Hereditary Spherocytosis—Defects in Proteins That Connect the Membrane Skeleton to the Lipid Bilayer

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The molecular causes of hereditary spherocytosis (HS) have been unraveled in the past decade. No frequent defect is found, and nearly every family has a unique mutation. In dominant HS, nonsense and frameshift mutations of ankyrin, band 3, and β-spectrin predominate. Recessive HS is most often due to compound heterozygosity of defects in ankyrin, α-spectrin, or protein 4.2. Common combinations include a defect in the promoter or 5′-untranslated region of ankyrin paired with a missense mutation, a low expression allele of α-spectrin plus a missense mutation, and various mutations in the gene for protein 4.2. In most patients' red cells, no abnormal protein is present. Only rare missense mutations, like ankyrin Walsrode (V463I) or β-spectrin Kissimmee (W202R), have given any insight into the functional domains of the respective proteins. Although the eminent role of the spleen in the premature hemolysis of red cells in HS is unquestioned, the molecular events that cause splenic conditioning of spherocytes are unclear. Electron micrographs show that small membrane vesicles are shed during the formation of spherocytes. Animal models give further insight into the pathogenetic consequences of membrane protein defects as well as the causes of the variability of disease severity.

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HEREDITARY spherocytosis (HS) is by far the most common congenital hemolytic anemia in northern European descendants. The hallmarks of the disease are anemia, intermittent jaundice (from hemolysis or biliary obstruction), and splenomegaly. Spherocytes are devoid of the normal surface surplus and rigid. They are trapped during their passage through the metabolically unfavorable splenic pulp and selectively destroyed. The disease was first described in 1871 by two Belgian physicians, Vanlair and Masius,[1] as microcythemia, referring to the decreased diameter of spherocytes in the blood smear.

Prevalence and Genetics

HS occurs in all ethnic groups. The highest frequency of 1: 5,000 is found in Northern European countries.

De novo mutations of ankyrin or other membrane protein genes are a frequent cause of sporadic HS. In about two thirds of patients, the disease is inherited in a dominant pattern and can be followed from generation to generation, mostly with the same severity. In the remaining cases, both parents are normal. About half of these sporadic cases are due to de novo mutations of the type associated with dominant inheritance[2]; the others are assumed to be due to recessive genes. If this assumption is correct, about 1% of the population carries recessive HS genes.

One asymptomatic parent may carry a germ-line mosaicism for an ankyrin mutation or β-spectrin. De novo mutations of ankyrin[3] or β-spectrin[4] genes can arise in one of the parental germ lines and be transmitted dominantly. Parental mosaicism also occurs and must be considered in genetic counseling. The inheritance of HS is truly recessive in only about 10% to 15% of families. In these cases, both parents have minor signs of the disease—usually only an increased osmotic fragility in incubated blood or a slight reticulocytosis.[5,6] Patients with recessive HS tend to more profound anemia,[7,8] but clinical severity varies greatly.

Homozygosity for dominant HS is (nearly) lethal. As will be described later, dominant HS is mostly due to null (nonsense or frameshift) mutations: virtually no abnormal protein is present. Homozygosity for these mutations is presumably lethal during fetal development, as homozygosity for dominant inheritance has only been described in two cases, neither of whom was a complete null mutation. A Portuguese baby with severe hydrops fetalis was homozygous for band 3 Coimbra (Y488M),[9] and an Italian baby was homozygous for band 3 Neapolis (16+2 T→C), in which a splicing defect impedes translation initiation.[10] A small amount of abnormal protein may be produced from both mutations. The parents of both patients had only mild HS, whereas both children had severe, life-threatening anemia.

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HS is due to mutations at different chromosome loci. Two genetic loci for HS have been identified by study of balanced chromosomal translocations or small deletions, and by linkage analysis. One of the loci, an interstitial deletion of chromosome 8p11.1-p21.2,[11] eliminates the gene for ankyrin.[12] The second locus is the gene for β- spectrin, which resides on chromosome 14q23-q24.2.[13] As will be described, other loci involve the genes for band 3, protein 4.2, α-spectrin, and possibly β-adducin and dematin.

