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*Original Research*

# A Mouse Model for Human Unstable Hemoglobin Santa Ana

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**In the present study, we described the phenotype, histologic morphology, and molecular etiology of a mouse model of unstable hemoglobin Santa Ana. Hematologic evaluation of anemic mice (*Anem*/+) discovered after N-ethyl-N-nitrosourea mutagenesis revealed moderate anemia with intense reticulocytosis and polychromasia, followed by anisocytosis, macrocytosis, hypochromia, and intraerythrocytic inclusion and Heinz bodies. The mice also demonstrated hemoglobinuria, bilirubinemia, and erythrocytic populations with differing resistance to osmotic lysis. Splenomegaly (particularly in older mutant mice) and jaundice were apparent at necropsy. Histopathologic examination revealed dramatically increased hematopoiesis and hemosiderosis in hematopoietic organs and intracellular iron deposition in tubular renal cells. These data are characteristic of a congenital hemolytic regenerative anemia, similar to human anemias due to unstable hemoglobin. Genetic mapping assigned the affected gene to mouse chromosome 7, approximately 50 cM from the *Hbb* locus. The sequence of the mutant *Hbb* gene exhibited a T→C transversion at nucleotide 179 in Hbb-b1, leading to the substitution of proline for leucine at amino acid residue 88 and thus homologous to the genetic defect underlying Santa Ana anemia in humans.**

In humans, hereditary abnormalities of hemoglobin synthesis are classified as those characterized by structurally abnormal hemoglobin variants (hemoglobinopathies) and those in which the production of one or more normal polypeptide chains of hemoglobin is decreased (thalassemias).[10] Due to the number of genes involved, mutations in the human β-globin chain (2 genes) are associated with more severe effects than are mutations in the α chain (4 genes) and result in abnormal proportions of total hemoglobin. The pattern of inheritance is autosomal dominant, and affected persons are almost exclusively heterozygous, with homozygosity being exceptionally rare. The clinical expression of unstable hemoglobin variants is quite variable. Some cases may manifest as severe persistent chronic anemia, whereas other patients show only slight hemolysis, which is exacerbated by exposure to oxidizing drugs or infections.[9,10,21] Conditions of unstable hemoglobin usually result from single amino-acid replacements, leading to the synthesis of structurally abnormal α- or β-globin chains and the presence of Heinz bodies. The pattern of inheritance is autosomal dominant, and the vast majority of affected persons is heterozygous for the abnormality.[21]

In mice, the organization of the mouse β-globin gene complex is generally similar to that of humans and other mammals, with considerable similarity in protein structure as well.[5,6] Most mice that are mutant for the β-globin gene complex develop signs that mimic β-thalassemia, with damaged erythrocytes that are prematurely destroyed by the reticuloendothelial cells of the spleen and liver, producing hemolytic anemia.[1,7]

Targeted knockin at the Presbyterian (Asnβ$^{-108}$- Lys) mutation in the β-globin locus was the first animal model for hemolytic anemia due to unstable hemoglobin.[18] In this model, heterozygous mice showed expression of the mutant protein, Hb Presbyterian, in 27.7% of total peripheral blood without any hematologic abnormalities, well mimicking human cases. In comparison, homozygous mice exclusively expressed Hb Presbyterian in 100% of peripheral blood, associated with hemolytic anemia, Heinz body formation, and splenomegaly. Furthermore, Presbyterian Hb was vulnerable to precipitation in isopropanol in a dose-dependent manner.[18]

Here we report a novel N-ethyl-N-nitrosourea–induced mutation provisionally designated *Anêmico* (anemic in Portuguese; symbol *Anem*[11]). Affected mice initially were identified at birth as being paler and smaller than their normal littermates. Breeding tests showed autosomal dominant inheritance, with intrauterine death of homozygous mutants. This mutation mimics the human hemoglobinopathy Santa Ana and causes unstable hemoglobin, followed by hemolytic anemia with Heinz body formation.

## Materials and Methods

**Mice and housing.** To comprehensively characterize the pathologic phenotype, 12 *Anem*/+ (5 females and 7 males) and 13 control (5 females and 8 males) BALB/c mice (*Mus musculus*), ranging from 3 to 8 mo in age, were used for the determination of the hematologic parameters and histopathologic evaluation. Mice were SPF for ectromelia virus, lymphocytic choriomeningitis virus, minute virus of mice, mouse hepatitis virus, mouse parvovirus, pneumonia virus of mice, reovirus, Sendai virus, Theiler murine encephalo-

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myelitis vírus, hantaviruses, cilia-associated respiratory bacillus, *Clostridium piliforme*, *Klebsiella pneumonia*, *Mycoplasma pulmonis*, *Pasteurella multocida, Pasteurella pneumotropica*, *Pseudomonas aeruginosa*, *Salmonella* spp, *Staphylococcus aureus*, *Streptobacillus moniliformis*, β-hemolytic *Streptococcus* spp, *Streptococcus pneumoniae*, endoparasites, and ectoparasites. Mice were bred and maintained in our animal facility (Department of Immunology, Institute of Biomedical Science, University of São Paulo, Brazil) and housed in IVC (Alesco Indústria e Comércio, Monte Mor, Brazil) under a 12:12-h light:dark cycle at 24 ± 1 °C. They had unrestricted access to acidified water and autoclaved commercial pelleted formulated according to the AIN-93M rodent diet[16] (Nuvilab, Quimtia S.A., Parana Brazil). Mice were euthanized by exsanguination after cardiocentesis under general anesthesia with isoflurane inhalation. Animal protocols were reviewed and approved by the Ethics Committees on Animal Use of the Institute of Biomedical Science and the School of Veterinary Medicine and Animal Science of the University of São Paulo (CEUA/ICBUSP number 037/2007 and CEUA/FMVZ number 2641/2012, respectively).

