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Hemolytic anemia in the mouse

Report of a new mutation and clarification of its genetics

Abstract: A new mutation causing spherocytic, hemolytic anemia has been discovered in the house mouse. It is inherited as a single autosomal recessive gene, allelic with both *sph* and *ha*, which, in turn, were shown to be allelic with each other. A revised nomenclature for the three apparent alleles is proposed: *sph* (formerly *sph*), sph^{ha} (formerly *ha*), and sph^{2Bc} (the new mutation). Like the other murine hemolytic anemias, sph^{2Bc} involves a defect in the red blood cell membrane protein, spectrin.

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FOUR SINGLE autosomal recessive mutations were reported to cause neonatal hemolytic anemia in the house mouse: jaundiced (*ja*)[14], spherocytosis (*sph*)[7], hemolytic anemia (*ha*)[1], and normoblastic anemia (*nb*)[2]. Each of these phenotypically similar conditions were considered to result from a mutation at different loci[3]. The present paper reports another occurrence of neonatal hemolytic anemia, its genetics, hematology, and red blood cell membrane state.

Materials and Methods

Anemic newborn mice appeared spontaneously during the fall of 1978 in the mouse colony of the Department of Medical Genetics at the University of British Columbia. The first affected animals, which were pale orange, were born to two pairs of sibs in the third intercross generation of a backcross-intercross system in which the open-eye gene opthalmatrophy (*oa*) was being transferred from a mixed strain background, including BALB/cGa, 129/J, and CBA/J, to the ICR/M1 stock. Initial crosses among offspring of these pairs suggested that the trait was determined by a single autosomal recessive gene, which assorted independently of *oa*. An anemia subline was created and maintained by brother-sister mating pairs. Inbreeding is now at the F_7 generation. All mice were maintained in the Zoology Research Unit 1, Animal Care Center, University of British Columbia. The animal rooms were kept on a 12-hour light (hours 0700 to 1700) and dark cycle. Mice were given Purina Mouse Chow and/or Purina Laboratory Chow and water ad lib.

During the routine weekly examination of the colony, cages containing pregnant females were noted and checked daily. For each newborn litter, the number of young (alive and dead), their sex and their condition with respect to anemia (seen as jaundice) was recorded. The litters were examined at approximately weekly intervals until weaning (21 days), and the survival of anemics was noted.

Mode of inheritance. Pairs that produced jaundiced newborns were designated carrier pairs. The numbers of jaundiced and normal newborns, observed within 24 hours of birth, were recorded from all such carrier-pair matings for the period from November 1978 to July 1979. The data were tested for fit to the hypothesis that anemia is inherited as a single autosomal recessive gene.

Allelism. Complementarity tests were used to test for allelism among the hemolytic anemia mutants. Two carrier males for each of *ja*, *ha*, *nb*, and one carrier male for *sph* (all on the C57BL/6J strain background) were sent by one of us (S.E.B.) from the Jackson Laboratory to the University of British Columbia (U.B.C.) where they were mated with heterozygotes for the new mutant. Any two mutants were considered to be allelic when matings between heterozygotes for each produced approximately 25 percent affected offspring.

Linkage. Preliminary data from linkage tests at the Jackson Laboratory indicated the

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possibility of linkage between *sph* and *Hba*, a marker on chromosome 11. These data and the result of the allelism test between *sph* and the new mutant (see Table II) led to a study at U.B.C. of linkage between the new mutant and *oe* (open eyelids—near the midpoint of chromosome 11). Homozygotes for *oe* were crossed with homozygotes for the new mutant and the assortment of phenotypes in the F_2 generation was analyzed.

Hematology. Data were obtained independently at U.B.C. and at the Jackson Laboratory. At U.B.C. they were obtained with the generous assistance of Dr. George Gray and Ms. Donna Siles of the Department of Hematology, Vancouver General Hospital. Venous blood was obtained from mice that were at least 8 weeks old by tapping the orbital sinus with heparinized microhematocrit tubes. Peripheral blood smears were made by the slide method and were stained with Wright's stain (Aimes Stain Pack). Reticulocyte smears were stained by mixing two parts of fresh blood with one part of 1 percent brilliant cresyl blue in isotonic saline. Basic hematological data, including the red and white blood cell counts (rbc, wbc), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), were obtained with a Coulter Counter Model F set for human blood cell determinations. Elevations in wbc concentration caused clouding in the samples drawn from the anemics and interfered with hemoglobin determination; the levels were corrected by the cyanmethemoglobin method[4]. In 1981, the new mutant was imported into the Jackson Laboratory where it is being maintained and hematological work has begun. The rbc counts were obtained there with a Coulter ZBI, hematocrits with a micro-hematocrit centrifuge, hemoglobin with the cyanmethemoglobin technique, and free erythrocyte protoporphyrin with a modified AVIV-ZPP meter[10].

