Mechanism of the Hereditary Anemia of $S^m$ Mutant Mice

By A. N. KALES, W. FRIED AND C. W. GURNEY

The mechanism of the anemia that occurs regularly in the C57BL/6-$S^mS^m$ mouse will be described in this report. $S^m$ is one of a series of $S$ alleles producing, in the homozygous condition, a macrocytic anemia associated with coat color change and sterility.\(^1\)\(^-\)\(^6\) The characteristics of the different $S$ mutants are compared in Table 1.

The triad of macrocytic anemia, coat color change, and sterility is known to occur also in $W$ series mutants. Although detailed studies of the mechanism of anemia in $S$ series mutants have not been published, the anemia of $W$ series mutants has been extensively studied.\(^7\)\(^-\)\(^14\) This is a pure red cell anemia associated with elevated reticulocyte counts.\(^7\) The marrow is hypoplastic, with a predominance of early erythroid forms, suggesting a maturation arrest.\(^*\) The anemia is gradually but completely cured following implantation of isologous fetal hematopoietic tissue from normal animals, suggesting that a defect in the marrow hematopoietic cells is responsible for the condition.\(^9\)\(^-\)\(^11\) Further support for this concept comes from recent work demonstrating an impaired ability to form spleen colonies of $W$ mutant marrow cells.\(^12\) The anemic animals are relatively resistant to erythropoietin, although large doses produce a small but apparently significant response.\(^13\)\(^,\)\(^14\) Exposure of the anemic mice to hypoxia produces an increase in the hematocrit comparable to that observed in normal mice.\(^7\)\(^,\)\(^14\)

Although the syndromes of the $S$ and $W$ series mutants are strikingly similar, the mutations occur at different genetic loci: the $S$ and $W$ genes segregate independently with no linkage between them.\(^1\) Moreover, we have found that the anemia of $S^mS^m$ animals has several characteristics not reported in $W$ series anemias. This paper describes the characteristics and mechanisms of the anemia of this mutant genotype.

Methods and Materials

Adult C57BL/6-$S^mS^m$, C57BL/6-$S^m$ and C57BL/6++ mice, derived from stock originally given us by Dr. Dorthea Miller, constituted the principal experimental animal. The gene mutation in these mice was shown to be allelic to that of $S^p$ mice by cross-matching with $S^p$ mice supplied by Dr. E. S. Russell. Animals used in individual experiments were matched for age and sex. Six 10-week-old CBA female mice from the Jackson Laboratory were used for erythropoietin assay. Retired CF No. 1 breeder females were used as red cell donors in producing polycythemia.

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Table 1.—Characteristics of S1 Series Mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>RBC</th>
<th>MCV</th>
<th>Pigmentation</th>
<th>Fertility</th>
<th>Viability</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>s1 s1</td>
<td>greatly decreased</td>
<td>greatly decreased</td>
<td>black-eyed white</td>
<td>no germ cells</td>
<td>lethal</td>
<td>1, 2</td>
</tr>
<tr>
<td>s1+ s1+d</td>
<td>greatly decreased</td>
<td>greatly increased</td>
<td>black-eyed white</td>
<td>sterile</td>
<td>decreased</td>
<td>3, 4</td>
</tr>
<tr>
<td>s1+ s1+o</td>
<td>greatly decreased</td>
<td>___</td>
<td>black-eyed white</td>
<td>sterile</td>
<td>severely decreased</td>
<td>5, 6</td>
</tr>
<tr>
<td>s1+ s1+m</td>
<td>greatly decreased</td>
<td>greatly increased</td>
<td>black-eyed white</td>
<td>sterile</td>
<td>decreased</td>
<td>5, 6</td>
</tr>
<tr>
<td>s1 +</td>
<td>decreased</td>
<td>slightly increased</td>
<td>dilute</td>
<td>fertile</td>
<td>normal</td>
<td>1, 2</td>
</tr>
<tr>
<td>s1+d +</td>
<td>decreased</td>
<td>increased</td>
<td>dilute</td>
<td>fertile</td>
<td>normal</td>
<td>3, 4</td>
</tr>
<tr>
<td>s1+o +</td>
<td>___</td>
<td>___</td>
<td>dilute</td>
<td>fertile</td>
<td>normal</td>
<td>5, 6</td>
</tr>
<tr>
<td>s1+m +</td>
<td>normal</td>
<td>normal</td>
<td>dilute</td>
<td>fertile</td>
<td>normal</td>
<td>5, 6</td>
</tr>
</tbody>
</table>

Erythropoietin was obtained from the United States Public Health Service, Hematology Section.