Clinical Presentation

Typical HS

In HS patients the family history is often positive for anemia, gallstones, or splenomegaly. In most cases the severity of HS is very similar in affected members of a family. Typically, there are increased number of spherocytes in the peripheral blood smear and increased osmotic fragility of the red blood cells. Other distinctive morphologic red cell alterations have been found in specific membrane defects[14] (Fig 1). Hemolysis (as evidenced by hyperbilirubinemia and ahaptoglobinemia) and reticulocytosis (a marker of compensatory erythropoiesis) are present.

HS typically presents in infancy or childhood, but may first manifest at any age.[15] In children, anemia is the most frequent complaint (50%), followed by splenomegaly, jaundice, and a positive family history (all 10% to 15%).

The majority of patients (60% to 75%) have incompletely compensated hemolysis and mild to moderate anemia. It is often difficult for parents to recognize the general symptoms of anemia (fatigue, mild pallor, or nonspecific findings such as "crabbiness"), and usually the true extent of reduced physical ability and school deficits only becomes apparent when positive behavioral changes following splenectomy.

Jaundice occurs in about half of patients, usually in association with viral infections. When present it is acholuric, characterized by unconjugated indirect hyperbilirubinemia and the absence of bilirubinuria.

The incidence of palpable splenomegaly varies from about 50% in young children to 75% to 95% in older children and adults.[16] The spleen is modestly enlarged (2 to 6 cm) but may be massive. Although likely given the pathophysiology and the response to splenectomy, there is no established correlation between spleen size and the severity of HS.

Classification According to Disease Severity

Disease severity of HS can be classified according to a few common clinical laboratory parameters. We classify HS into mild, moderate, moderately severe, and severe categories based on the hemoglobin and bili-

