Mechanisms of anemia in SHP-1 protein tyrosine phosphatase-deficient “viable motheaten” mice

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Objective. Viable motheaten mice (abbreviated gene symbol me)v are deficient in SHP-1, a critical negative regulator of signal transduction in hematopoietic cells. These mice exhibit severe immune dysfunction accompanied by hyperproliferation of myeloid cells, widespread inflammatory lesions, and regenerative anemia. The aim of this study was to investigate the mechanisms underlying anemia in me/v mice.

Materials and Methods. Multiple hematologic parameters, osmotic fragility, and erythropoietin levels were measured to characterize the anemia in me/v mice. B-cell–deficient me/v Igh-6null mice were generated to assess the role of anti-erythrocyte antibodies. Coombs assays and flow cytometry were carried out for detection of anti-erythrocyte antibodies. Oxidant production by macrophages, glutathione levels, and lipid peroxidation products in erythrocytes were measured, as was the impact of oxidant on the ultrastructure of me/v erythrocytes. Erythroid maturation and erythrocyte plasma membrane integrity were assessed with flow cytometry by evaluating CD71 expression and annexin V labeling.

Results. The regenerative anemia of me/v mice was associated with erythrocyte changes that were independent of the presence of anti-erythrocyte antibodies. Erythrocytes from me/me mice had increased fragility and heightened susceptibility to oxidant damage. Macrophages from me/me mice demonstrated a higher basal level of oxidant production and enhanced production after stimulation. Oxidant damage in me/me erythrocytes was evidenced by a significant elevation of lipid peroxidation and diminished levels of glutathione.

Conclusion. Our results support the hypothesis that as a consequence of severe inflammatory disease, me/me erythrocytes are subject to exceptionally high oxidative stress resulting in oxidation of phospholipids in the erythrocyte membrane with subsequent hemolysis. © 2003 International Society for Experimental Hematology. Published by Elsevier Science Inc.

There are three fundamental mechanisms of anemia: blood loss, accelerated rate of erythrocyte destruction, and impaired erythrocyte production [1]. Accelerated destruction (hemolysis) can be caused by inherited disorders of the erythrocyte membrane, abnormalities in hemoglobin synthesis, or specific enzyme deficiencies. Additionally, acquired hemolytic anemias can be caused by pathogenic organisms, exposure to abnormal levels of reactive oxygen species, or as a sequela of immune-mediated disease [2,3]. In this article, we describe the nature of the hemolytic anemia in viable motheaten mice.

The autosomal recessive viable motheaten (Hcphme-v) mutation disrupts the structural gene (Hcph) for hematopoietic cell phosphatase. This gene, located on mouse chromosome 6, encodes a cytoplasmic protein tyrosine phosphatase commonly called Src-homology 2-domain phosphatase-1 (SHP-1). SHP-1 is expressed primarily in hematopoietic cells [4] and functions as a critical negative regulator of signal transduction in a number of immune and hematopoietic signaling pathways [5,6]. Viable motheaten mice (abbreviated gene symbol me-v) are deficient in SHP-1 and express approximately 20% wild-type activity. These mice have a regenerative anemia and severe immune dysfunction with greatly increased numbers of myeloid cells resulting in multifocal pyogranulomas of the skin, lung, and elsewhere.
and consequent agammaglobulinemia [15]. C57BL/6J-(abbreviated gene symbol Mt1/Mt2) mice transgenic for metallothionein, C57BL/6J-TgN(Mt1)174Bri mice was also postulated to be a consequence of anti-erythrocyte antibodies [8].

Studies have shown that colony-forming unit erythroid (CFU-E) progenitor cells in some patients with polycythemia vera (PV) have reduced expression of SHP-1. This myeloproliferative disorder is characterized by hyperproliferation of erythroid cells, myeloid cells, and megakaryocytes. The erythroid progenitor cells of PV patients with reduced expression of SHP-1 have an enhanced proliferative capacity and are hypersensitive to erythropoietin (Epo) [9]. Similarly, there are increased splenic CFU-E progenitors in me/l me mice, and these erythroid progenitor cells are also hypersensitive to Epo [10]. However, unlike PV, the increase in CFU-E progenitors in me/l me mice is not reflected by erythrocytosis, but paradoxically these mice develop a regenerative anemia.