Pathology. Adult mice were killed by prolonged exposure to ether. Touch smears of liver and spleen tissue and bone marrow from the femur were stained with Wright's stain (Aimes Stain Pack). Liver and spleen tissues were stored in Bouins fixative; histological sections were stained with hematoxylin and eosin.

Blood extraction. Normal (+/+) and homozygous anemic mice were anesthetized with pentobarbitol (approximately 0.25 ml of a 50 mg/ml solution, i.p.). They were then exsanguinated by cardiac puncture, (approximate yield: 1.2 cc whole blood per animal), and their blood was collected into separate heparinized syringes. Because the blood of the anemic strain was prone to rapid coagulation, an additional dose of heparin (25 μl of a 1000 units/ml solution) was injected directly into the heart just prior to exsanguination.

Membrane preparation. The heparinized blood was centrifuged and the packed red cells were washed three times in phosphate buffered saline and hemolyzed with 10 volumes of ice-cold 5mM sodium phosphate, pH 8.0, containing 1mM diisopropyl fluorophosphate (DFP) (Sigma) to minimize proteolysis. The membranes were collected by centrifugation (50,000G, 12 minutes) and washed repetitively until no further hemoglobin was removed.

In contrast to normal animals, small amounts of hemoglobin remained bound to the mutant's red cells despite persistent washing.

Polyacrylamide gel electrophoresis. The protein concentration of the isolated membranes was estimated by the method of Lowry et al.[8]. Appropriate aliquots (50 mg each) were added to an equal volume of sodium dodecyl sulfate (SDS) containing 1mM DEP and solubilized at 100°C for 3–5 minutes. The solubilized samples were then electrophoresed on 5 percent SDS-polyacrylamide gels under the conditions described by Steck[13] until the Pyronin-Y dye front had reached the bottom edge of the gel. The gels were fixed in $MeOH/H_2O$ for 12 hours, stained in fixative containing 2.5 percent w/v Coomassie brilliant blue for 1 hour and destained electrophoretically in 10 percent acetic acid for 45 minutes.

Results

Natural history

Affected mice are easily detected in the neonatal period by their body color—pale pink at birth and progressing to yellow-orange within a few hours—which contrasts sharply with the deep pink of their phenotypically normal littermates. Anemic mice become harder to distinguish as they approach weaning. Adult albino anemics can be detected relatively easily by the yellow-orange tinge to their ears, tails, and genital regions, and by their translucent peach-colored eyes; pigmented anemic adults are more difficult to detect.

Anemic mice have a very poor prognosis: approximately one-half (32/63) of those born live survive the first week, one-third (19/63) survive the second week, one-quarter (15/63) survive to weaning, and about two-thirds (10/15) of those weaned die before the age of 6 months. Young anemic adults are much smaller than their normal littermates. Two anemic females have had litters and 10 anemic males have sired litters.

Genetics

The new mutant fits the inheritance pattern of a single autosomal recessive gene. Among the offspring of matings between putative heterozygotes, 0.24 (185/761) were anemic; this proportion is not significantly different from the 0.25 expected ($P > 0.05$). Furthermore, a mating between two anemic mice produced 29/29 affected offspring (three litters). The sex ratio among anemic newborns, 1:1.26 (82♀:103♂), is not significantly different from 1:1 ($P > 0.05$).

The results of the complementarity tests performed at U.B.C. are shown in Table I. Because no affected offspring were observed in crosses between heterozygotes for the new mutation and either *ja*/+ or *nb*/+, it was concluded that the new mutation was at a locus separate from both *ja* and *nb*. However, all crosses between heterozygotes for the new mutation and both *ha*/+ and *sph*/+ produced affected offspring, indicating that the new mutation was likely at the same locus as both *ha* and *sph*. The total proportions of affected mice in crosses between heterozygotes for the new mutation and both *ha*/+ (6/25) and *sph*/+ (24/86) fitted that expected for a single autosomal recessive gene (0.25).