Red cell and white cell counts were determined in the standard manner on blood from a tail vein. Hematocrits were measured by the microhematocrit method and hemoglobin levels by the cyanmethemoglobin method. The reticulocyte percentage was determined by counting 1000 cells on a smear prepared with brilliant cresyl blue stain. Platelets were counted by the modified direct method of Skirmont et al.15

Gastrointestinal blood loss was estimated in the following manner. C57BL/6++ mice were injected intraperitoneally with 1.5 μC Fe\(^{59}\)Cl\(^*\) and exsanguinated 3 days later to obtain Fe\(^{59}\) labeled red cells. Two-tenths ml. of a 50 per cent suspension of labeled red cells in saline was injected intravenously into each animal to be tested. The mice were kept in individual metabolism cages and the stools collected daily for 2 weeks. The radioactivity of the stools was determined in a well-type scintillation counter. The specific activity of the blood was measured on the first and last days. The specific activity of the blood on intermediate days was estimated by interpolation on semi-log paper. Daily blood loss in ml. was then calculated as the dividend

\[
\text{total activity of the stools on day } x \div \text{specific activity of the blood on day } x
\]

Fe\(^{59}\) incorporation into the circulating red cells, as a test of erythropoietic response or as an erythropoietin assay, was determined by the method of DeGowin et al.16 Mice were injected subcutaneously with erythropoietin or plasma no sooner than 2 days after being made polycythemic, and 58 hours later 0.5 μC Fe\(^{59}\). Cl in 0.2 ml. saline was injected intravenously. Seventy-two hours later a measured amount of blood was obtained by cardiac puncture or from a tail vein and counter in a well-type scintillation counter. The percentage of Fe\(^{59}\) incorporation was calculated on the basis of an estimated blood volume in plethoric mice of 7 per cent of body weight.

*Specific activity 36 mc per mg Fe.
Table 2.—Peripheral Blood Parameters of Normal and Slm Mutant Mice
Values Represent the Average of 10 Animals ± 1 S.D.

<table>
<thead>
<tr>
<th></th>
<th>++</th>
<th>Slm +</th>
<th>Slm Slm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hgb (gm %)</td>
<td>15.4 ± 1.0</td>
<td>15.2 ± 0.7</td>
<td>11.5 ± 1.9</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>50 ± 1</td>
<td>48 ± 3</td>
<td>39 ± 5</td>
</tr>
<tr>
<td>RBC x 10^6/mm^3</td>
<td>8.16 ± 0.88</td>
<td>7.89 ± 0.66</td>
<td>5.42 ± 1.06</td>
</tr>
<tr>
<td>MCV (μ³)</td>
<td>61 ± 4</td>
<td>61 ± 4</td>
<td>73 ± 7</td>
</tr>
<tr>
<td>Retic (%)</td>
<td>3.6 ± 1.0</td>
<td>2.5 ± 1.7</td>
<td>7.6 ± 4.8</td>
</tr>
<tr>
<td>WBC/mm³</td>
<td>11,000 ± 3,000</td>
<td>15,000 ± 7,000</td>
<td>20,000 ± 9,000</td>
</tr>
<tr>
<td>Platelets x 10^6/mm³</td>
<td>1.5 ± 0.2</td>
<td>1.5 ± 0.2</td>
<td>1.5 ± 0.2</td>
</tr>
</tbody>
</table>

Mice were made polycythemic either by intravenous injection of 0.5 ml. of an 80 per cent suspension of washed red cells in saline or by intraperitoneal injection of 1.0 ml. of such a suspension of 2 consecutive days. Booster transfusions were given as necessary to maintain the hematocrits between 55 and 65 per cent. Animals with hematocrits outside these limits were excluded from the experiment.