**Genetic mapping.** Heterozygous mice of the BALB/c mutant substrain *Anemic* (*Anem*/+) were crossed with C57BL/6 (+/+) mice, generating F1 hybrids. The *Anem*/+ CB6 F1 mice were then testcrossed with normal (+/+) C57BL/6 mice to produce an N2 generation, which was used for mapping. Genetic mapping was achieved by using selected microsatellite markers that were distributed over the entire mouse genomic map and that readily discriminated polymorphisms between the BALB/c and C57BL/6 strains. To refine the genetic localization, additional tests were carried out by using additional markers in the same region. Genes in which mutations might explain the anemic syndrome (candidate genes) were sequenced by using the Sanger method[17] (primers 5′ ATG GTG CAC CTG ACT 3′ and 5′ TTA GTG GTA CTT GTG AGC C 3′).

**Hematologic analysis.** Given the preliminary hemogram results, we followed a clinical flowchart for the diagnosis of unstable hemoglobin.[9]

Blood samples obtained by cardiocentesis after general anesthesia of mice by isoflurane inhalation were collected into tubes containing 10% EDTA for analysis of CBC counts and RBC osmotic fragility and containing lithium heparin for the other tests.

Blood samples for hematology (CBC count, RBC count, Hgb, Hct, MCV, MCH, MCHC, RBC distribution width, total leukocyte count, and platelet count) were evaluated on an automated hematology analyzer (BC2800-Vet, Mindray, Shenxhen, China). Blood smears were stained with May–Grünwald–Giemsa for morphologic analyses and leukocyte differential count on optical microscopy. Reticulocyte counts were performed on blood smears prepared with brilliant cresyl blue supravital stain, and results are presented as percentages and absolute values. For results presentation, analyses are separated by cell type (RBC, leukocytes, platelets).

RBC osmotic fragility consisted of challenging small-volume blood samples in large excesses of buffered saline solutions of varying concentration (0% to 8.5% NaCl through 16 tubes). A cumulative curve of hemolysis was obtained by plotting the optical density of the supernatant in each tube at 540 nm against the NaCl concentration in the tube, considering that the last tube (0% NaCl) resulted in 100% hemolysis.

**Hemoglobin analysis.** Abnormal hemoglobin can cause alterations in electric charge due to amino acid substitutions in the molecule; these changes can be studied through cellulose acetate electrophoresis. In this study, control and mutant hemolysates underwent hemoglobin electrophoresis in alkaline pH with Tris buffer (pH 8.5), staining with Ponceau S, and densitometry analysis by using VisionWorks software (UVP, Upland, CA). To separate and quantify the α- and β-globin chains in the blood samples and thus establish which chain is affected, control and mutant hemolysates underwent cellulose acetate electrophoresis with Tris–urea buffer (pH 8,0), staining with Ponceau S, and densitometry analysis with VisionWorks software.

When hemoglobin is submitted to heat under physiologic pH or is dissolved in isopropanol, the van der Waals bonds are weakened and the molecular stability decreases. For heat tests of stability[14], lysates were diluted in (0.1 M) disodium hydrogen phosphate solution, incubated at 60 °C, and observed for precipitation every 10 min for a total of 60 min. Only unstable hemoglobin structures precipitates whereas stable hemoglobin proteins remain in solution. For isopropanol tests[14], lysates were diluted in Tris–isopropanol buffer (pH 7.4), incubated at 37 °C, and observed for precipitation every 10 min for a total of 60 min.

Methemoglobin might be formed due to a globin defect. Therefore hemolysates from control and mutant mice were diluted in a phosphate buffer ($Na_2HPO_4 \cdot 12\ H_2O$, 9.0 g; $KH_2PO_4$, 5.7 g; per 1 L) at pH 6.6.[14] The optical densities of the samples from 480 to 630 nm were read every 10 nm, with that of the buffer normalized to 0 at each wavelength. An analysis curve was obtained by plotting the optical density of the sample against the wavelength used. The absorption spectrum of methemoglobin due to a globin chain defect differs from that of normal methemoglobin.

**Histopathologic evaluation and bone marrow cytology.** Organs were weighed, and samples of spleen, liver, and kidney tissue were fixed in nonbuffered 10% formalin and routinely processed with paraffin impregnation for histologic study. Histologic sections were stained with hematoxylin and eosin for morphologic analysis and with Perl stain to detect the presence of iron in tissues.

Bone marrow cells obtained from mouse femurs were counted by using a hemocytometer, and smears stained with May–Grünwald–Giemsa were used for morphologic analysis.

**Statistical analysis.** For all parameters, descriptive statistics were applied. The values for the erythroid series are presented as mean, standard deviation 1 SD, and *P* values from one-tailed *t* tests. Data regarding platelets and WBC are presented as median, first quartile, and third quartile values, with the preparation of boxplot graphs by using the statistical analysis software program Minitab 16.0 (State College, PA).