The hypothesis that *sph* and *ha* were themselves allelic was tested both at U.B.C. (Table II) and at the Jackson Laboratory (Table III). At U.B.C., a C57BL/6J male carrying *ha* and another carrying *sph* were crossed with females of the ICR/M1 stock. The offspring were identified as carriers by crosses with proven carriers of the new mutation. Carriers of *ha* identified in this way were confirmed by crosses with carrier sibs. Crosses then made between *ha*/+ and *sph*/+ produced 9/59 (0.15) affected offspring, a proportion that is not significantly different from the 0.25 expected ($P > 0.05$) if *ha* and *sph* are alleles. At the Jackson Laboratory, matings between

Table I. Results of allelism tests between the new mutant and *ja*, *sph*, *ha*, and *nb*

Sire*	Sire's strain	No. litters	Proportion affected
ja/+	C57BL/6J	3	0/32
	WB/B6 F1	2	0/40
sph/+	C57BL/6J	4	17/57 (0.30)
	WB/B6 F1	2	7/29 (0.24)
ha/+	C57BL/6J	4	6/25 (0.24)
nb/+	C57BL/6J	2	0/43

* Dams were heterozygous for the new mutant

ha/+ and *sph*/+ also produced a large number of affected newborns (34/308 = 0.11). Earlier tests of allelism between C57BL/6J-*ha*/+ and C57BL/6J-*sph*/+ had not indicated allelism (Table III). Subsequent matings at U.B.C. between *sph*/+ and both *ja*/+ (0/19) and *nb*+ (0/18) added to the above evidence that *ja* and *nb* are at loci distinct from *sph*. It now seems clear that *sph*, *ha*, and the new mutation are all alleles. Subsequently, we shall refer to them as *sph*, sph^{ha}, and sph^{2Bc}, respectively.

Table II. Results of allelism tests between *sph and *ha** from U.B.C.**

Dam's no.	Sire's no.	No. affected/total† (at birth)
sph/+ 23	*ha*/+ 9	2/12
sph/+ 24	*ha*/+ 8	3/ 9
sph/+ 27	*ha*/+ 8	2/14
ha/+ 1	*sph*/+ 19	2/13
ha/+ 2	*sph*/+ 17	0/11
		9/59 = 0.15 (P >0.05)

* F_1's from an ICR/MI × C57BL/6J cross
† One litter per pair

The assortment of the anemic and open eye traits (sph^{2Bc} and *oe*) in the F_2 generation of a cross between homozygotes of each is shown in Table IV. The evidence for nonlinkage between these loci is strong, and sph^{2Bc} is not near the midpoint of chromosome 11. The class with open eyes alone was deficient whereas the class with both open eyes and anemia was not. This suggests that the presence of sph^{2Bc}/sph^{2Bc} increases the penetrance of *oe/oe*.

In spite of an extensive search at the Jackson Laboratory for the location of the spherocytic anemia genes, no linkage group has yet been identified for any of them[3]; efforts to locate these mutants are continuing.

Hematology

The quantitative hematology results for young adult mice of genotypes sph^{2Bc}/sph^{2Bc}, $sph^{2Bc}/+$, *and* +/+ are given in Tables V and VI. The $sph^{2Bc}/+$ was similar to the +/+ in all values. The erythrocyte (rbc) count was low in sph^{2Bc}/sph^{2Bc}, in the order of half that in the normals. In sph^{2Bc}/sph^{2Bc}, the hematocrit was reduced by half, the hemoglobin level by somewhat more; the other rbc values (MCV, MCH, MCHC) were close to the control. The

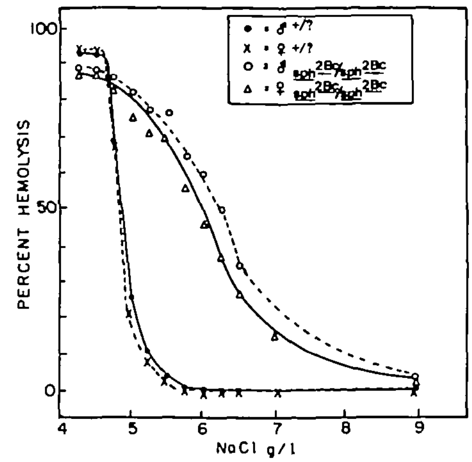

FIGURE 1 Osmotic fragility of erythrocytes from sph^{2Bc}/sph^{2Bc} mice and their +/? littermates.