Hypoxia was produced in the hypobaric chamber described by DeGowin et al.\(^7\)

RESULTS

The peripheral blood parameters of adult SlmSlm (mutant homozygote), Slm+ (mutant heterozygote) and ++ (normal) mice are recorded in Table 2. Slm+ mice and ++ mice differ little in these parameters. In contrast, the values for SlmSlm animals reveal a macrocytic anemia with an elevated reticulocyte count. The reduction in cell number is limited to the erythrocytes, there being no depression of the leukocyte or platelet count. The bone marrows are normocellular with a normal ratio of myeloid to erythroid cells.

The elevation of the reticulocyte count suggested that either hemolysis or bleeding might be the basis of the anemia. Occult blood was demonstrable by the benzidine method in stools of the anemic animals, where a strikingly more prompt and intense response was noted than in control mice, and it therefore seemed important to compare quantitatively the amount of gastrointestinal blood loss in normal and mutant mice. As shown in Figure 1, Slm+ and ++ mice lost radioiron equivalent to 3.1 to 3.3 microliters of blood daily. In contrast, SlmSlm mice lost radioiron equivalent to 34 microliters daily, over 10 times as much as the normals. Since the test animals averaged 20 Gm. in total body weight, this amount approximated 3.5 per cent total blood volume daily, assuming a blood volume of 5 per cent of body weight.

In order to test whether blood loss of this magnitude could entirely account for the anemia, we subjected normal mice to blood loss approximating that observed in SlmSlm mice. An amount of blood equal to 3.5 per cent of the estimated blood volume (again assuming a blood volume of 5 per cent of body weight) was taken from a tail vein every day. Within 20 days the blood
parameters had stabilized at new steady-state values. These values are compared to those of the anemic mice in Figure 2. The normal mice respond to what is presumed to be comparable blood loss with a greater reticulocytosis and do not become as anemic as the mutant animals.

Failure of normal erythropoietic response to bleeding might result from a failure to elaborate sufficient quantities of erythropoietin, from rapid inactivation of erythropoietin, or from failure of the marrow to respond normally to the erythropoietin produced. In order to determine if there was a reduction in the rate of production or an increase in turnover of erythropoietin, we measured the plasma erythropoietin levels of the anemic animals. As shown in Figure 3, SI^m SI^m plasma produces a striking erythropoietic response in normal mice, demonstrating that even under normal conditions the plasma of SI^m SI^m mice is characterized by high concentrations of erythropoietin.

Failure of normal response to erythropoietin might result if after prolonged bleeding the mutant animals developed iron deficiency of sufficient magnitude to depress erythropoiesis. We therefore administered iron to the anemic animals in an attempt to ameliorate the anemia. One mg. of iron, in the form of a saccharated iron oxide preparation,* was injected intraperitoneally on day 0 and an additional 2 mg. on day 23. Hemoglobin levels and reticulocyte counts were measured at intervals, as indicated in Figure 4. The anemic animals failed to respond to iron with elevations either of reticulocyte count or hemoglobin level.

We next tested the response of the mutant animals to exogenous erythropoietin. Erythropoietin was administered to plethoric test animals and the resulting Fe^{59} incorporation into red cells was determined. The results are

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*Proferin, produced commercially by Merck, Sharpe and Dohme.
ANEMIA OF S\textsuperscript{Im} MUTANT MICE

Blood Parameters of ++ Mice Subjected to Chronic Blood Loss Compared to Blood Parameters of S\textsuperscript{Im} S\textsuperscript{Im} Mice

Plasma Erythropoietin Levels (Assayed in Polycythemic Normal Mice as % Fe\textsuperscript{59} Incorporation)

Fig. 2.—Average ± 1 S.D. () = number of test animals.

Fig. 3.—Average ± 1 S.D. () = number of assay animals. Volume of plasma assayed = 0.5 ml.

shown in Figure 5. In Experiment A, plethoric ++ and S\textsuperscript{Im}++ mice were given 3 injections of 0.33 units of erythropoietin at 5 hour intervals. The normal mice responded with Fe\textsuperscript{59} incorporation several times that of the mutant heterozygotes. In Experiment B, a single injection of 3 units of erythropoietin produced a markedly greater erythropoietic response in normal mice than in the mutant
Reticulocyt. Count

Reticulocyte Count

Hemoglobin

Days

Fig. 4.—The brackets indicate the standard error of the mean. n = number of test animals.