## Results

**Genetic mapping.** Mutants from N2 generation were identified according to the reticulocyte count, which was clearly increased in all cases. Linkage with chromosome 7 was identified by analyzing 10 N2 mice for 39 microsatellites that were evenly distributed throughout the mouse genome. Once the linkage to chromosome 7 was discovered, we used additional polymorphic markers to genotype 85 N2 mutant mice regarding the 4.5-cM region between *D7Mit301* (46.5 cM) and *D7Mit131* (51 cM).

The phenotypic data gathered after analysis of the anemic mutant mice indicated the *Hbb* locus as a top candidate. This locus encodes the β-major (Hbb-b1) and β-minor (Hbb-b2) globins. Genetic sequencing disclosed a T179G transversion in the *Hbb-b1* gene, resulting in a Leu88Pro substitution (Figure 1). This genotype is strictly homologous to the mutation of hemoglobin Santa Ana, a rare unstable hemoglobin described in humans.[21]

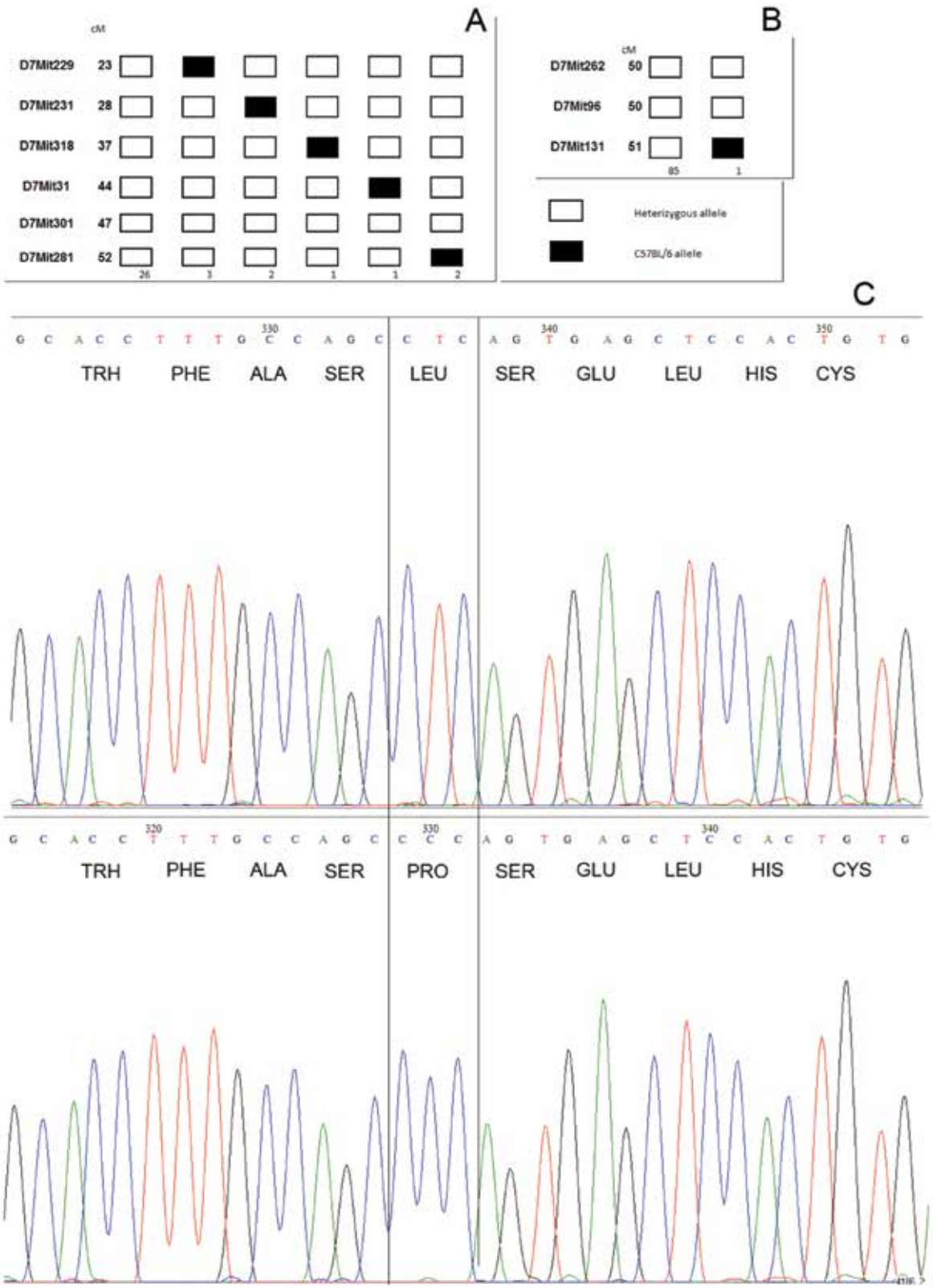


**Figure 1.** Positional cloning of the *Hbb–b1* Santa Ana allele. (A) The chromosome 7 haplotypes from 35 backcross mice are indicated. (B) The haplotype of 86 mice from the same backcross are indicated. Panels A and B respectively show 2 recombinant mice and 1 recombinant mouse that were critical for determining the minimal region for the *Hbb-b1* gene. (C) Point mutation in the *Hbb-b1* gene from anemic mice. A T→C transition causes an amino acid change from leucine to proline at residue 88. This point mutation is located in the 2nd exon of the *Hbb-b1*gene of anemic mice.