Humans and experimental animals with autoimmune disease and concurrent anemia commonly have a hemolytic anemia secondary to anti-erythrocyte antibodies [2,11–13]. Anemia in me/l me mice was also postulated to be a consequence of anti-erythrocyte antibodies [8]. However, the variable presence of these antibodies in me/l me mice suggests that other mechanisms contribute in significant ways to the development of anemia. In this study, we investigated the mechanisms by which erythrocytes are destroyed in me/l me mice. We hypothesize that as a consequence of persistent inflammatory disease, me/l me erythrocytes are subject to exceptionally high oxidative stress resulting in oxidation of phospholipids in the erythrocyte membrane with subsequent hemolysis.

Materials and methods

Mice

Viable motheaten mice were raised by mating C57BL/6J- +/+Hcph(me/v) (+/me) heterozygotes. Homozygous (me/me) offspring were identified at 4 to 5 days of age by the presence of multifocal alopecia. Heterozygotes (+/me) were identified by polymerase chain reaction as previously described [14]. The targeted disruption of the membrane exon of the immunoglobulin μ gene (Igh-6null) results in a deficiency of mature B cells and consequent agammaglobulinemia [15]. C57BL/6J-Igh-6null mice were mated with C57BL/6J- +/+ me mice to produce mice doubly homozygous for the me mutation and the Igh-6null allele. Mice transgenic for metallothionein, C57BL/6J-TgN(Mt1)174Bri (abbreviated gene symbol Mt1/m1), which carry 112 copies of the Mt1 transgene [16], mice homozygous for targeted mutation of the metallothionein 1 and 2 genes, 129S7/SvEvBrd-Mt1tm1H11001/H9262m2H11034 (abbreviated gene symbol Mt1/m2) [17], and NZB/B1NJ mice were obtained from The Jackson Laboratory Animal Resources colony. All mice were bred and housed at The Jackson Laboratory under standard specific pathogen-free conditions and were provided pasteurized food and acidified water ad libitum.

Hematology

Blood was collected from the retro-orbital sinus with EDTA-coated capillary tubes and placed directly into Isoton II diluent (Coulter, Miami, FL, USA). Hematologic values were determined using the ADVIA120 hematology system (Bayer, Norwood, MA, USA). In some studies, erythrocytes were enumerated using a Z1 Coulter counter. Blood smears were air dried, fixed in methanol, and stained with Wright-Giemsa. For demonstration of reticulocytes, equal volumes of blood and new methylene blue stain were incubated for 15 minutes at room temperature.

Flow cytometry

Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). Cells were prepared in cold Hank’s balanced salt solution (HBSS, Sigma Chemical Corp., St. Louis, MO, USA) containing 5% fetal bovine serum (FBS, Sigma) and 0.1% sodium azide (FACS buffer) and aliquoted at 104 cells per 100 μL in wells of a 96-well microtiter plate. Nonspecific binding of antibody was blocked by incubation with rabbit IgG at 1 mg/mL (Sigma). After blocking, cells were washed in FACS buffer, optimized dilutions of antibodies were added, and the cells were incubated for 1 hour at 4°C. Cells were washed twice in FACS buffer and resuspended in cold HBSS containing 0.1% sodium azide. At least 10,000 cells were analyzed per sample. Annexin V and antibodies were obtained from BD PharMingen Inc. (San Diego, CA, USA) as fluorescein isothiocyanate or phycoerythrin conjugates: anti-IgM, clone R6-60.2; anti-Ig, polyclonal; anti-pan erythrocyte, clone TER-119; anti-CD47, clone miap301; anti-MAC-1, clone M1/70; anti-Gr-1, clone RB6-8C5, and anti-CD71, clone C2.

Scanning electron microscopy

For ultrastructural studies, erythrocytes were fixed overnight in 2% glutaraldehyde and 2% paraformaldehyde in cacodylate buffer (pH 7.3). Cells were washed with buffer, and a thin film of cells was pipetted onto poly-L-lysine coated coverslips. The coverslips were air dried, coated with gold, and were imaged on a JEOL 35C scanning electron microscope at 30 kV. For some studies, erythrocytes were incubated in 10 μM H2O2 for 60 minutes prior to fixation.