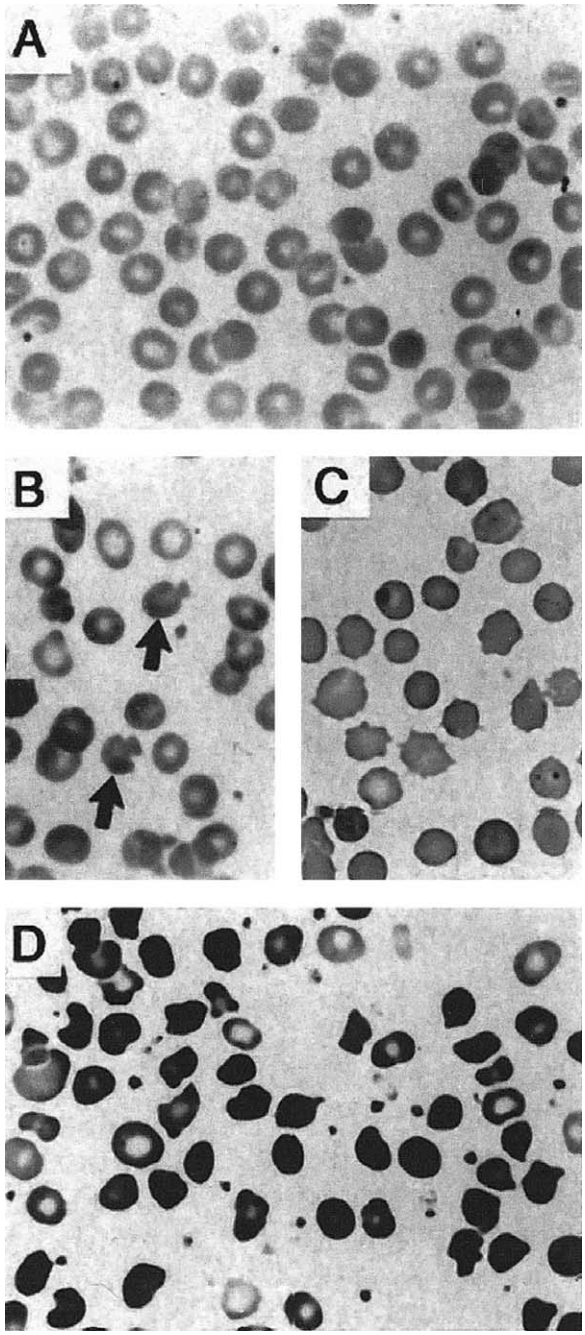

Figure 1. Abnormal red cell morphology in HS due to different membrane defects. (A) Ankyrin defects: in most blood smears spherocytes and anisocytosis are the typical morphologic change. (B) Band 3 defects: a small number of mushroom shaped (or "pincered") red cells (arrows) are usually seen. (C) β-Spectrin defects: a moderate proportion of acanthocytes and echinocytes are present. (D) α-Spectrin defects: these severely affected patients have numerous spherocytes, contracted, dense cells, and bizarre poikilocytes. (Reprinted with permission from Walensky LD, et al: Disorders of the red blood cell membrane, in Handin RI et al (eds): Blood: Principles and Practice of Hematology (ed 2). © 2003 Lippincott Williams & Wilkins.)

Table 1. Classification of Spherocytosis and Indications for Splenectomy

	Mild HS	Moderate HS	Moderately Severe HS	Severe HS*
Hemoglobin (g/dL)	11-15	8-11.5	6-8	<6
Reticulocytes (%)	3-8	≥8	≥10	≥10
Bilirubin (mg/dL)	1-2	≥2	>2-3	≥3
Osmotic fragility				
Fresh blood	Normal or slightly ↑	Distinctly ↑	Distinctly ↑	Distinctly ↑
Incubated blood		Distinclty ↑	Distinctly ↑	Markedly ↑
Transfusions†	0-1	0-2‡	≥3	Regular
Splenectomy§	Generally not necessary	If vitality is decreased	Necessary (at >5 yr)	Necessary (at > 3 yr)

*Patients depend on regular transfusions.
†One transfusion during an aplastic crisis and neonatal exchange transfusions are not counted.
‡Some patients need 1 or 2 transfusions during infancy.
§May be a total or subtotal splenectomy.

rubin concentrations and the reticulocyte count[5] (Table 1). Asymptomatic carriers of a recessive HS gene represent a separate group. The semiquantitative evaluation of the osmotic fragility test in fresh and incubated blood as well as quantitation of spectrin by specific enzyme-linked immunsorbent assay (ELISA) or radioimmunoassay (RIA) also can contribute to this classification.

Mild HS. Patients with mild HS have compensated hemolysis. About one third of patients have a mild form of HS, with slight reticulocytosis (3% to 8%). Red blood cell production and destruction are balanced or nearly equivalent,[17] and the erythrocyte production index[18] is increased by no more than three- to fourfold to achieve a state of "compensated" hemolysis. Patients are not anemic or barely anemic and are usually asymptomatic. The drive for increased erythropoiesis is still not fully understood. Possibly the dehydrated, rigid spherocytes do not adequately perfuse the juxtaglomerular renal vessels, where erythropoietin is produced, even when the hemoglobin is normal. This hypothesis is consistent with recent observation that serum erythropoietin is inappropriately high in patients with mild HS, maintaining bone marrow hyperplasia.[19] The diagnosis may be difficult because spherocytes are obvious in only two thirds of cases. Osmotic fragility is often increased only after the blood is preincubated for 24 hours (the incubated osmotic fragility test). The concentration of spectrin is at least 80% of normal.

Parvovirus infection, pregnancy, exercise, and splenic enlargement may exacerbate mild HS. Hemolysis may become more severe in patients with mild HS who develop illnesses that cause splenomegaly, such as mononucleosis, or during pregnancy[20] or with endurance sports. Some patients are diagnosed when they develop aplastic crises, typically caused by parvovirus B19. Symptomatic gallstones may be the first manifestation of mild HS in an otherwise asymptomatic patient.