**Table 1.** Hematologic indices of BALB/c and mutant (*Anem*/+) mice

| | Male | | Female | |
|---|---|---|---|---|
| | BALB/c | *Anem*/+ | BALB/c | *Anem*/+ |
| RBC (x $10^6$/μL) | 9.9 ± 0.7 | 6.6 ± 0.4 | 9.6 ± 0.5 | 6.2 ± 0.3 |
| Hgb (g/dL) | 14.0 ± 0.9 | 11.0 ± 0.6[a] | 15.0 ± 0.8 | 11.0 ± 0.3[a] |
| Hct (%) | 43.3 ± 2.6 | 34.5 ± 1.5[a] | 43.9 ± 2.4 | 35.8 ± 1.0[a] |
| MCV (fL) | 44.0 ± 1.5 | 53.0 ± 2.7[a] | 45.0 ± 0.5 | 58.0 ± 2.3[a] |
| MCH (pg) | 14.6 ± 0.6 | 16.6 ± 0.8[a] | 15.9 ± 0.3 | 18.0 ± 0.7[a] |
| MCHC (%) | 33.0 ± 1.1 | 32.0 ± 1.0[a] | 35.0 ± 1.0 | 31.0 ± 0.3[a] |
| RBC distribution width (%) | 13.3 ± 0.5 | 17.6 ± 1.1[a] | 13.6 ± 0.4 | 18.8 ± 1.4[a] |
| Reticulocytes (x $10^3$/μL) | 184.0 ± 198.5 | 3837.0 ± 858.5[a] | 196.0 ± 88.8 | 3978.0 ± 632.8[a] |
| Reticulocytes (%) | 1.9 ± 2.1 | 58.5 ± 13.0[a] | 2.0 ± 0.8 | 64.1 ± 11.2[a] |

[a]$P < 0.001$

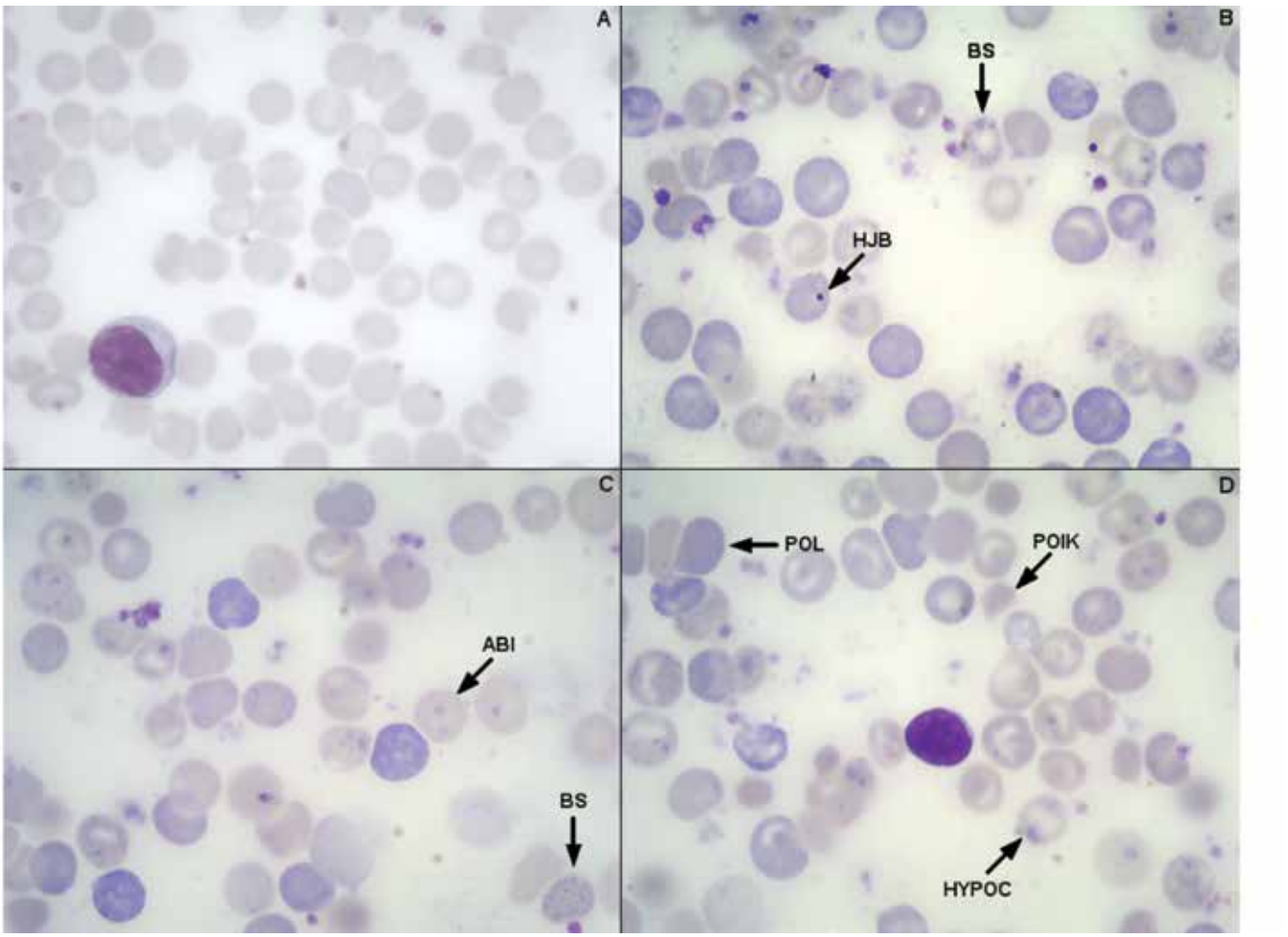


**Figure 2.** Blood smears from control and anemic mice. (A) Control mice. Note the uniformity of RBC size, shape, and color. (B–D) Anemic mice. Note the variation in RBC size, shape, and color. ABI, amorphous basophilic inclusion; BS, basophilic stippling; HJB, Howell–Jolly body; HYPOC, hypochromia; POIK, poikilocyte; POL, polychromasia. May–Grümwald–Giemsa stain; magnification, 1000×.