Direct and indirect Coombs assays

The indirect Coombs assay was carried out on plasma. Blood was obtained from the retro-orbital sinus of mice through heparin coated microcapillary tubes. After removal of the cells by centrifugation, 25 μL of the plasma was added to U-bottomed microtiter wells containing 25 μL of 1% BSA in phosphate-buffered saline (PBS). The samples were serially diluted in 1% BSA/PBS. To each well, 25 μL of C57BL/6J erythrocytes [prepared from heparinized blood in PBS as 1% (v/v)] were added. The microtiter plate was tapped to mix the cells with the plasma dilutions, covered, and incubated at 4°C for 2 hours. Agglutination was assessed as the inhibition of the formation of a tight cell button in the bottom of the wells and is expressed as the last dilution to give a positive agglutination reaction. The direct Coombs assay was carried out with heparinized blood cells that were washed with 1% FBS/PBS. Fifty microliters of a 1% solution of blood cells in 1% FBS/PBS was added to 50 μL of rabbit anti-mouse Ig (heavy and light chain specific). After mixing, the wells were incubated as described earlier and scored for agglutination.
Osmotic fragility
Erythrocytes were collected from the retro-orbital sinus through heparinized capillary tubes, washed with PBS, and resuspended to 1% (v/v). Aliquots (50 μL) of the erythrocyte suspension were added to 50 μL of PBS that had been serially diluted with distilled water in a 96-well V-bottomed microtiter plate. The cell suspensions were allowed to stand at room temperature for 30 minutes and then centrifuged to pellet intact cells. A 50-μL sample of the supernatant was transferred to a flat-bottomed microtiter plate and the optical density at 523 nm was determined with a Spectromax microplate reader (Molecular Devices, Menlo Park, CA, USA). Values were normalized to complete erythrocyte lysis in distilled H2O.

Oxidant production
Oxidant production by Mac-1+ bone marrow cells was determined by flow cytometry using dihydrorhodamine-123 (DH-123, Molecular Probes, Eugene, OR, USA) as previously described [18]. Briefly, 25 × 10^6/mL bone marrow cells were incubated with 1 mg/mL rabbit IgG (Sigma) for 1 hour at 4°C. Cells were rinsed, optimized dilutions of cell lineage-specific antibodies were added, and the cells incubated for 1 hour at 4°C. Cells were washed once in FACS buffer and resuspended at 5 × 10^6/mL, and DH-123 was added for a final concentration of 1 μM. Replicate aliquots were stimulated with phorbol 12-myristate 13-acetate (PMA, Sigma) at time zero, and all aliquots were transferred to a 37°C water bath. Cells were analyzed by flow cytometry at time zero and at 10-minute intervals for 1 hour. At least 10,000 cells were analyzed per sample following incubation with antibodies for the cell lineage-specific markers Gr-1 and Mac-1.

Erythrocyte glutathione levels
Blood was drawn into EDTA capillary tubes, centrifuged at 1,000g for 5 minutes, and 20 μL of packed erythrocytes was collected. The cells were lysed in ice-cold HPLC-grade H2O, centrifuged at 10,000g for 15 minutes at 4°C, and the supernatants (erythrocyte lysates) collected and stored on ice. The erythrocyte lysates were deproteinated and assayed for glutathione (GSH) according to the manufacturer’s instructions using a glutathione assay kit (Cayman Chemical, Ann Arbor, MI, USA). Optical density was measured at 405 nm in a Spectromax microplate reader.

Assay for thiobarbituric reactive substrates
Measurement of thiobarbituric reactive substrates (TBARS) was done essentially as described by Jain [19]. 1,1,3,3-tetramethoxypropane [malondialdehyde (MDA)] was used as a standard for these assays. Optical density measurements were made at 532 and 600 nm using a Spectromax microplate reader. Quantification was based upon a molar extinction coefficient 1.56 × 10^5 M^-1 cm^-1.

Epo levels
Serum Epo levels were determined using a radioimmunoassay as previously described [20].

Statistical analysis
Data were analyzed using the Prism Statistical Software package (GraphPad Software, San Diego, CA, USA). Unpaired t-tests were done to assess statistical significance. p < 0.05 was considered statistically significant.