Moderate HS. This is the largest group of HS patients, comprising about 60% to 70% of the total; most have typical HS as described above. The hemoglobin level is between 8 and 11 g/dL and reticulocytes are nearly always above 8%. The diagnosis is easy if there is spherocytosis and increased osmotic fragility. Family history is positive in about two thirds of cases. The amount of spectrin is usually below 80%. Ankyrin mutations are frequent causes of the disease (see below).

Moderately severe HS. A small group of patients, about 10%, have a low hemoglobin level beyond infancy (6 to 8 g/dL) and require occasional transfusions.[21,22] In some cases the symptoms are more evident during infancy and early childhood and much improve at school ages. Physical activities may be normal or impaired. Careful evaluation can show slightly retarded psychomotor development, especially motor coordination (Eber S, personal observation). Moderately severe HS is distinguished from moderate HS mostly by the low hemoglobin levels and the patient's intermittent need for transfusions. In addition, reticulocytosis is greater (often >15%) and bilirubinemia more pronounced (often >3 mg/dL).

Severe HS. Only 3% to 4% of HS patients have life-threatening anemia and require regular transfusions to maintain a hemoglobin level above 6 g/dL. In most cases inheritance is autosomal recessive (but we have observed a few dominant cases with severe HS). Patients develop hemosiderosis that may lead to organ failure. Continuous iron chelation therapy is usually necessary, starting around 4 years of age. Without regular transfusions or splenectomy, the patients may suffer growth retardation, delayed sexual maturation, or thalassemia-like facies.

Silent carrier state. Heterozygous parents or relatives of patients with recessive HS are carriers of an asymptomatic trait. They do not have anemia, splenomegaly, or hyperbilirubinemia.[5,23] Some carriers can only be detected by scrupulous testing for minor laboratory signs of HS. Some have a slight reticulocytosis (1.5% to 3%) or signs of slightly increased hemolysis such as a decreased haptoglobin level. The incubated osmotic fragility test is probably the most sensitive method for detecting carriers, particularly the 100% lysis point; the acidified glycerol lysis test (AGLT) may also be useful. In carriers, the halftime for hemolysis in the test may lie between the values for patients with overt HS ($AGLT_{50} < 5$ minutes) and normal controls (>30 minutes).

From the estimated prevalence of recessive HS (1 in 40,000 or about 12.5% of all HS, which is 1 in 5,000 in the United States), about 1% of the population can be estimated to be silent carriers. Indeed, screens of normal Norwegian[24] or German[25] blood donors with osmotic fragility or AGLT tests show a 0.9% to 1.1% incidence of previously undetected HS carriers.

HS in pregnancy. Transfusion is rarely necessary. Unsplenectomized pregnant patients with HS have no significant complications except for anemia, which is aggravated by the plasma volume expansion that occurs normally in pregnancy,[26] and sometimes by increased hemolysis[19] or megaloblastic crises. Transfusions are rarely necessary but should be instituted if the hemoglobin drops to less than 8 g/dL in order to guarantee optimal oxygen supply of the fetus. Folic acid at 1 mg daily should be provided to prevent vitamin deficiency and megaloblastic crises.

HS in the neonate. Increased hemolysis should be suspected when a neonate develops precocious or prolonged icterus, in Western Europe and the United States not infrequently due to HS. Approximately half of infants with HS develop severe neonatal jaundice, compared with 8% of normal newborns, and 91% of infants discovered to have HS in the first week of life have hyperbilirubinemia (bilirubin > 10 mg/dL).

Newborns with the combination of HS and the trait for Gilbert's syndrome frequently have hyperbilirubinemia. The presence of the trait for Gilbert's syndrome, a TATA box polymorphism in the bilirubin uridine diphosphate (UDP) glucuronyltransferase gene (*UGT1A1*), raises the frequency and severity of hyperbilirubinemia in newborns with glucose-6-phosphate dehydrogenase deficiency[27] and probably also with HS. Kernicterus is a risk if hyperbilirubinemia is not controlled. In most patients phototherapy is sufficient, but occasionally an exchange transfusion is required.

