion with PEM and determined the level of phagocytosis for each interval. Fig. 2 shows the combined results of three separate hypertransfusion experiments. The degree of preferential phagocytic uptake (expressed as the ratio of the uptake of the hypertransfused to the normal mouse erythrocyte populations) correlated with mean mouse erythrocyte age.

Antibody-Dependent Phagocytosis of Aged Mouse Erythrocytes. If phagocytosis by PEM required threshold levels of erythrocyte-bound antibody and if the antibody accumulated in an age-dependent manner, then there should be circulating erythrocytes that bear threshold concentrations of antibody and this subpopulation of cells should increase during the hypertransfusion procedure. If this hypothesis is correct, then the addition of an antibody to mouse immunoglobulin should enhance the uptake of erythrocytes, particularly those from mice in the last stages of the hypertransfusion procedure. We added anti-mouse IgG and anti-human IgA (control) antibodies to suspensions of normal and hypertransfused mouse erythrocytes and determined the proportion ingested by PEM. Table 1 shows that while rabbit anti-human IgA had no appreciable effect on the uptake of erythrocytes from either normal or hypertransfused mice, the anti-mouse IgG reagent caused an approximate 2-fold increase in the uptake of erythrocytes in both suspensions. However, while the anti-IgG increased the uptake of 30-day-old erythrocytes from 3 to 6%, it caused an increase of >20% in the phagocytosis of erythrocytes with a mean age of 58 days, indicating that blood from hypertransfused mice contained about 4-fold more autologous antibody-coated erythrocytes than did blood from normal mice.

To investigate further, we selected three concentrations of the antibodies and tested their influence on the phagocytic

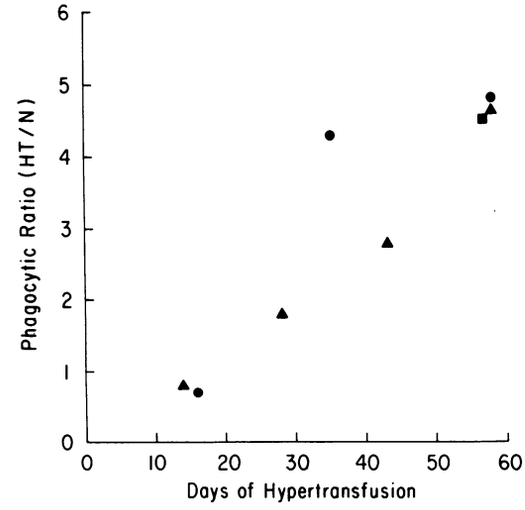

FIG. 2. Ratio of ingestion of erythrocytes from hypertransfused (HT) mice to that of erythrocytes from normal (N) mice as a function of days of hypertransfusion. ^{51}Cr-labeled erythrocytes (4 × 10^5) from normal and hypertransfused mice were mixed with 10^6 PEM, and the proportion ingested was determined 100 min later. Each assay was performed in triplicate with the mean phagocytic values used to calculate the phagocytic ratio HT/N. The symbols ●, ▲, and ■ represent three separate hypertransfusion experiments.

Table 1. Effect of anti-immunoglobulin antibodies on the phagocytosis of mouse erythrocytes

Mean erythrocyte age, days	Anti-human IgA	Anti-mouse IgG	% phagocytosis
30	−	−	2.2 ± 0.2
30	+	−	2.9 ± 0.4
30	−	+	6.0 ± 0.3
58	−	−	9.3 ± 0.4
58	+	−	12.1 ± 1.2
58	−	+	22.0 ± 2.3

Suspensions of erythrocytes (4×10^5) from normal mice (mean erythrocyte age of 30 days) or 56-day hypertransfused mice (mean erythrocyte age of 58 days) were mixed with 16×10^5 PEM in a final volume of 60 μl containing the indicated antibody (40 μg/ml). The proportion of ^{51}Cr-labeled erythrocytes ingested was determined 100 min later. The data are means and ranges for triplicate samples from a single representative experiment.

susceptibility of erythrocytes from mice hypertransfused for 33 and 56 days (estimated mean ages of 47 and 58 days). Table 2 shows that the anti-mouse IgG reagent did not increase the selective uptake of erythrocytes with mean ages of 47 days, but it did enhance the uptake of 58-day-old erythrocytes. Taken together, the data in Tables 1 and 2 are consistent with the idea that antibody accumulates on erythrocytes as a function of cell age.