**Hematologic analysis.** Erythrocyte parameters (RBC, Hgb, Hct) from anemic mice of both sexes were lower than those of control groups, characterizing anemia. In addition, the MCV of anemic mice was greater (indicating larger erythrocytes, macrocytosis) when compared with control values and the MCHC of anemic mice was decreased, suggesting hypochromic anemia. In the mutant mice, the increased mean RBC distribution width (indicating anisocytosis) and numerous immature RBC (characterizing reticulocytosis) were indicative of erythroid regeneration (Table 1).

Blood smear analysis revealed several changes in RBC morphology in the anemic mice, including anisocytosis, polychromasia, hypochromia, Howell–Jolly bodies, basophilic stippling and

**Table 2.** Relative (%) and absolute (cells/mL) first quartile (Q1), median, and third quartile (Q3) values for WBC populations and platelets in control and anemic mice

| | Leukocytes (/mL) | Neutrophils | | Lymphocytes | | Monocytes | | Eosinophils | | Platelets (x $10^3$/mL) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | % | /mL | % | /mL | % | /mL | % | /mL | |
| Female | | | | | | | | | | |
| Control | | | | | | | | | | |
| Q1 | 4700 | 17 | 846 | 73 | 1820 | 1 | 59 | 1 | 59 | 355 |
| Median | 5200 | 18 | 1003 | 76 | 3572 | 2 | 68 | 3 | 120 | 446 |
| Q3 | 5900 | 20 | 1156 | 79 | 4779 | 3 | 141 | 3 | 141 | 453 |
| Anemic | | | | | | | | | | |
| Q1 | 9650 | 10 | 1235 | 79 | 7624 | 4 | 390 | 0 | 0 | 463 |
| Median | 9750 | 13 | 1475 | 82 | 7790 | 4 | 424 | 1 | 97 | 483 |
| Q3 | 10600 | 19 | 1834 | 83 | 9328 | 5 | 475 | 1 | 98 | 619 |
| Male | | | | | | | | | | |
| Control | | | | | | | | | | |
| Q1 | 5375 | 17 | 1215 | 69 | 3761 | 2 | 138 | 1 | 41 | 450 |
| Median | 6300 | 22 | 1242 | 74 | 4522 | 4 | 220 | 1 | 71 | 470 |
| Q3 | 7150 | 25 | 1442 | 78 | 5560 | 5 | 299 | 1 | 97 | 521 |
| Anemic | | | | | | | | | | |
| Q1 | 8400 | 12 | 1233 | 70 | 5493 | 1 | 89 | 0 | 0 | 299 |
| Median | 9600 | 16 | 1353 | 82 | 7200 | 1 | 122 | 0 | 0 | 392 |
| Q3 | 10900 | 27 | 1689 | 87 | 9586 | 2 | 208 | 1 | 58 | 517 |

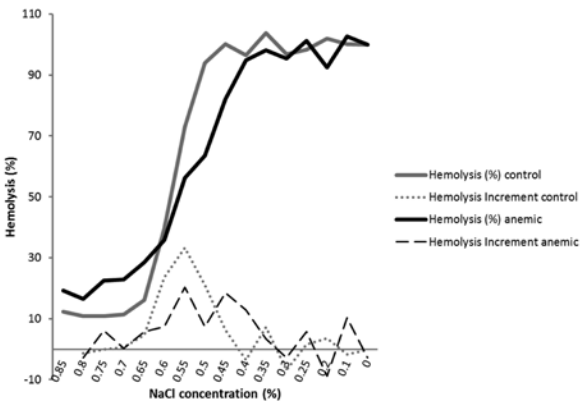


**Figure 3.** Cumulative and derivative curves for RBC osmotic fragility. The continuous lines represent the percentage of hemolysis; the dotted lines indicated the increment of hemolysis according to NaCl concentration.

abnormal hemoglobin precipitation, and Heinz bodies (Figure 2). Anemic mice presented increased leukocyte counts due to neutrophilia and marked lymphocytosis. Platelet numbers did not differ between anemic and normal mice (Table 2).

The results of RBC osmotic fragility revealed that even before testing began, hemolysis was greater in anemic blood samples than in control samples and presented a more gradual curve with a delayed hemolysis plateau; these characteristics indicate that the mutant mouse has multiple RBC populations and at least one is more easily broken and other is more resistant to hemolysis than are normal RBC. For control mice, the peak of hemolysis occurred at 0.5% NaCl concentration for the major RBC population, with another smaller peak at 0.35% NaCl concentration, which presumably was due to the reticulocyte population. Compared with normal RBC, anemic mature RBC are more sensitive to osmotic lysis, with the first peak at 0.55% NaCl, but the largest peak occurred at 0.40% NaCl, likely due to the larger immature RBC population of anemic mouse blood (Figure 3).