The natural history of HS during the first year of life was recently re-evaluated.[28] The hemoglobin concentration was normal at birth (>15 g/dL) in 57% of HS patients, consistent with the absence of intrauterine hemolysis. The normal intrauterine survival of spherocytes is not completely understood but may be due to the functional hyposplenia in neonates. The number of pocked red blood cells is increased in normal neonates up to the level of splenectomized older children, indicating impaired splenic phagocytic function at birth. Within the first weeks of life, splenic function improves. The infrequency of anemia in neonates with HS contributed to the low detection rate of HS at birth (approximately two thirds of affected neonates are undiagnosed). The hemoglobin value sharply decreased during the first 3 weeks of life, often necessitating transfusion,[28] and underscoring the desirability of early detection of HS. Due to current practice of early discharge of neonates from the nursery, physicians, midwives, and parents with a family history of HS must be alert for a drop of hemoglobin, especially if hyperbilirubinemia was present; it is not uncommon for infants with HS to be admitted at the age of 4 to 6 weeks with severe anemia.

Many neonates with HS have transient sluggish erythropoiesis. Many HS infants with only mild to moderate anemia at birth develop a transiently severe anemia (hemoglobin nadir of ≤6 g/dL) at the age of 4 to 8 weeks, probably due to increased hemolysis as splenic function improves after birth, combined with the "physiological" reduction of erythropoiesis in the oxygen-rich environment outside the uterus. In about 10% to 15% of cases, the erythropoietic response remains sluggish and reticulocyte counts do not rise appropriately for the degree of anemia during the first year. A moderate to severe anemia persists throughout infancy in these patients, who may need repeated red blood cell transfusions up to the age of 9 months. In a recent series,[28] only 24% of HS infants escaped transfusion: 34% needed a single transfusion, usually before 2 months of age; 24% required multiple transfusions for up to 9 months to reach transfusion independence; and 18% had severe HS and required chronic transfusions. In our combined experience of about 1,000 HS patients, the number of infants who required transfusions was much lower, and less than 3% of infants remained transfusion-dependent after the first year of life.

If a child is otherwise well, we allow the hemoglobin to fall to about 5 to 6 g/dL before we transfuse, to help to stimulate erythropoiesis. In addition, we only transfuse to a hemoglobin of 9 g/dL to avoid suppressing the desired marrow response. Regular subcutaneous administration of erythropoietin is recommended by some for infants with HS and sluggish erythropoiesis to avoid blood transfusions.[29] However, considering that subcutaneous injections may need to be administered for months and that antibod-

ies to erythropoietin may develop, further study of the use of this hormone is needed in anemic infants with HS before routine use can be recommended

Close observation of infants with HS is necessary. It is important to monitor hemoglobin levels and reticulocyte counts in infants with HS at least monthly during the first 6 months of life in order to detect and treat late anemia. The observation interval may be prolonged to 6 to 8 weeks after 6 months of life, and to 3 to 4 months in the second year of life. During childhood, each patient should have hemoglobin, reticulocyte, and bilirubin levels checked every 6 to 12 months up to the age of 5 years, and approximately yearly thereafter.

Complications of HS

Anemic crises. Most patients with typical moderate HS suffer a few hemolytic crises, often triggered by viral infections, and characterized by increased jaundice and anemia. Abdominal pain, vomiting, and tender splenic swelling are other typical features. Increased hemolysis is probably due to enlargement of the spleen during infections as well as activation of the reticuloendothelial system. For most patients with hemolytic crises, just supportive care is needed. Red blood cell transfusions are only required if the hemoglobin level falls below 5 to 6 g/dL.

