Fetal Compensation of the Hemolytic Anemia in Mice Homozygous for the Normoblastosis (nb) Mutation

By Luanne L. Peters, Connie S. Birkenmeier, and Jane E. Barker

The mouse autosomal recessive mutation nb causes a deficiency of erythroid ankyrin and generates a life-threatening hemolytic anemia in adult mice; however, at birth, nb/nb mice appear to be robust and show no pallor. In our study, the time of disease onset was sought by comparison of nb/nb and +/+ mice both in utero and postnatally. Erythroid ankyrin messenger RNA (mRNA) is expressed in fetal erythroid progenitors from normal mice, but is reduced to 10% of normal levels in mutant fetuses. Despite the deficiency of erythroid ankyrin mRNA, 16 and 18 day nb/nb fetuses have normal levels of red blood cells (RBCs) and the RBCs are morphologically normal by scanning electron microscopy. The earliest signs of any clinical anomaly are an increase in the number of circulating reticulocytes and the deposition of minor amounts of iron just before birth in the 18 day fetal nb/nb liver, suggesting that RBCs are being destroyed. Within 24 hours after birth, nb/nb neonates have a slight but significant decrease of their RBC counts. During the next 5 days, the nb/nb RBC counts decrease markedly, the reticulocyte counts assume the mutant adult levels of 60%, the erythrocytes become microcytic and fragmented, and iron deposits accumulate in the liver. The rapid onset of clinical disease postnatally, coupled with our findings that the erythroid ankyrin gene is transcribed in fetal erythroid cell precursors from normal mice, suggest that mechanisms exist in the nb/nb fetus to compensate for the erythroid ankyrin deficiency.

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MATERIALS AND METHODS

Animals. All mice were produced at The Jackson Laboratory (Bar Harbor, ME), where there is strict adherence to the regulations established by the American Association for the Accreditation of Laboratory Animal Care. The nb mutation was maintained by forced heterozygosity on both the WB/Rc (WB) and C57BL/6J (B6) inbred strains. Heterozygous (nb/+), mice from both stocks were crossed to generate WBB6F1-nb/nb mice and normal littermate controls.

Timed pregnancies were established by observing females from a mating pair daily for the presence of a vaginal plug, designated day 0. Females allowed to go to term invariably delivered their pups on the night of day 18. B6-+/+ mice were mated together to provide normal controls. To generate litters with nb/nb fetuses, the following matings were established: (1) WBB6F1-nb/nb females and males were bred together, but because of the reduced vitality of the mutant mice, few productive matings were obtained; and (2) WBB6F1-nb/+ females were transplanted with the ovaries of WBB6F1-nb/+ males. Because the nb/nb fetuses and newborns derived either from recipients of ovarian transplants or from crosses between nb/nb mice were indistinguishable, all data from these two groups were combined.

Tissues and cells also were obtained from B6-+/+ and WBB6F1-nb/nb adult mice to determine blood cell counts, tissue iron accumulation, and blood cell morphology for comparison with the fetal and neonatal values.

Collection of fetal and newborn cells. Fetuses were killed by cervical dislocation in the morning of either day 16 or 18 of pregnancy. Fetuses were detached from the placenta and placed in sterile phosphate-buffered saline (PBS) without Ca2+ or Mg2+ (PBS; GIBCO 310-4200AG, pH 7.4; GIBCO, Grand Island, NY). Fetuses and neonates were blotted dry and decapitated. Blood was collected in heparinized microhematocrit tubes for blood cell counts,
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from fetuses of time mated bearing nblnb ovaries were mated to WBBGFl-nb/+ males to generate nb/nb and nb/+ fetuses. Individual fetuses were retrieved for RNA collection. The Northern transfers were probed with a sterile Pasteur pipette.