**Hemoglobin analysis.** Densitometry after hemoglobin electrophoresis in cellulose acetate at alkaline pH revealed 2 main peaks for the BALB/c hemoglobin (Figure 4 A); the $\alpha_2\beta_{dmaj2}$ globin chains constitute the main hemoglobin of BALB/c adult mice, with another β chain forming the $\alpha_2\beta_{dmin2}$ protein. The hemoglobin migrations of control and anemic mice are the same, but the anemic sample contains less $\alpha_2\beta_{dmaj2}$ protein than does the control. Note the 2 bands represented by peaks in the graphic, with each one corresponding to a different hemoglobin (Figure 4 A). The first peak is the hemoglobin composed of 2 α and 2 $\beta d_{minor}$ globin molecules (Hb $\alpha_2\beta_{dmin2}$), whereas the largest peak corresponds to the hemoglobin comprising 2 α and 2 $\beta d_{major}$ globins (Hb $\alpha_2\beta_{dmaj2}$).

The β globins ($\beta_{dmajor}$ and $\beta_{dminor}$) of BALB/c mice could not be separated from each other by electrophoresis, but separation of the α from the β chain showed that anemic mice have less β chain than do controls. The first peak represents all of the β chains in mouse hemoglobin proteins, with no differentiation of $\beta_{dmin2}$ and $\beta_{dmaj2}$ chains. The highest peak is the α chain globin (Figure 4 B).

Unlike control samples, blood samples from anemic mice showed turbidity at 10 min during the hemoglobin heat-stability test. Unstable hemoglobin proteins may flocculate after 10 min and then precipitate at later time points, whereas normal hemoglobin is expected to be stable throughout the process or present discrete precipitation after 30 to 45 min.[14] Both control and anemic samples precipitated at 30 min and remained as such until the end of the 60 min of observation. In the isopropanol test of hemoglobin stability, both control and anemic samples were turbid at 10 min. The anemic sample showed flocculation at 20 min and precipitation at 60 min, whereas the control sample produced flocculation at 30 min and precipitated at 60 min.

Methemoglobin formation was similar between control and anemic hemoglobin, because both showed peak absorbance at

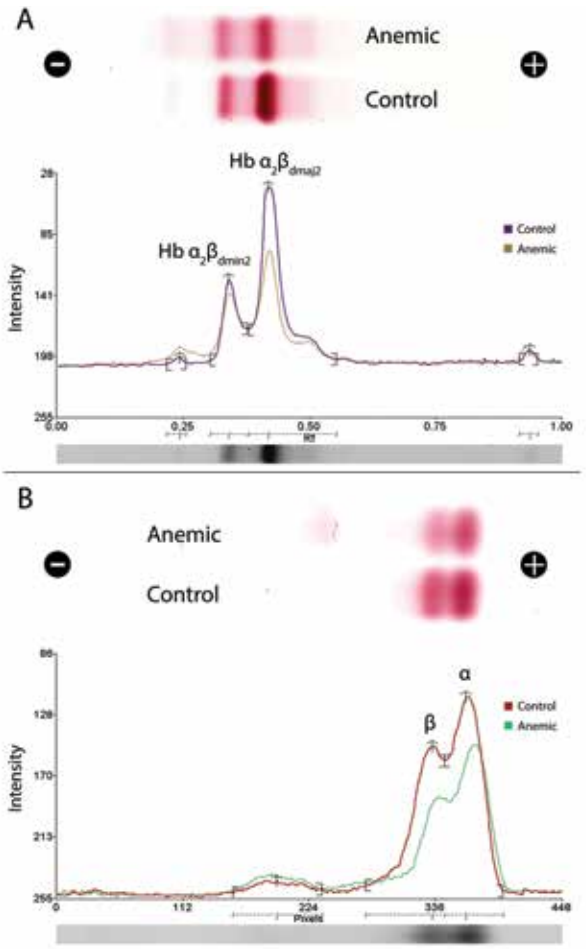


**Figure 4.** (A) Hemoglobin cellulose acetate and (B) hemoglobin polypeptide chain electrophoresis in alkaline pH (Ponceau S stain) showing electrophoretic curves of blood from anemic and control mice.

500 nm (Figure 5), although the hemoglobin concentration differed between the two phenotypes.

**Histopathologic evaluation and bone marrow cytology.** Abdominal palpation of anemic mice revealed an easily palpable, enlarged spleen. At necropsy, anemic mice showed intense dark-red coloration of both liver and spleen and icteric subcutaneous tissue pigmentation when compared with control animals. The spleens of anemic mice were 6 to 8 times larger than those in normal mice. Histologically the spleens of anemic mutant mice showed marked hematopoietic activity, with greatly increased macrophage iron deposition and numerous germinal centers scattered throughout the tissue. The liver and kidney samples of anemic mice showed no tissue or cell architectural changes, but iron deposition in Kuepfer cells and renal tubular cells, respectively, were evident on Prussian blue or Perl staining (Figure 6). These iron deposits confirm the presence of hemosiderosis in spleen, liver, and kidneys and tended to increase with advancing age.