The characteristic rash of parvovirus infection is absent in patients with hemolytic anemias and aplastic crises. With rare exceptions, severe aplastic crises occur only once in life. Most aplastic crises are caused by infection with parvovirus B19,[30] which confers life-long immunity. The virus infects and kills erythroid precursors, leading to a 10- to 14-day suppression of erythropoiesis.[31] The characteristic laboratory finding is a low number of reticulocytes (<2%) despite severe anemia. The earliest laboratory sign is an increase in the serum iron level due to the loss of erythroblasts and decreased hemoglobin synthesis. Loss of erythropoiesis in a patient with hemolysis can rapidly lead to a severe drop of hemoglobin (<3 g/dL), and may be fatal. The authors know of several children who died during an aplastic crisis, probably due to cardiac failure. Children with aplastic crises should be carefully observed and transfused if their hemoglobin decreases below 5 to 6 g/dL.

Parents should be advised to watch for pallor, extreme lassitude, and white conjunctivae after mild nonspecific signs of infection such as fever, vomiting, and abdominal pain. Children with HS should avoid contact with children with erythema infectiosum or fifth disease. There are anecdotal reports of the advantage of using intravenous immunoglobulin, which contains anti-parvovirus antibodies, early during the infection.[32] However, in most patients the aplastic crisis is discovered at the start of clinical symptoms, when the patient is producing his own antibodies to parvovirus; hence, the clinical benefit would be only marginal. Immunoglobulin therapy cannot be recommended before clinical efficacy is proven by a formal study.

Even though megaloblastic crises due to folate deficiency are very rare in developed countries, where nutrition and prenatal care are good, this complication can occur in patients who are malnourished or pregnant. Folic acid intake may be inadequate to minimize plasma homocysteine levels even in normal individuals.[33] Due to the higher demand for folic acid to support increased erythropoiesis, the risk of folate deficiency is increased in HS. We recommend 1 mg/d of folic acid for all patients with HS.

Gallstones. Bilirubin (pigment) gallstones are found in at least 5% of children less than 10 years of age with HS; the frequency reaches 40% to 50% in the second to fifth decade, with the increased incidence mostly during the second and third decades. Patients who inherit both HS and homozygosity for the mutation in the promoter of the *UGT1A* gene that characterizes Gilbert's syndrome[34] have a four- to fivefold increased rate of gallstone formation compared to cases with normal bilirubin clearance.[35]

The best technique to detect gallstones is ultrasonography. We recommend an abdominal ultrasound every third to fifth year and before splenectomy. About 40% to 50% of patients with gallstones eventually develop symptoms of gallbladder disease or biliary obstruction. The treatment of gallbladder disease in HS is debatable, especially in patients with mild HS or asymptomatic gallstones. Surgery is clearly necessary if there are recurrent episodes of cholecystitis. Isolated laparoscopic cholecystectomy is recommended mostly for children with mild to moderate HS. Children with more severe disease, who will need a splenectomy later, may profit from simultaneous subtotal splenectomy (see below) and cholecystectomy. A recent Markov analysis suggested that splenectomy (total or subtotal) and cholecystectomy should be done in adults who are less than 39 years old and have no gallbladder symptoms.[36] If the patient has occasional biliary colic, both operations are recommended up to age 52. Cholecystectomy alone is preferred in older patients with biliary colic.

Other complications. Leg ulcers and extramedullary hemopoietic tumors are rare complications that occur mostly in adults. Spinocerebellar degeneration and myocardiopathy have been described in association with HS, but the relationship, if any, to the hemolytic anemia is unclear.

Diagnosis and Laboratory Features

For patients with typical HS, the diagnosis is established by increased red blood cell osmotic fragility and spherocytosis on the blood smear, and if the direct Coombs test is negative. The combination of a high mean cellular hemoglobin content (MCHC), a widened red cell distribution width, and shifts in distribution curves are often sufficient to suggest HS.[37] The MCHC is greater than 36 g/L in half of patients.