reticulocyte count in +/+ and $sph^{2Bc}/+$ animals was between 5 and 10 percent. By contrast, anemic mice had reticulocyte counts of more than 90 percent, the absolute number being in the order of $3.32 \times 10^{12}/1$. Adult anemics have various abnormal forms of rbc's, including spherocytes, schistocytes, anisocytes, poikilocytes, erythroblasts, stipple cells, and elliptocytes. The form of rbc's in $sph^{2Bc}/+$ appeared to be normal. Osmotic fragility results (Figure 1) show that the rbc's from mutant homozygotes hemolyse at higher NaCl concentrations than do those of their unaffected littermates, i.e., they are more fragile.

The total white blood cell (wbc) count for young adult sph^{2Bc}/sph^{2Bc} mice was extremely high (Tables V and VI), several times that of the controls. The morphology of wbc's of sph^{2Bc}/sph^{2Bc} was normal except for the presence of smudge forms caused by shearing of fragile wbc's. No disturbance in the wbc differential in anemics was observed. The platelets appeared normal in number and morphology.

Table III. Results of allelism tests between *sph* and *ha* from the Jackson Laboratory

Year	Dam	Sire	No. affected/total† (at birth)
1979	B6*+/*ha*	B6 +/*sph*	0/18
1981	B6 +/*ha*	B6 +/*sph*	1/39
	B6 +/*sph*	B6 +/*ha*	0/26
	B6 +/*sph*	WB†+/*ha*	2/15
	WB +/*ha*	WB +/*sph*	2/12
1982	B6 +/*ha*	B6 +/*sph*	10/73
	B6 +/*sph*	WB +/*ha*	6/41
	WBB6F1 +/*sph*	WBBB6F1 +/*ha*	13/84
			34/308 = 0.11 (P <0.01)

* B6 = C57BL/6J
† WB = WB/ReJ

Table IV. Phenotype distribution of F_2 newborns from a cross between sph^{2Bc}/sph^{2Bc} and *oe/oe*: a test for linkage

Phenotype: anemia	Phenotype: eyes	Proportion and no. exp. if no linkage		No. newborns obs.	χ^2
normal	normal	9/16	175.5	184	0.41
anemic	normal	3/16	58.5	66	0.96
normal	open-eyed	3/16	58.5	41	5.23
anemic	open-eyed	1/16	19.5	21	0.12
Total				312	6.72
					for df = 3, P >0.05

Pathology

Hyperbilirubinemia was seen by direct observation as orange deposits in the liver and intestines of sph^{2Bc}/sph^{2Bc} animals. Histological sections of the liver showed erythropoietic foci, and bile pigments congesting the liver cords. Touch smears of the liver revealed the presence of darkly stained fragmented rbc's and more lightly stained erythroid precursors. Splenomegaly is a prominent feature of sph^{2Bc}/sph^{2Bc} adults: spleens were from 8 to 20 times normal size. In contrast to the healthy red color of normal spleens, the spleens of affected animals were a deep purple and