Results
Viable mev/mev mice display significant hematologic anomalies
Evaluation of blood smears from mev/mev mice revealed the presence of anisocytosis and polychromasia. These changes in erythrocyte morphology and staining properties are reflective of reticulocytes, which are larger than mature erythrocytes and contain residual RNA (Fig. 1A and B). Specific staining for reticulum confirmed the presence of greatly elevated numbers of reticulocytes in mev/mev smears (Fig. 1C and D). Increased reticulocyte numbers are indicative of a responding bone marrow and a normal erythropoietic response. These findings are consistent with a regenerative anemia. Young adult mev/mev mice (5–7 weeks of age) do not exhibit significant abnormalities in erythrocyte numbers or in hematocrit values (Table 1). However, these mice show erythrocyte anomalies consistent with a compensated regenerative anemia. Erythrocytes from 5- to 7-week-old mev/mev mice have increased mean cell volumes, decreased mean cell hemoglobin levels, and include a two-fold higher percentage of reticulocytes when compared to littermate controls. By 12 weeks of age, mev/mev mice are unable to compensate for their anemia as evidenced by a decrease in erythrocyte numbers despite a 10-fold increase in reticulocyte percentage. This inability to balance erythrocyte loss with erythrocyte production is not due to reduced Epo levels, as mev/mev mice have a 50-fold increase in plasma Epo when compared to littermate control mice (250 mU/mL for mev/mev vs 5 mU/mL for littermate control; pooled samples of six mice for each genotype).

Figure 1. Blood cell morphology. (A,B) Wright-Giemsa stained blood smears. (A) +/- erythrocytes exhibit normal size and staining. (B) mev/mev erythrocytes exhibit polychromasia and anisocytosis. (C,D) New methylene blue stained blood smears. (C) Normal numbers of reticulocytes in +/- blood. (D) mev/mev blood film shows increased numbers of reticulocytes. Reference bar = 20 μm.
Table 1. Erythrocyte values for me/mem and littermate control mice

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Genotype</th>
<th>RBC × 10⁶/mL</th>
<th>Hematocrit (%)</th>
<th>Mean cell volume (fL)</th>
<th>Mean cell hemoglobin (g/dL)</th>
<th>Reticulocyte (%)</th>
</tr>
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<tbody>
<tr>
<td>5–7</td>
<td>+/-</td>
<td>9.3 ± 0.2</td>
<td>47.6 ± 1.0</td>
<td>51.2 ± 0.3</td>
<td>30.1 ± 0.3</td>
<td>7.9 ± 1.4</td>
</tr>
<tr>
<td>5–7</td>
<td>mem/mem</td>
<td>8.9 ± 0.2</td>
<td>49.2 ± 1.6</td>
<td>55.4 ± 1.0</td>
<td>27.3 ± 0.3</td>
<td>17.6 ± 2.8</td>
</tr>
<tr>
<td>&gt;12</td>
<td>+/-</td>
<td>9.5 ± 0.3</td>
<td>46.9 ± 2.0</td>
<td>49.2 ± 1.0</td>
<td>32.4 ± 0.5</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>&gt;12</td>
<td>mem/mem</td>
<td>6.9 ± 1.0*</td>
<td>44.8 ± 6.3</td>
<td>65.2 ± 2.5†</td>
<td>25.0 ± 3.6</td>
<td>30.4 ± 5.8†</td>
</tr>
</tbody>
</table>

Data are given as mean ± SEM of values for 4–8 individual mice.

Comparison of age-matched mem/mem and +/- blood values show statistically significant differences: *p < 0.05; †p < 0.01; ‡p < 0.001.

Anti-erythrocyte antibodies in me/mem mice
Viable motheaten mice develop an autoimmune disease characterized in part by the presence of high levels of circulating autoantibodies [8]. To determine if autoantibody directed against erythrocyte plasma membrane determinants was present in me/mem mice, indirect and direct antiglobulin (Coombs) assays were carried out. The indirect Coombs assay, which detects anti-erythrocyte antibodies in the plasma, was positive in 11 of 11 me/mem mice tested. Younger me/mem mice had the lowest titers, whereas most me/mem mice over 70 days of age exhibited higher titers (Fig. 2A). Anti-erythrocyte autoantibodies were not detected in plasma from any littermate control mice tested (data not shown). The direct Coombs assay, which detects endogenous antibodies attached to the erythrocyte membrane, was negative in all me/mem and littermate control mice tested (data not shown). We also used flow cytometry to detect any erythrocyte-bound immunoglobulin. Erythrocytes from me/mem mice did not display bound IgM or polyclonal Ig at levels higher than littermate controls (Fig. 2B). Blood from an aged NZB mouse was used as a positive control [21].