Diagnostic Findings

Required diagnostic criteria are at least one sign of increased hemolysis: reticulocytosis, increased indirect bilirubin, increased lactate dehydrogenase, or decreased haptoglobin; increased spherocytes, subtle in mild cases, and anisocytosis; increased red blood cell osmotic fragility, especially after 24-hour preincubation of the blood and increased MCHC or a right shift in the MCHC histogram. All required signs must be present, with the exception that two optional signs can substitute for one of the required signs other than increased hemolysis and osmotic fragility. Optional diagnostic findings include a positive family history; spleen enlargement (a slight increase may escape detection); anemia (about one third of HS patients are not anemic); and decreased concentrations of spectrin, or spectrin and ankyrin, or band 3, or protein 4.2 in red blood cell membranes. Except for the most severe variants, anemia and hemolysis are virtually cured in HS patients by splenectomy. If significant hemolysis persists after splenectomy, a faulty diagnosis must be considered.

Detection of hyperdense red cells is a new diagnostic tool. Patients with HS consistently have a subpopulation of hyperdense red blood cells (MCHC >40 g/dL) in MCHC histograms. However, the measurement can only be made with modern-generation laser-based blood counters (such as the Bayer Technicon/Advia).[38] Similar information can be obtained from data generated by aperture impedance (Coulter) analysis.[37] These tests are appropriate to screen family members.

Osmotic gradient ektacytometry is a very sensitive method for diagnosing HS: it detects the characteristic decrease of membrane surface area in all cases.[39] The deformability index of the red cell is measured as a function of the osmolality of the suspending medium, which is continuously varied (Fig 2). The osmolality at which the red cell deformability index reaches a minimum is the same as the osmolality where 50% of the red blood cells hemolyze in an osmotic fragility test.[40,41] Unfortunately, the equipment needed for this technique is only available in a small number of laboratories.

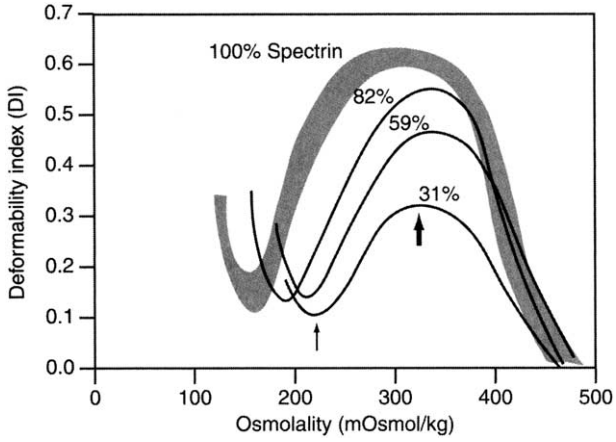

Figure 2. Osmotic gradient ektacytometry of red blood cells with varying degrees of spectrin deficiency. In the spectrin-deficient cells, the minimum deformability index observed in the hypotonic region (*thin arrow*) is shifted to the right of the control (*shaded area*), indicating a decrease in the cell surface area-to-volume ratio. The maximum deformability index (DImax) associated with the spectrin-deficient cells (*thick arrow*) is less than that of control cells, implying reduced surface area. The more pronounced the spectrin deficiency, the greater is the loss of surface area and the lower is the DImax. The osmolality in the hyperosmolar region at which the DI reaches half its maximum value is a measure of the hydration state of the red cells. It is decreased in the patient with the lowest spectrin content, indicating cellular dehydration. (Reprinted with permission from Walensky LD, et al: Disorders of the red blood cell membrane, in Handin RI et al (eds): Blood: Principles and Practice of Hematology (ed 2). © 2003 Lippincott Williams & Wilkins.)

Differential Diagnosis of HS

There are only a few diseases that can be confused with HS.

As noted earlier, the diagnosis of HS can be difficult in newborns. Only 35% of affected neonates have a reticulocyte count greater than 10% (normal: <8% on the first day of life) and 33% of neonates do not show distinct spherocytosis.[42] In addition, the osmotic fragility of fetal/neonatal red cells is diminished if adult control values are used; control values for neonates have been determined and should be employed for neonatal osmotic fragility tests.[42] The AGLT,[43] with modifications,[25] offers an easier approach to the diagnosis of HS in neonates.