Guanylum-thiocyanate-phenol-chloroform reticulocyte (pA+)RNA from adapted (PA+) RNA was isolated by oligo(dT)-cellulose chromatography, was 12P-radiolabeled by the random hexamer method. Polyadenylated (pA+) RNA was isolated by oligo(dT)-cellulose chromatography, RNA was separated by electrophoresis in agarose-formaldehyde gels, transferred to nylon filters, and hybridized under the conditions specified for Zetabind filters (AMF CUNO, Meridan, CT) but with 1% sodium dodecyl sulfate (SDS). The hybridization probe was a 4.6-kb mouse cDNA (mAnk-1) encoding a portion of the 5' repeat sequences, the spectrin and Band 3 binding domains, and portions of the alternatively spliced sequences in the regulatory domain of erythroid ankyrin. mAnk-1 was 32P-radiolabeled by the random hexamer method. After hybridization, the filters were washed once in 2x SSC (0.3 mol/L sodium chloride, 0.03 mol/L sodium citrate), 0.1% SDS at room temperature for 15 minutes, twice in 0.1x SSC/0.1% SDS at 65°C for 30 minutes each, and exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) with an intensifying screen at -70°C.

Peripheral blood counts and iron stains. A 1 μL aliquot of whole blood withdrawn directly into heparinized microhematocrit tubes was diluted in 100 mL of 0.9% NaCl in H2O and the erythrocytes were counted in a Coulter Counter Model ZBI (Coulter, Hialeah, FL). Blood smears were prepared and stained with Wright’s-Giemsa using an automatic Hemastainer (Geometric Data Corporation). Reticulocytes were counted after exposure to new Methylen Blue. For histologic examinations, liver and kidney were fixed in Bouin’s fixative, embedded in paraffin, and sectioned at 5 μm. Sections were stained with Gomori’s iron stain.

Scanning electron microscopy. Heparinized whole blood was collected and placed on ice. Samples were centrifuged (4°C) for 3 minutes at 350g and the cell pellet washed gently three times in sterile PBS. The cells were resuspended in 1 mL of fixative (glutaraldehyde, paraformaldehyde, cacodylate) and deposited onto polylysine-coated glass cover slips, dried in CO2, sputter coated with Platinum-palladium by standard techniques, and screened at 20 kV with the JEOL 35C scanning electron microscope (JEOL, Ltd, Tokyo, Japan).

Statistical analysis. Numerical results were analyzed by the two-tailed Student’s t-test for significance of difference between mean values. Values expressed as a percentage were transformed before statistical analyses.

RESULTS

Identification of nb/nb pups by Ank-1 expression. Adult mice homozygous for the mutation nb have severe hemolytic anemia caused by a defect at the erythroid ankyrin (Ank-1)

Table 1. Blood Parameters in Adult Normal and Mutant Mice

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>RBC Count (x 10^6/L)</th>
<th>Reticulocyte (%)</th>
<th>Absolute No. Reticulocytes (x 10^7/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WB6BFl+/+</td>
<td>10.6</td>
<td>3.03</td>
<td>0.32</td>
</tr>
<tr>
<td>WB6BFl-nb/nb</td>
<td>5.4</td>
<td>57.72</td>
<td>3.12</td>
</tr>
</tbody>
</table>

Fig 1. Northern blot of erythroid ankyrin RNA. Normal females bearing nb/nb ovaries were mated to WB6BFl-nb/+ males to generate nb/nb and nb/+ fetuses. Control +/+ RNA was isolated from fetuses of time mated B6-+/+ females. Individual fetuses were retrieved for RNA collection. The Northern transfers were probed with mAnk-1: lane 1, 10 μg total liver RNA from a 16 day nb/+ fetus; lane 2, 10 μg total liver RNA from a 16 day nb/nb fetus; lane 3, 4 μg reticulocyte (pA+)RNA from a 16 day nb/+ fetus; lane 4, 4 μg reticulocyte (pA+)RNA from a 16 day nb/nb fetus.

Fig 2. RBC counts in (●) normal (B6) and (○) mutant (nb/nb) mice. Counts are given for the indicated day of fetal (F) or postnatal (P) life. Numbers in parentheses represent the number of mice analyzed; bars indicate the standard error of the mean (SEM). *P < .05, **P < .001 for B6-/+ versus nb/nb at the same age.