Response of S/m S/m Mice to Iron

Response of S/m S/m S/m+ and ++ Mice to Erythropoietin

Fig. 5.—Average ± 1 S.D. ( ) = number of test animals.

homozgygotes. The homozgygotes showed no greater Fe59 incorporation after this dose of erythropoietin than did normal controls given no erythropoietin.

To determine if the homozgygotes were capable of responding to larger doses of erythropoietin, plethoric homozgygotes were given 20 units of erythropoietin on 4 successive days. This elicited a reticulocytosis of 1.2 per cent on the fourth day after the last injection (Fig. 6).
ANEMIA OF Slh mutant mice

Fig. 6.—The brackets indicate the standard error of the mean. n = number of test animals.

To further characterize the erythropoietic defect in the mutant mice, we tested the ability of SlhSlh mice to respond to a hypoxic stimulus. SlmSlm and + + mice were made comparably polycythemic by hypertransfusion and placed in a hypoxic chamber at 0.5 atm. Serial reticulocyte counts and plasma erythropoietin determinations were used to follow the response to hypoxia. The results are shown in Figure 7. As compared to normal animals, SlhSlh mice were markedly deficient in their reticulocyte response, despite the production of high plasma levels of erythropoietin.

In a companion experiment, we compared the responses of Slh and + + mice to hypoxia. Since Slh+ mice are not anemic, it was not necessary to transfuse the animals in order to assure comparable hemoglobin levels. The animals were placed in the hypoxic chamber at 0.5 atm. and their response followed by reticulocyte counts and hemoglobin determinations. The results are shown in Figure 8. After 4 days of hypoxia, normal mice exhibited a significantly greater rise (p < 0.05) in reticulocyte count than did the heterozygotes. By 10 days, however, this difference had disappeared. The rise in hemoglobin levels during the 10 days was not significantly greater in the normals than in the heterozygotes.

DISCUSSION

These studies suggest that two etiologic factors, gastrointestinal bleeding and erythropoietin defect, underlie the anemia of SlhSlh mice. The existence of gastrointestinal bleeding is postulated on the basis of evidence indicating that large amounts of radioactivity appear in the stools after transfusion of Fe59 labeled red cells into the anemic animals. It should be pointed out that we have actually demonstrated only that Fe59 is lost into the gastrointestinal tract, not that red cells are. However, if there is no bleeding, then the appear-
Response of Polycythemic $^m$ and $^{++}$ Mice to Hypoxia

A. Reticulocyte Counts

B. Plasma Erythropoietin Levels (Assayed in Polycythemic CBA Mice as % Fe$^{59}$ Incorporation)

Fig. 7.—The brackets indicate the standard error of the mean. $(\bar{X}) = \text{number of test animals. Volume of plasma assayed} = 0.5 \text{ ml.}$

Response of $^m$ and $^{++}$ Mice to Hypoxia

A. Reticulocyte Counts

B. Hemoglobin Levels

Fig. 8.—The brackets indicate the standard error of the mean. Number of test animals = 16.

ance of Fe$^{59}$ in the stools must be explained by the coincidence of two defects, one leading to removal of Fe$^{59}$ from the red cells and the other to transport of Fe$^{59}$ into the intestinal lumen. We would also need to hypothesize a defect leading to transport of Cr$^{51}$ into the intestinal lumen, for when Cr$^{51}$ labeled red cells are employed the results obtained are similar to those with Fe$^{59}$ labeled red cells.$^2$ We therefore favor the simpler hypothesis that a gastrointestinal bleeding defect exists.

We have been unable to find a cause for the gastrointestinal bleeding. The
gastrointestinal tract appears grossly normal throughout its length, and several random sections of the stomach and intestine failed to show erosion or ulceration. A detailed histologic study of the entire gastrointestinal tract remains to be undertaken. There is no evidence of bleeding in sites other than the gastrointestinal tract, and the platelet count is normal.

Despite their continued bleeding, iron deficiency will not completely explain the impaired erythropoiesis of S\textsuperscript{m}S\textsuperscript{m} mice, since administration of iron does not ameliorate their anemia.