Absence of immunoglobulin production does not prevent anemia in me/mem mice
As another measure of the contribution of anti-erythrocyte antibodies to the anemia of me/mem mice, we examined the hematologic characteristics of me/mem mice that also were homozygous for a targeted disruption of the membrane exon of the immunoglobulin μ gene (Igh-6mev) (abbreviated gene symbol Igh-6null). This targeted mutation results in B-cell deficiency [15]. As previously reported, Igh-6null mice express little or no serum immunoglobulin with the exception of small amounts of IgA [22]. In our laboratory, adult Igh-6null mice (14–30 weeks old) had less than 3.1 μg/mL of serum Ig by enzyme-linked immunosorbent assay (data not shown). This targeted mutation was combined with the me mutation by selective breeding. Mice homozygous for both me/mem and the Igh-6null allele showed significant changes in erythrocyte parameters similar to age-matched me/mem mice (Table 2). Erythrocytes from 8-week-old me/mem Igh-6null mice had increased mean cell volumes, decreased mean cell hemoglobin levels, and a higher percentage of reticulocytes than did age-matched +/me Igh-6null controls (Table 2). These results are consistent with a compensated anemia, as is seen in young me/mem mice. This confirms that the presence of anti-erythrocyte autoantibody is not essential for the development of anemia in me/mem mice.

![Figure 2](image-url)
Evidence of membrane damage in $me^v/me^v$ erythrocytes

Normal shape and deformability are critical to erythrocyte survival in vivo. Because defects in the structural integrity of the erythrocyte membrane render them susceptible to premature removal from circulation, we examined erythrocytes from $me^v/me^v$ mice by electron microscopy. Erythrocytes from littermate control mice had a normal discoid shape, whereas erythrocytes from $me^v/me^v$ mice showed mild deformation of the erythrocyte membrane (Fig. 3A and B). Moderate deformation of the erythrocyte membrane was evident in erythrocytes held for 1 hour at room temperature prior to fixation (Fig. 3C and D). Blood samples from mice of both genotypes contained echinocytes, but echinocytes were more numerous in $me^v/me^v$ blood. These membrane alterations were exacerbated by incubation of the erythrocytes with H$_2$O$_2$, a strong oxidant, prior to fixation. Blood from littermate control mice subjected to H$_2$O$_2$ incubation resulted in a small number of echinocytes, whereas H$_2$O$_2$-treated $me^v/me^v$ blood contained a large proportion of echinocytes (Fig. 4A and B). Erythrocytes from $+/Igh-6^{null}$ mice showed minimal morphologic changes, whereas blood from $me^v/me^v$ Igh-6$^{null}$ mice contained a large proportion of echinocytes similar to the numbers observed in $me^v/me^v$ blood (Fig. 4C and D). Because metallothionein is an important antioxidant in erythrocytes, we also explored the impact of overexpression and targeted disruption of met-

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</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>$+/me^v$ Igh-6$^{null}$</td>
<td>8.6 ± 0.2</td>
<td>43.8 ± 0.6</td>
<td>51.4 ± 1.1</td>
<td>32.0 ± 0.3</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>8</td>
<td>$me^v/me^v$ Igh-6$^{null}$</td>
<td>9.2 ± 0.5</td>
<td>53.4 ± 2.4*</td>
<td>57.9 ± 1.2*</td>
<td>28.5 ± 0.2†</td>
<td>11.0 ± 3.2</td>
</tr>
</tbody>
</table>

Data are given as mean ± SEM of values for 3–5 individual mice.

* $p < 0.05$; † $p < 0.01$.

Figure 3. Erythrocyte ultrastructural features. Erythrocytes were fixed immediately following collection or following 1-hour incubation at room temperature and examined by scanning electron microscopy. Most wild-type erythrocytes had a normal discoid appearance (A), whereas many $me^v/me^v$ erythrocytes showed mild deformation of the erythrocyte membrane (B). These differences in membrane morphology were exacerbated by an hour-long incubation at room temperature (C, wild-type and D, $me^v/me^v$). Reference bar = 10 μm.

Figure 4. Erythrocytes treated with H$_2$O$_2$ developed membrane anomalies. (A) Erythrocytes from $+/Igh-6^{null}$ mice were minimally affected by H$_2$O$_2$. A marked increase in echinocyte (e) formation was observed in blood from (B) $me^v/me^v$ or (D) $me^v/me^v$ Igh-6$^{null}$ mice incubated with H$_2$O$_2$. Metallothionein levels also affected H$_2$O$_2$ susceptibility. Erythrocytes from $Mt1$ transgenic mice were only slightly affected by H$_2$O$_2$ (E), whereas erythrocytes from mice with a targeted disruption of metallothionein 1 and metallothionein 2 were completely destroyed by oxidant treatment (F). Reference bar = 10 μm.
allothionein gene expression on oxidant-induced echinocyte formation. Erythrocytes from metallothionein-1 (Mt1) transgenic mice appeared resistant to oxidant induced injury (Fig. 4E), whereas erythrocytes from mice with a targeted disruption of the Mt1 and Mt2 genes were exquisitely sensitive to oxidant-induced injury as demonstrated by the absence of intact erythrocytes (Fig. 4F).