Fig 3. Reticulocyte counts in (●) normal (B6) and (○) mutant (nb/nb) mice. Reticulocyte numbers for the indicated fetal (F) or postnatal (P) age (days) are presented as (A) percent of total circulating cells and (B) as absolute reticulocyte counts (total number of cells x % reticulocytes). Numbers in parentheses represent the number of mice analyzed; bars indicate SEM. *P < .05, **P < .001 for B6-/+ versus nb/nb at the same age.
locus that leads to decreased levels of *Ank-1* messenger RNA (mRNA). During a recent study, we noted that *nb/nb* mice did not appear anemic in utero or soon after birth. To determine whether *Ank-1* is expressed in fetal *nb/nb* erythroid tissues, Northern blot analyses were performed on 16 day fetal liver RNA generated from a cross in which the expected frequency of *nb/nb* pups is 50%. Of 27 fetuses tested, 13 (48%) showed markedly reduced expression (to $\leq 10\%$ of normal levels) of erythroid ankyrin compared with their littermates. The expression patterns of fetal liver RNA and of peripheral blood (pA+)RNA from 16 day +/+ and *nb/nb* fetuses are compared in Fig 1. Northern analysis was used to identify the phenotype of fetuses and pups from ovarian transplant recipients in the ensuing experiments.

**Time of onset of the anemia as determined by peripheral blood counts.** The blood parameters of adult normal and mutant *nb/nb* mice were published in 1980. Those studies were repeated to ascertain whether any variability appeared in the mice during the ensuing years of forced heterozygous. The results are presented in Table 1 and show that there has been no variation with time in the blood parameters of WBB6F1-+/+ and WBB6F1-nb/nb mice. The adult *nb/nb* mice have a severe microcytic anemia and pronounced reticulocytosis.

The observation that the *nb/nb* mice are not anemic in

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**Fig 4. Deposition of iron in the tissues.** Sections were stained with Gomori's iron stain. Tissues are from (A) a normal adult kidney; (B) an *nb/nb* adult kidney, arrows indicate iron deposits in the kidney glomerulus and arrowheads indicate iron in the proximal convoluted tubules; (C) a normal adult liver; (D) an *nb/nb* adult liver, arrowheads indicate typical iron deposits; (E) a 16 day normal fetal liver; (F) an 18 day normal fetal liver; (G) a less than 24 hour neonatal normal liver; (H) a 3 to 5 day postnatal normal liver; (I) a 16 day *nb/nb* fetal liver; (J) an 18 day *nb/nb* fetal liver; (K) a less than 24 hour neonatal *nb/nb* liver; and (L) a 3 to 5 day postnatal *nb/nb* liver. The arrowheads denote typical iron deposits in J through L.
utero or at birth, as judged by their lack of pallor, was investigated further. RBC counts in fetal nb/nb mice at 16 and 18 days of development did not differ from B6-/+ controls (Fig 2). Within the first 24 hours after birth, RBC counts in nb/nb mice were significantly lower than normal and continued to decrease dramatically during the early neonatal period.

Reticulocyte counts from 16 day fetal nb/nb mice did not differ from control fetuses. Within 24 hours after birth, a slight but significantly increased reticulocytosis was detected in nb/nb pups (Fig 3). The difference in the percentage of circulating reticulocytes in nb/nb versus +/+ mice further increased during the early postnatal period to attain the level (60%) observed in adult mutant mice. Interestingly, however, there appeared to be a crisis in 2- to 3-day-old nb/nb mice when the production of reticulocytes did not fully compensate for the loss of RBCs. This was reflected in the absolute reticulocyte counts of mutants and normal mice at 2 to 3 days postnatally and indicated a compromised erythrogenerative capacity at this time in development. At 5 to 6 days postnatally, the absolute number of reticulocytes in nb/nb mice was elevated, but still much lower than adult values (Table 1). The reasons for reduced erythropoiesis in neonatal anemic nb/nb mice are unknown, but probably account for the large number of deaths that occur in mutant mice during this period.11

Onset of tissue iron accumulation and of morphologic abnormalities in nb/nb mice. Adult nb/nb mice showed extensive deposition of hemosiderin in liver macrophages and kidney proximal convoluted tubules (Fig 4). Similar findings were described in the sph/sph mice with α spectrin deficiency20 and in human beings with hemolytic anemia.21 In nb/nb fetuses and neonates, iron deposits were observed in the liver but not in spleen and kidney. At fetal day 16, trace amounts of hemosiderin were detected in the liver as well, but there was no apparent difference between the mutant and normal mice. By fetal day 18, liver iron deposits were more obvious in the mutant than in the normal, but were still insignificant. After birth, there was a dramatic increase in liver iron deposits in the mutant, far exceeding that seen in the +/+ control.