Gastrointestinal bleeding has not been reported in \textit{W} series mutants. However, our demonstration of an erythropoietic defect in \textit{S\textsuperscript{m}} mutants shows that their similarity to \textit{W} series animals is more than superficial. The erythropoietic defect renders \textit{S\textsuperscript{m}} mutants, the heterozygote as well as the homozygote, relatively refractory to erythropoietin. We have, in fact, been unable to demonstrate a significant response to a single injection of erythropoietin in the homozygotes. However, repeated high doses do induce a clear-cut reticulocytosis in plethoric homozygotes. This relative insensitivity to erythropoietin is also a characteristic of \textit{W} series mutants.

\textit{S\textsuperscript{m}} mutants are defective in their response to hypoxia as well as to erythropoietin. In the homozygotes there is markedly less erythropoietic response to hypoxia than in normal animals despite the production of high plasma concentrations of erythropoietin. In the heterozygotes the defect is much less apparent and is evidenced only by a lag in achieving a reticulocyte peak. Thus, although the normal mouse shows a greater reticulocytosis than the heterozygote after 4 days of hypoxia, by 10 days the difference in reticulocyte count no longer exists.

Keighley et al.\textsuperscript{13} have reported normal response to hypoxia in \textit{WW\textsuperscript{v}} mice, despite a markedly decreased sensitivity to erythropoietin. While the apparently normal response of \textit{WW\textsuperscript{v}} mice to hypoxia as contrasted to the abnormal response of \textit{S\textsuperscript{m}S\textsuperscript{m}} mice may represent another difference between these mutants, it may also be attributable to differences in experimental conditions. The experiments with \textit{WW\textsuperscript{v}} mice were conducted with anemic animals and compared the effect of hypoxia on these animals to its effect on mice with normal hematocrits. It is possible that an already anemic animal would be sufficiently sensitive to hypoxia to respond with production of enough erythropoietin to stimulate even a defective marrow to a high rate of erythropoiesis. Our experiments were conducted in plethoric animals where comparable hematocrits excluded the possibility that different cell volumes might contribute to the results observed.

In \textit{W} series mutants, it has been established that the observed erythropoietic defect is an intrinsic cellular one, since implantation of hematopoietic tissue from normal animals abolishes the anemia.\textsuperscript{9-11} Experiments to determine if this is also true for \textit{S\textsuperscript{m}} mutant animals are in progress.

**Summary**

The anemia of mutant homozygote C\textit{57BL/6S\textsuperscript{m}S\textsuperscript{m}} mice is due to a gastrointestinal bleeding defect and to an erythropoietic defect. The anemic animals lose approximately 3.5 per cent of their blood volume into the gastrointestinal...
tract daily. The cause of this blood loss has not been established. The anemic animals also have an erythropoietic defect which renders them unable to respond normally to erythropoietin or to hypoxia. Although the homozygotes appear refractory to erythropoietin in moderately large single doses, they do respond if sufficiently large doses are given repeatedly.

The erythropoietic defect detected in the homozygote is also evident in the heterozygote. However, the defect remains latent in the heterozygote, for although this animal is subnormally responsive to erythropoietin and to hypoxia, it is not anemic.

**Summario in Interlingua**

Le anemia de mutante muses homozygotico C57BL/6Sl"Sl" es causate per un defecto sanguinatory gastrointestinal in combination con un defecto erythropoietico. Le animales afficite de anemia perde omne die approximativemente 3,5 pro cento de lor volumine de sanguine ad in le vias gastrointestinal. Le causa de iste perdita de sanguine ha non ancora essite identificate. Le animales afficite de anemia etiam ha un defecto erythropoietico le qual rende illos incapace de responder normalmente a erythropoietina o a hypoxia. Ben que le animales homozygotico pare esser refractori a erythropoietina in moderatemente grande doses individual, illos responde si sufficientemente grande doses es administrate repetitemente.

Le defecto erythropoietico detegite in le animales homozygotico es etiam evidente in le animales heterozygotico. Tamen, le defecto remane latente in le heterozygoticos, proque—ben que iste animales es subnormalmente responsive a erythropoietina e a hypoxia—illos non es anemic.

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