**Increased osmotic fragility of me/+me+ red blood cells**

Because subjecting me/+me+ erythrocytes to exogenous oxidant resulted in membrane alterations far more dramatic than those observed with wild-type erythrocytes, we evaluated the osmotic fragility of erythrocytes from me/+me+ and littermate control mice. Whole blood was incubated in serial dilutions of increasingly hypotonic saline to assess fragility. Erythrocytes from me/+me+ mice were significantly more fragile than control erythrocytes at both 60% and 50% (v/v) PBS. At 40% and 30% (v/v) PBS, erythrocytes from me/+me+ and control mice were equally sensitive to hypotonic lysis (Fig. 5).

**Oxidant production by Mac-1⁺ bone marrow cells in me/+me+ mice**

An important mechanism by which myeloid cells kill invading microbial organisms is dependent upon the generation of reactive oxygen intermediates. The overproliferation and chronic activation of myeloid cells in me/+me+ mice suggest that these cells might be a potent source of exogenous oxidant. In order to examine this possibility, we labeled bone marrow cells from me/+ me+ and littermate control mice with DH-123, a cell permeant and nonfluorescent probe that is converted to fluorescent rhodamine-123 in the presence of oxidants (Fig. 6) [18]. Mac-1⁺ bone marrow cells from me/+ me+ mice had significantly elevated basal levels of oxidant production compared to littermate control mice. After stimulation with PMA, Mac-1⁺ cells from both me/+ me+ and littermate control mice demonstrated a substantial increase in oxidant production; however, oxidant production from PMA-stimulated me/+ me+ Mac-1⁺ cells was markedly elevated compared with PMA-stimulated control Mac-1⁺ cells.

**Erythrocytes from me/+me+ mice have higher levels of lipid peroxides**

The breakdown of polyunsaturated fatty acids in the lipid bilayer of membranes produces MDA, which is a sensitive indicator of lipid peroxidation. To assess oxidant damage in me/+me+ erythrocytes, we used the TBARS assay in which MDA reacts with thiobarbituric acid to form a colored complex that is used to quantify the level of lipid peroxidation. Erythrocytes from me/+me+ mice had a significantly higher level of lipid peroxides compared with erythrocytes from control mice (Fig. 7).

**Glutathione levels in me/+me+ erythrocytes**

The observation of increased oxidant production in me/+me+ bone marrow cells and lipid peroxidation of me/+me+ erythrocyte membranes suggested that accumulating oxidant damage might affect the intracellular pool of antioxidant. To evaluate erythrocyte antioxidant levels, total glutathione (GSH)
was measured. GSH levels were significantly depleted in me/mem\textsuperscript{e} erythrocytes compared to littermate control erythrocytes (4.15 ± 0.51 μM GSH/mg protein in me/mem\textsuperscript{e} erythrocytes vs 6.52 ± 0.57 in normal erythrocytes, \(p < 0.05\)).

**Externalization of phosphatidylserine on immature erythrocytes of me/mem\textsuperscript{e} mice**

To determine if the decreased survival of erythrocytes in me/me mice affected mature or immature erythroid populations disproportionately, we examined erythrocytes from me/mem\textsuperscript{e} and littermate control mice for phosphatidylserine (PS) exposure. Externalization of PS on the outer leaflet of the plasma membrane has been proposed as an important signal for erythrocyte removal from the circulation [23,24]. Peripheral blood was immunolabeled with the erythroid-specific TER-119 antibody and the transferrin receptor (CD71) antibody. CD71 is expressed by erythroid precursors and reticulocytes but is not expressed on mature erythrocytes [25]. The cells then were incubated with annexin V, which binds exposed PS. After gating on the TER-119\textsuperscript{+} erythrocyte lineage cells, cells from me/mem\textsuperscript{e} and littermate control mice were analyzed for anti-CD71 and annexin V binding. Peripheral blood from me/mem\textsuperscript{e} mice had a higher percentage of immature erythrocytes, and an increased percentage of these erythrocytes had PS on the external leaflet of the plasma membrane compared with littermate control blood (2.82% ± 0.82% me/me erythrocytes were CD71\textsuperscript{+} AnV\textsuperscript{+} vs 0.55% ± 0.05% on control erythrocytes, \(p < 0.05\)) (Fig. 8). Intriguingly, me/me mice also had a lower percentage of mature erythrocytes with PS exposure (0.86% ± 0.34% me/mem\textsuperscript{e} erythrocytes were CD71\textsuperscript{−} AnV\textsuperscript{−} vs 2.45% ± 0.10% on control erythrocytes, \(p < 0.05\)).