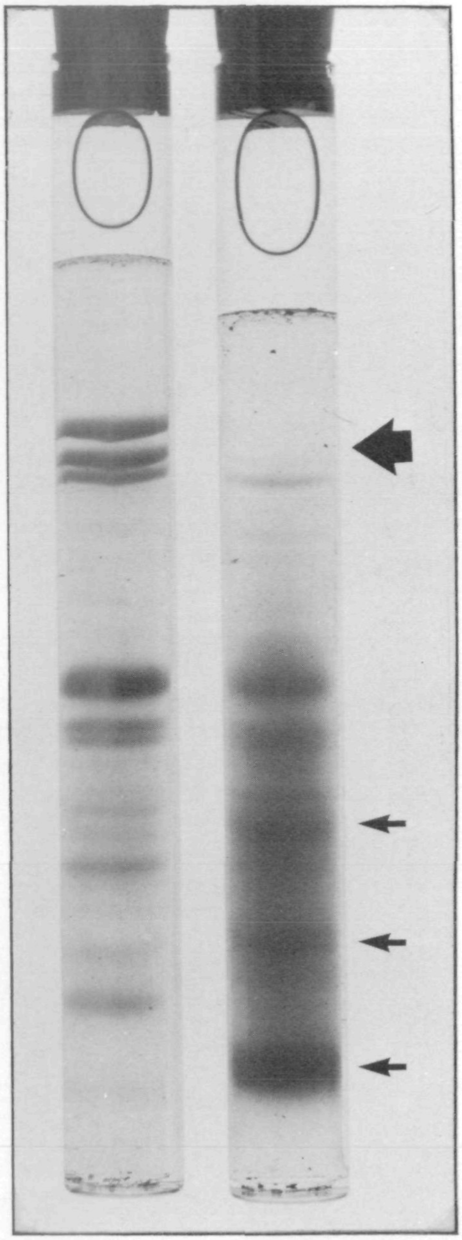

FIGURE 2 SDS polyacrylamide gel electrophoresis of +/+ (left) and sph^{2Bc}/sph^{2Bc} (right) erythrocytes. The large arrow indicates the position of the deficient spectrin polypeptides. The smaller arrows denote adsorbed cytoplasmic proteins.

histological examination showed many groups of darkly staining rbc's identified as late normoblasts. Touch smears also showed many late normoblasts, lymphocytes, and early granulocytes. Erythroid hyperplasia was observed as increased numbers of nucleated rbc's in touch smears of bone marrow from anemics. Normal mice had a myeloid:erythroid ratio of 3:1, anemics of 1:1. Lymphocytes were not found to be infiltrating the bone marrow of anemic mice as would be expected if the high wbc count indicated a chronic lymphocytic leukemia.

Red blood cell membrane protein composition

Normal erythrocytes had a pattern of membrane polypeptides on SDS gels that closely resembles the pattern observed in human erythrocytes (Figure 2)[9]. The anemic (sph^{2Bc}/sph^{2Bc}) mouse membranes showed distinctive and reproducible differences, particularly in the high molecular weight region. In particular, in over a dozen separate experiments, both of the spectrin bands (Figure 2, arrow) were markedly diminished. Typically, as shown, the top band (band 1), which corresponds to the larger or α-subunit, was not detectable, while a trace of material was evident in the position of the smaller β-subunit (band 2). Whether the latter material represents residual spectrin or another minor component that migrates in this position was not determined. Control experiments with normal erythrocytes, enriched in reticulocytes (80 percent) by repeated centrifugation, excluded the possibility that the changes were simply due to presence of a population of young red cells. Similar spectrin deficiency has been observed in mice bearing the *sph/sph* and sph^{ha}/sph^{ha} mutations[6,12].

In contrast to spectrin, the other major membrane polypeptides were present in approximately normal quantities (relative to the integral membrane protein 3). However, sph^{2Bc}/sph^{2Bc} membranes invariably contained residual adsorbed cytoplasmic proteins (small arrows, Figure 2) including hemoglobin (bottom arrow). In addition, a diffuse background haze was present, suggesting that some proteolysis had occurred despite the presence of a potent protease inhibitor (1mM DFP) during membrane preparation and solubilization. It is clear that the observed spectrin deficiency is not simply a proteolytic artifact, however, since the protein also was absent in gels of *intact* sph^{2Bc}/sph^{2Bc} erythrocytes (data not shown).

The membranes of the mutant rbc's were very fragile and, unlike normal, vesiculated spontaneously during preparation. Similar fragility has been observed in the other spectrin-deficient mutants.

Discussion

Hematologic and pathologic investigations indicate that the new mutation is expressed in the homozygote as a severe spherocytic, hemolytic anemia. The phenotype includes a low hemoglobin concentration, a high reticulocyte count, an elevated bilirubin level, erythroid hyperplasia in the bone marrow, extramedullary hematopoiesis in the liver, and an en-