During the collection of bone marrow samples, it was observed that the femur was darker in anemic mice than in controls. Bone marrow cellularity was increased in both sexes of mutant mice, with values of 21,703 cells/mm$^3$ in control male mice compared with 48,660 cells/mm$^3$ in anemic males and 22,433 cells/mm$^3$ in control females compared with 40,343 cells/mm$^3$ in anemic female mice. In addition, mutant mice showed dramatically increased iron deposition in bone marrow macrophages, which further supports the hemosiderosis scenario.

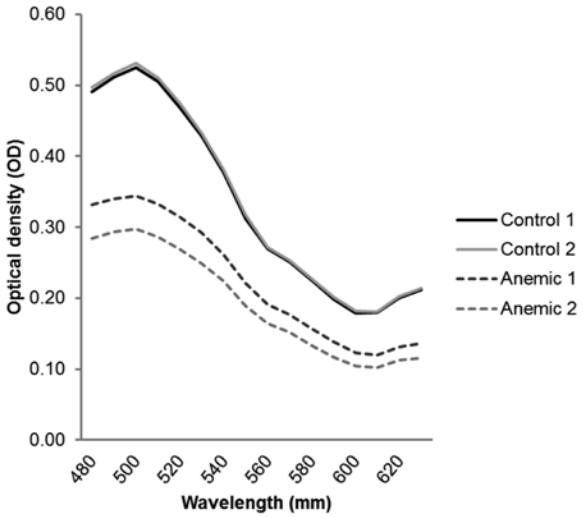


**Figure 5.** Methemoglobin curve of control and anemic blood. Data from 2 different of 10-wk-old anemic and control female mice.

## Discussion

Unlike the majority of mouse hemoglobin mutants, which affect the β-globin gene and thus provide models for the study of β-thalassemia in humans,[8,13] our mutant can be seen as a model for the study of the physiology and treatment of unstable hemoglobinopathies.

Mice affected by the Hb Santa Ana syndrome very likely arose as a novel N-ethyl-N-nitrosourea–induced mutation. The mutant was initially identified at birth, when mutant pups were paler and smaller than their normal littermates. The mutation shows an autosomal dominant mode of inheritance, with intrauterine death of homozygous mutants. Anemic mutants also demonstrate splenomegaly and reticulocytosis, indicating the presence of chronic hemolytic anemia and increased erythropoiesis.

The quantitative and qualitative changes in the RBC of anemic mice classified the hematologic profile as a congenital chronic regenerative hemolytic anemia with intraerythrocitic inclusions and Heinz bodies.[20] The presence of numerous reticulocytes in mutant mice of all ages contributes to an increased RBC distribution width and polychromasia, and there is a tendency to macrocytosis as indicated by the higher MCV in anemic mice than in controls, and discrete hypochromia, as indicated by the lower MCHC in mutant mice. This regenerative character reveals the intense erythropoietic response to the anemic process, which is reflected in the larger volume and increased cellularity of the hematopoietic organs, including spleen, bone marrow and liver, in mutant mice when compared with the control group. In particular, the cell count of the bone marrow was approximately twice that of controls, whereas that of the spleen was at least 6 times increased in mutants.

The presence of intraerythrocytic basophilic amorphous inclusions indicates intracellular precipitation of hemoglobin. These inclusions eventually progress to become well-defined Heinz bodies, characteristic of oxidative damage to hemoglobin. This feature implies a decreased RBC half-life and leads to the induction of