**CD47 expression on me/mem\textsuperscript{e} erythrocytes**

Retention of erythrocytes in the circulation is associated with expression of CD47 on the plasma membrane [26]. This integrin-associated protein is expressed on the external leaflet of the erythrocyte plasma membrane and interacts with macrophage signal regulatory protein-α (SIRP-α) on phagocytic cells. Ligation of the inhibitory SIRP-α by CD47 on the erythrocyte surface prevents engulfment by phagocytes [26]. This inhibitory signal is mediated by SHP-1. Binding of SHP-1 to SIRP-α results in the interruption of signaling through tyrosine kinase-dependent activation pathways. To determine if interaction between SIRP-α and CD47 contributed to the anemia of SHP-1–deficient me/me mice, we examined TER-119\textsuperscript{+} cells for CD47 expression. Levels of CD47 were found to be higher on me/mem\textsuperscript{e} erythrocytes than on littermate control erythrocytes (Fig. 9).

**Discussion**

SHP-1 deficiency in motheaten and viable motheaten mice results in a wide variety of immune defects. Extensive studies of the immune system in these mutant mice have provided valuable insight into intracellular signaling pathways in the hematopoietic system [8,27,28]. However, the mechanisms underlying the progressive anemia in these mice have not been clearly established [7].

Previous reports have suggested that abnormalities in erythrocyte proliferation and differentiation are linked to decreased SHP-1 activity [10,29]. Reduced SHP-1 expression in erythroid progenitor cells has been demonstrated in a subset of patients with PV, a clonal myeloproliferative disorder that results in erythroid cell hyperproliferation [9]. However, an earlier report failed to find alterations in SHP-1 protein expression in granulocytes from PV patients [30]. Although it is difficult to assess the role of SHP-1 in the pathogenesis of PV, nevertheless, me/mem\textsuperscript{e} mice and PV patients exhibit similar erythroid defects and may share similar mechanistic anomalies in the regulation of erythropoiesis. SHP-1 has been
shown to be important in the regulation of erythropoiesis by inhibiting signaling through the Epo receptor [31]. SHP-1–deficient me/me mice have a dramatic increase in the CFU-E precursor population of the spleen. These precursor cells are much more sensitive to Epo, with a subpopulation of these cells exhibiting Epo-independent proliferation [10]. The increased numbers of erythroid progenitors accompanied by increased responsiveness to Epo would predict that me/me mice develop an erythrocytosis, but the cellular composition of the blood in these mice instead reflects a progressive anemia.

The severity of this anemia suggests that it is not solely a consequence of the low levels of anti-erythrocyte autoantibodies that can be detected in the plasma of me/me mice by indirect Coombs assay. We found no evidence that these antibodies bind in significant amounts to me/me erythrocytes in vivo. Neither direct Coombs assays nor flow cytometric analysis of TER-119+ erythroid cells detected anti-erythrocyte antibodies on the surface of me/me erythrocytes at levels in excess of normal littermate control levels. In contrast, autoimmune hemolytic anemia in NZB mice is associated with significant amounts of anti-erythrocyte antibodies bound to the surface of circulating erythrocytes. Additional evidence that autoantibodies are not responsible for anemia in me/me mice is that anemia persists despite the absence of antibodies in me/me Igh-6null mice.

Erythrocytes are subjected to wide fluctuations in oxygen exposure, ranging from approximately 40 mmHg oxygen pressure (P02) in the tissues to 104 mmHg P02 as they pass through the lung vasculature [32]. Normally cellular antioxidant systems offset the deleterious effects of oxygen. Erythrocytes use several antioxidant mechanisms, including glutathione [33], the principal nonprotein thiol in the cell, and metallothionein [34], the principal thiol-containing protein in the cell. Moreover, a number of anti-oxidant enzyme systems limit erythrocyte oxidative damage.