FCM Analysis of Autologous IgG on Aged Mouse Erythrocytes. To measure directly the accumulation of anti-erythrocyte autoantibody as a function of erythrocyte age, we examined suspensions of mouse erythrocytes by FCM for the presence of surface-bound immunoglobulin during the course of erythrocyte aging in the hypertransfused mouse. In the first series of experiments (Table 3), we tested for the proportion of cells bearing detectable immunoglobulin, using a FITC-labeled rabbit anti-mouse immunoglobulin reagent. The results show that <1% of erythrocytes from mice hypertransfused for 16 and 33 days bore detectable immunoglobulin, while about 10% of the erythrocyte suspension with a mean cell age of 58 days had detectable immunoglobulin. These results agree with those in Table 2 showing that anti-IgG causes an additional ingestion of about 10% of erythrocytes from 56-day hypertransfused mice.

Since macrophages bear isotype-specific Fc receptors (12, 13), we next tested whether or not the accumulated autologous immunoglobulin found on 58-day-old mouse erythrocytes was isotype restricted. Three of the isotypes (IgG1, IgG2a, and IgG2b) were present on between 8% and 19% of

Table 2. Influence of anti-mouse immunoglobulin on the net phagocytosis of aged mouse erythrocytes

Erythrocyte age, days	Antiserum, μg/ml	Net phagocytosis*	
		Anti-IgA	Anti-IgG
47	5	9 ± 1	7 ± 1
	10	9 ± 1	9 ± 3
	20	9 ± 3	7 ± 5
58	5	9 ± 1	18 ± 3
	10	10 ± 1	16 ± 4
	20	12 ± 3	16 ± 5

Suspensions containing 4×10^5 ^{51}Cr-labeled erythrocytes from normal mice and mice hypertransfused for 33 days (mean age of 47 days) and 56 days (mean age of 58 days) were mixed with 16×10^5 PEM in the presence of the indicated antisera. The proportion of cells ingested was determined 100 min later. Net phagocytosis values were determined by subtracting the uptake of normal mouse erythrocytes incubated with the antibodies from the values obtained with erythrocytes from hypertransfused mice.

*All assays were performed in triplicate, and the means and ranges are presented.

Table 3. FCM analysis for autologous IgG on aged mouse erythrocytes

Mean erythrocyte age, days	% IgG$^+$ mouse erythrocytes
38	<1.0
47	<1.0
58	10.3

Suspensions of mouse erythrocytes were mixed with FITC-conjugated rabbit anti-mouse IgG antiserum, and the washed cells were examined by FCM. The results are the means of three determinations from a single hypertransfusion experiment; the percentages of IgG$^+$ (positive) cells present in suspensions of erythrocytes from normal mice (mean erythrocyte age of 30 days) have been subtracted.

the cells, whereas about 1% of the cells bore the IgG3 isotype (data not shown). Hence, the distribution of isotypes on the erythrocytes reflected their relative content in serum (14).

Phagocytosis via a Trypsin-Sensitive Receptor on PEM. Our previous studies showed that the recognition of normal mouse erythrocytes by a macrophage cell line is abolished by treating the macrophages with trypsin (unpublished data). We next determined the effect of trypsin on the ability of PEM to ingest erythrocytes aged in hypertransfused mice. Incubation for 10 min with enzyme at 500 μg/ml reduced the uptake of 58-day-old mouse erythrocytes by about 90% (Table 4). However, the enzyme-treated macrophages were able to ingest antibody-sensitized heterologous erythrocytes (data not shown) through trypsin-resistant Fc receptors. Also, consistent with previous studies (unpublished data), the uptake of the susceptible subpopulation of erythrocytes from normal mice was sensitive to the enzyme (Table 4).

DISCUSSION

Macrophages are the effectors of hematologic homeostasis (3, 16), but the means by which they recognize senescent erythrocytes remains obscure. Because older erythrocytes appear to be smaller, denser, and less deformable (17), some investigators propose that they become trapped in the sinusoidal network in the spleen, where their decreased deformability (18) increases their chances of binding to macrophages. Others suggest that older erythrocytes have a decreased sialic acid content, which can be detected by macrophages (3). Still others propose that aging erythrocytes accumulate an autoantibody that, on reaching a threshold level, targets the cell for removal (3–6).