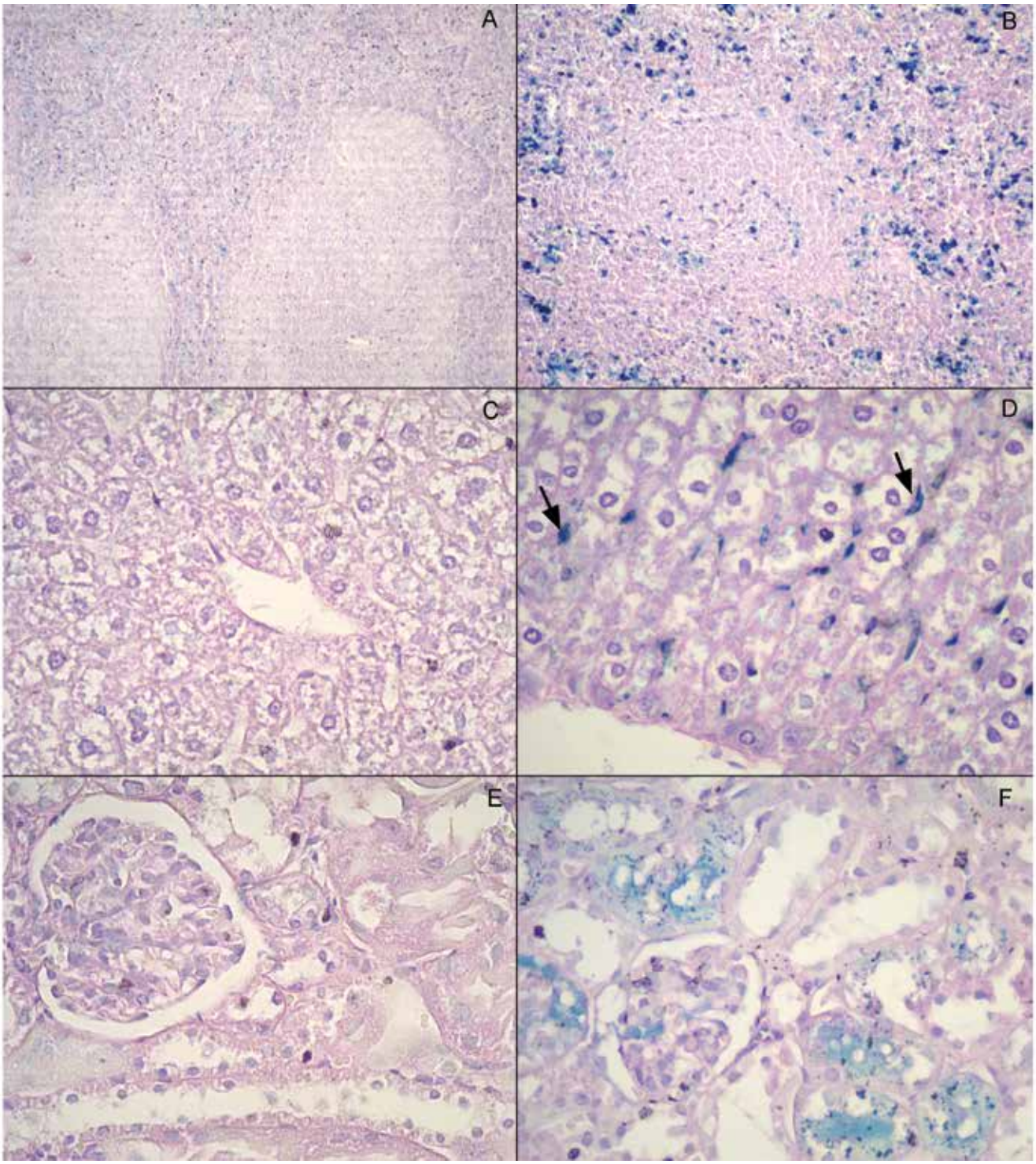


**Figure 6.** Histologic sections obtained from spleen, liver, and kidney of control and anemic mice and stained with Perl iron stain. (A) Spleen, control (magnification, 100×): discrete distribution pattern of iron deposits revealed in blue. (B) Spleen, anemic (magnification, 100×): moderate quantity of iron (blue). (C) Liver, control (magnification, 400×): no iron deposits present. (D) Liver, anemic (magnification, 400×): moderate quantity of iron in Kupfer cells (arrows). (E) Kidney, control (magnification, 400×): no iron deposits. (F) Kidney, anemic (magnification, 400×): note the blue staining (indicating iron deposits) in tubular cells.

hemolysis. The hemolytic condition in our mice is supported by the presence of hemoglobinuria, increased plasma phosphorus and bilirubin, and increased indirect bilirubin, which permeates tissues including subcutaneous tissue and which is clinically observed as jaundice. Symptoms in human patients with Hb Santa Ana include congenital compensated hemolytic anemia with intraerythrocytic inclusions, nonspherocytic anemia, splenomegaly, jaundice, dark urine, and bilirubinemia. Our mutant mice have similar symptoms

except that Heinz bodies are present naturally, whereas they are seen only after splenectomy in human patients.[19]

Hemoglobin electrophoresis at alkaline pH revealed the different hemoglobin β chains of BALB/c mice. Specifically, the proportions of Hb $\alpha_2\beta_{dmin2}$ and Hb $\alpha_2\beta_{dmaj2}$ differed between anemic and control mice, indicating that anemic mice have less β-major chain than do controls. We were unsuccessful in separating mouse $\beta d_{major}$ from $\beta d_{minor}$ by using hemoglobin polypeptide chain electrophoresis under alkaline conditions. In contrast, this method reveals an additional band in the position of a free α-chain when human β-unstable hemoglobins are examined.[15] However, compared with that of control mice, the stability of the mutant hemoglobin was decreased according to both the heat and isopropanol tests.

The hematologic changes were similar among mutant mice of all ages, without aggravation of the symptoms over time despite the hemosiderosis in the spleen and liver. Hemosiderosis was apparent in all of the organs analyzed from anemic mice, in addition to extramedullary erythropoiesis in the spleen. Similar to our mutant mice, patients with Santa Ana anemia occasionally have dark urine, due to hematuria.[15]

The human form of this mutation was first observed in 1968 in a family from Santa Ana, CA. Other human cases of unstable hemoglobin were documented in Minnesota,[3] France,[2] Philadelphia,[19] Japan,[12] and Brazil.[4] The replacement of leucine by proline at position B88 in Hb Santa Ana occurs in a helical portion of the molecule (F4 region). This replacement may not necessarily alter the structural conformation of the main chain, but may modify its side chain, which contacts the heme group, given that leucine occurs in this position in all known mammalian hemoglobins. Considering the structural contribution made by each of the exons of the β-globin gene, the second exon, where the mutation is located, provides the heme pocket (E and F helices) and the $\alpha_2\beta_2$ sliding contacts (helix C and the FG corner).[21] Such a loss of hydrophobic contact allows water to enter the heme pocket, causing the formation of methemoglobin and denaturation of the globin, resulting in intracellular precipitation.[19]

These data led to our conclusion that the anemic BALB/c mutant mouse we developed presents the same genotype as and a similar phenotype to that of the human Santa Ana unstable hemoglobin syndrome. As such, this model is the first mutant mouse described for the study of Hb Santa Ana. Although some of the symptoms observed in affected humans are not evident in the mutant mice, this anemic mouse model is appropriate for the study of unstable hemoglobinopathies.

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