Scanning electron microscopy indicated dramatic morphologic changes in adult nb/nb RBCs; many spherocytes and microspherocytes but almost no normal, biconcave shaped cells were seen (Fig 5). RBCs of day 18 fetal nb/nb mice, on the other hand, appeared morphologically similar to +/+ RBCs. Within 24 hours after birth, the nb/nb blood cells differed dramatically from +/+ blood cells and showed the presence of many microspherocytes, putative evidence of extensive hemolysis.

DISCUSSION

Our results indicate that the mice with normoblastosis are not anemic in utero, despite a deficiency of Ank-1 expression. The first appearance of the disease occurs just before birth with the accumulation of minor iron deposits in the liver. At this time, there is no indication of decreased blood cell counts and the blood cell morphology is indistinguishable from normal, suggesting that the defect is fully compensated during in utero development. By 24 hours after birth, there is a significant decrease in blood cell counts, a significant increase in reticulocytosis, and increased deposits of iron in the liver. It appears that disease onset occurs just before birth and rapidly accelerates in the nb/nb neonate.

The mechanism whereby the nb/nb mice escape disease in utero is unknown. It might be argued that erythrocytes are subjected to low mechanical stress in the fetal circulation or that the fetus is incapable of destroying defective erythrocytes because of an underdeveloped reticuloendothelial system. We believe these are not the reasons because of the following observations. Mice heterozygous for the mutation Nan (neonatal anemia) exhibit classic symptoms of marked hemolytic anemia at the time of birth and Nan homozygotes die at 10 to 11 days of fetal development.22,23 In the β-spectrin-deficient jaundiced (ja) mutants, homozygotes are detected at 15 days in utero by their pallid
appearance; however, actual fetal RBC counts were not performed. Newborn hea/hea (hereditary erythroblastosis anemia) mice also show marked hemolytic anemia at birth, with a near 50% reduction of the RBC count. With the exception of ja, the underlying defect in all of these hemolytic anemia mutants is unknown. However, the important point is that hemolytic anemia in mice can be manifested in utero.

Another explanation for the normal blood picture in the mutant fetuses is that erythroid ankyrin mRNA/protein is not essential. This seems unlikely in light of our studies. Erythroid ankyrin has binding sites for both the transmembrane anion exchanger and l-spectrin and functions as a strong linker of the spectrin-based skeleton to the erythrocyte membrane. Both erythroid ankyrin and l-spectrin are synthetized in early reticulocytes of adult rats and humans. We have shown that erythroid ankyrin is also expressed at high levels in the erythroid-committed progenitors from normal fetuses of 10 days' gestation through birth (Fig 1). The fact that the nb/nb fetuses are not anemic despite the lack of erythroid ankyrin expression suggests they can compensate for its loss through alternative mechanisms.

It is possible that the mutant nb/nb mice use a different product to bind the spectrin-based skeleton to the cell membrane. The onset of overt anemia after birth and subsequent exacerbation postnatally in nb/nb mice are reminiscent of the P-thalassemia syndromes and suggest that unique fetal products may exist. In human beings with P-thalassemia, caused by a defective l-globin gene, appearance of clinical symptoms occurs at age 1 to 2 years and is coincident with the normal decline in production of fetal l-globin chain. Moreover, persistent elevated expression of the fetal l-globin gene is a compensatory mechanism in those patients. In the murine P-thalassemia model, there is an increased production of a normally occurring adult l-globin. Our more recent studies suggest that the ankyrin composition of fetal and adult reticulocytes may differ substantially. Whether the products we detect are generated from alternatively spliced erythroid and brain ankyrin genes or from unique fetal genes is currently being investigated.

**ACKNOWLEDGMENT**

The authors thank Nancy Hambley for maintaining the mouse breeding colony and assisting with the timed matings. We also thank Drs Robert A. White and Sam Lux for providing the mAnk-I cDNA, and our reviewers, Drs Eva Eicher and Les Kozak, for their helpful comments on a previous version of the manuscript.

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