To examine the last possibility, we adapted the hypertransfusion procedure of Ganzoni *et al.* (8) to mice. The availability of *in vivo*-aged mouse erythrocytes, coupled with the finding that their selective uptake is mediated by a trypsin-sensitive surface structure on a macrophage cell line (unpublished data), prompted us to test directly whether PEM selectively recognize erythrocytes aged in hypertransfused

Table 4. Trypsin sensitivity of selective phagocytosis

Trypsin, μg/ml	% phagocytosis of mouse erythrocytes*	
	Normal mice	Hypertransfused mice
0	1.6 ± 0.2	9.5 ± 1.2
50	1.4 ± 0.5	10.7 ± 0.7
500	0.1 ± 0.3	1.3 ± 0.7

Suspensions of PEM were incubated with trypsin for 10 min at 37°C, washed, and then 1×10^6 cells were mixed with 4×10^5 ^{51}Cr-labeled erythrocytes from normal or 56-day hypertransfused mice (mean erythrocyte age of 58 days). The amount of mouse erythrocytes ingested was determined 100 min later.

*The means and ranges are given for triplicate samples from a single representative experiment.

mice and, if so, whether their preferential uptake depends upon the accumulation of antibody.

In experiments to confirm our studies (unpublished data), only a subpopulation of erythrocytes from either normal or hypertransfused mice was susceptible to phagocytic uptake (Fig. 1). We also found that selective ingestion of the subpopulation of erythrocytes from hypertransfused mice increased as a function of erythrocyte age (Fig. 2) and was mediated by a trypsin-sensitive surface structure (Table 4).

FCM analysis of erythrocyte samples from mice undergoing hypertransfusion (Table 3) showed that about 10% of the cells obtained at 56 days of hypertransfusion (mean erythrocyte age of 58 days) bore detectable immunoglobulin. Consistent with this is the theoretical pattern of erythrocyte removal illustrated in Fig. 3, in which it can be seen that the rate of removal began to increase rapidly only after 50 days of hypertransfusion. This rate assumes that erythropoiesis is completely inhibited by hypertransfusion and that all 60-day-old erythrocytes are removed from the circulation. The experimental value for 58-day-old erythrocytes shown in Table 3 agrees well with the calculated proportion of mouse erythrocytes that would be susceptible to phagocytic removal on day 56 of hypertransfusion (Fig. 3). Since these IgG-bearing erythrocytes appear only after their mean age is greater than 47 days, it is highly unlikely that the antibody was induced in response to a hypertransfused erythrocyte-associated epitope.

Surprisingly, we failed to detect preferential binding of an IgG isotype to erythrocytes from hypertransfused mice, despite finding that the ingestion of these cells was completely abolished when the PEM were treated with trypsin (Table 4). Whether the removal of senescent erythrocytes depends on the binding of a particular isotype is unclear, but apparently the protease-resistant Fc receptor plays little, if any, role in the process.

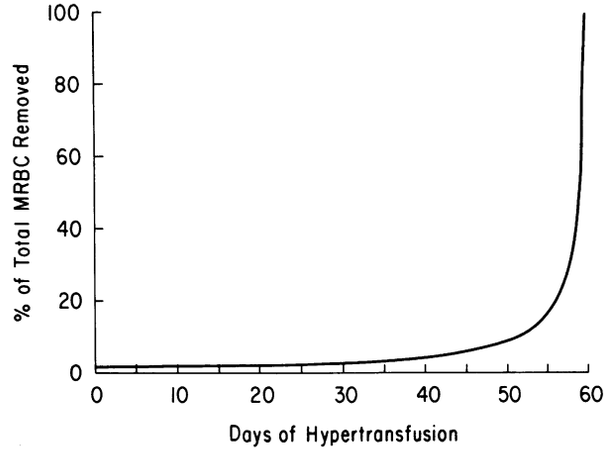

FIG. 3. A computer-generated curve of the proportion of mouse erythrocytes (MRBC) removed from the circulation as a function of hypertransfusion time. The model assumes the life span of all mouse erythrocytes is 60 days, erythropoiesis is completely inhibited by hypertransfusion, and all 60-day-old mouse erythrocytes are removed from the circulation. Thus, on day 1 of hypertransfusion, 1.7% of the cells are removed, and by day 60 all remaining erythrocytes are cleared. The graph is based on the following calculation:

$$\% = [0.0167/1 - (0.0167 \cdot D - 1)],$$

where D = days of hypertransfusion.

Our data directly support the concept that the accumulation of autoantibodies to a senescent epitope(s) signals the removal of old erythrocytes from the circulation. Whether the reactivity of these autoantibodies is limited to epitopes on the anion transporter, protein 3 (19, 20), or is directed to terminal galactose residues on erythrocyte membrane glycoproteins (15, 21) remains unclear. Equally unclear is whether the removal of aged mouse erythrocytes depends on the presence of a particular isotype of antibody; however, a trypsin-sensitive recognition structure on the surface of macrophages appears to play an essential role in the phagocytic process.

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