Table V. Hematological values for +/+, $sph^{2Bc}/+$, and sph^{2Bc}/sph^{2Bc} mice at U.B.C*

	sph^{2Bc}/sph^{2Bc}	$sph^{2Bc}/+$	+/+
Erythrocytes ($\times 10^{12}/l$)	3.7 ± 0.1 (7)	7.5 ± 0.02 (2)	8.1 ± 0.3 (8)†
Hematocrit (%)	18.8 ± 1.1 (5)	35.5 ± 2.1 (2)	37.4 ± 1.9 (8)
Hemoglobin (g/d)	6.9 ± 0.3 (7)	16.5 ± 0.7 (2)	16.6 ± 0.5 (8)
MCV (fl)	50.6 ± 1.0 (5)	48.0 ± 2.0 (2)	46.3 ± 1.3 (8)
MCH (pg)	18.3 ± 0.8 (7)	22.0 ± 0.4 (2)	20.7 ± 1.1 (8)
MCHC (%)	37.1 ± 1.3 (4)	46.5 ± 1.1 (2)	44.5 ± 1.2 (8)
White blood cells ($\times 10^9/l$)	61.3 ± 4.5 (6)	13.2 ± 3.1 (2)	9.7 ± 0.6 (8)

* Young adults of both sexes from same stock
† Mean ± SE(M) (n)

Table VI. Hematological values for +/? and sph^{2Bc}/sph^{2Bc} mice at the Jackson Laboratory*

	sph^{2Bc}/sph^{2Bc}	+/? Littermates
Erythrocytes ($\times 10^{12}/l$)	4.49 ± 0.10 (12)	10.92 ± 0.10 (12)†
Hematocrit (%)	28.96 ± 0.72 (12)	52.41 ± 0.45 (12)
Hemoglobin (g/dl)	5.87 ± 0.14 (12)	16.30 ± 0.14 (12)
MCV (fl)	64.55 ± 1.12 (12)	48.00 ± 0.47 (12)
MCHC (%)	20.30 ± 0.32 (12)	31.11 ± 0.22 (12)
Free protoporphyrin (μg/dl Hg)	1206.0 ± 69.4 (12)	138.7 ± 20.5 (12)
White blood cells ($\times 10^9/l$)	34.10 ± 2.52 (12)	9.60 ± 0.60 (12)

* 7-week-old males and females
† Mean ± SE(M) (n)

larged spleen. The rbc morphology and the increased osmotic fragility suggest a decreased rbc survival time. There also is an elevated wbc count, the relevance of which is unclear. The basic defect appears to be in the rbc membrane. This hypothesis is supported by the rbc membrane protein pattern seen by SDS PAGE, indicating that the homozygote is deficient in the membrane component, spectrin.

The four known murine mutants with hemolytic anemia (*ja*, *sph*, *ha*, and *nb*) were considered to be caused by mutations at different loci. However, the present investigations show that the new mutation observed at U.B.C. is at the same locus as both *sph* and *ha*, which in turn are allelic with each other. The question then arises: are these three mutations different alleles, or are they repeat mutations of the same kind? Because *ha* and *sph* have been transferred to common genetic backgrounds—both are on two inbred strains, C57BL/6J and WB/Re—differences in phenotype can be attributed to allele differences. Differences between *sph* and *ha* in free protoporphyrin, osmotic fragility, and spectrin, indicate that these two mutations are in fact different alleles[3]. Because the new mutation, sph^{2Bc}, arose in, and has been maintained in, a different mouse stock, no comparisons in phenotype between it and the other four mutations have been made in this report. The new mutation is in the process of being transferred to the C57BL/6J and WB/ReJ strain backgrounds. At present, it is not possible to say whether sph^{2Bc} is a repeat mutation or a new allele with different phenotypic expression. It is, however, suggested that the most useful working hypothesis is that it is a new allele.

In conclusion, the genetic evidence presented in this paper leads us to suggest that three hemolytic anemia mutants—spherocytosis, hemolytic anemia, and the new mutant—are all at one locus and that jaundiced and normoblastic anemia are not. We recommend that the three allelic mutants be renamed in accordance with the standard rules of nomenclature (*Mouse News Letter* No. 61, 1979) and assigned the symbols *sph*, sph^{ha}, and sph^{2Bc}, respectively.

Addendum: A report of yet another phenotypically similar murine hemolytic anemia has come to our attention[11] since this manuscript was submitted. It was given the provisional gene symbol *sph*(H) in 1964 but has, to our knowledge, never been tested for allelism with any of the other hemolytic anemias. This mutant still exists (H. Scheufler, pers. comm.).

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