The presence of echinocytes in the blood of me/me mice is consistent with oxidative damage to the plasma membranes [35]. Erythrocytes exposed to room air at room temperature experience oxidation by dissolved oxygen from the atmosphere. Erythrocytes from me/me mice developed echinocyte morphology during a 1-hour incubation, and the me/me erythrocyte population was highly enriched for echinocytes following incubation with exogenous H2O2.

The severe morphologic changes observed following exposure of me/me erythrocytes to hydrogen peroxide suggested increased fragility of me/me erythrocytes, a conjecture that was borne out by the increased sensitivity of me/me erythrocytes to hypotonic lysis. Exposure of erythrocytes to hypotonic saline at 60% of normal tonicity caused dramatic hemolysis of me/me erythrocytes, whereas littermate control erythrocytes demonstrated little hemolysis. This fragility may be a consequence of chronic exposure to exogenous oxidant produced by the persistent activation of myeloid lineage cells in me/me mice. Macrophages from me/me mice have an elevated level of oxidant production compared to normal macrophages, and they respond to phorbol ester activation with a more robust oxidative burst. These observations are consistent with previous studies demonstrating that me/me neutrophils display higher than normal levels of oxidant production [27].

Our findings suggest that the me/me erythrocyte plasma membrane may be damaged as a consequence of exposure to exogenous oxidants. Erythrocyte plasma membranes from me/me mice contained markedly elevated levels of lipid peroxides (as measured by the TBARS assay) compared with littermate control erythrocytes. Reflective of persistent severe oxidative stress, the primary nonprotein thiol antioxidant in cells, glutathione (GSH), was significantly depleted in me/me erythrocytes.

Identification of erythrocytes for clearance from the circulation by splenic macrophages is mediated by reduced expression of CD47 on the erythrocyte plasma membrane. This integrin-associated protein functions as a marker of self on erythrocytes [26]. Binding of CD47 to macrophage SIRP-α inhibits erythrocyte phagocytosis [26]. Signaling through SIRP-α is mediated by SHP-1 [36]. SHP-1–deficient me/me mice have an accelerated rate of clearance of opsonized erythrocytes; however, there is no difference in the clearance of unopsonized erythrocytes in me/me mice compared with that in wild-type mice [36]. These data suggest that even though the signaling through the CD47/SIRP-α complex is attenuated in me/me mice, it still is sufficient to prevent phagocytosis of erythrocytes in the absence of other phagocytic stimuli. Our observation that me/me erythrocytes express elevated levels of CD47 may reflect the fact that there is a larger percentage of immature erythrocytes in me/me mice, as aged erythrocytes demonstrate reduced CD47 expression [37].

The presence of anisocytosis and polychromasia in blood smears from me/me mice is consistent with an increased proportion of immature erythrocytes in me/me blood. This is supported by the increased number of reticulocytes in me/me blood smears and by the increased frequency of TER-119+ erythrocytes that coexpress CD71+, a marker of immature erythrocytes. The mechanism by which immature me/me erythrocytes are removed from the circulation appears to be associated with PS exposure, because these cells show elevated annexin V binding compared to littermate control erythrocytes. Alternatively, a study has demonstrated that annexin V will also bind to MDA on the plasma membrane [38]. This product of lipid peroxidation of the plasma membrane was shown to be elevated in me/me erythrocytes by the TBARS assay. Recognition of membrane damage in me/me erythrocytes and consequent removal by phagocytes may be MDA dependent. These data suggest that in me/me mice, either immature erythroid cells are more susceptible to oxidant induced membrane damage or cumulative membrane damage prevents most me/me erythroid cells from reaching maturity.

Our results support the hypothesis that me/me erythrocytes are exposed to a highly oxidative environment sec-
ondary to myeloid proliferation and that this environment causes oxidative damage with subsequent removal of erythrocytes from the circulation. Other anemias, such as sickle cell, β-thalassemia, malaria, and erythropoietic protoporphyria have a hemolytic component secondary to oxidative damage [3]. Moreover, exposure to a wide variety of oxidative toxicants can result in anemia [39]. Viable motheaten mice may prove to be a useful model of these forms of anemia. Additionally, the role of oxidant in the progression of this anemia may be susceptible to manipulation by antioxidants such as N-acetylcysteine or vitamin E. Viable motheaten mice may be an excellent model for assessment of the therapeutic effect of antioxidants.

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