ABO incompatibility is important in the differential diagnosis of HS in neonates because it is four times more often the cause of hyperbilirubinemia; it can be difficult to distinguish from HS, especially in the rare cases of Coombs-negative ABO incompatibility with spherocytosis.

In older children and adults an autoimmune hemolytic anemia (AIHA), especially with IgG (warm) antibodies or the biphasic IgG antibodies of the Donath-Landsteiner type, must be excluded by a negative Coombs test. The demonstration of a subnormal

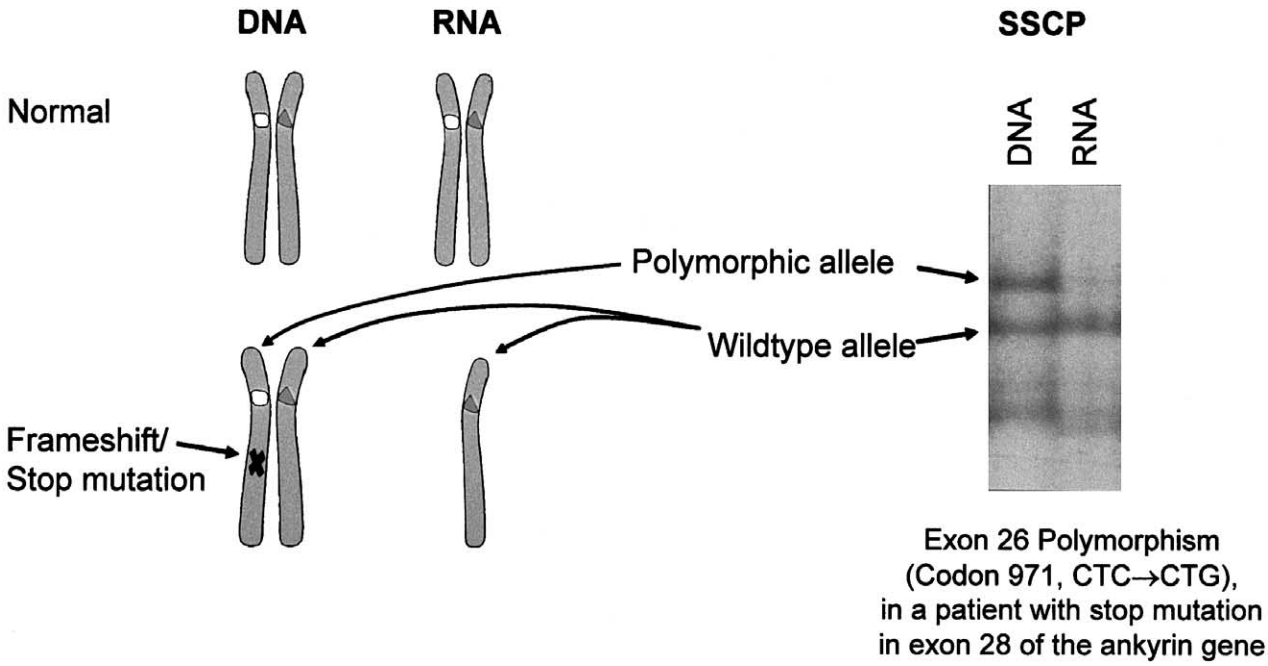

Figure 3. Reduced expression of one cDNA allele in an ankyrin nonsense mutation. SSCP analysis of genomic DNA and mRNA (as cDNA) of a patient with a nonsense mutation (stop codon in exon 28 of ankyrin) on a chromosome with a polymorphic ankyrin marker.[48] While the patient is heterozygous in the genomic DNA, one of the two alleles is (nearly) missing in the cDNA, demonstrating reduced expression of one cDNA allele. (Modified with permission from Özcan R, et al: Simultaneous (AC)n microsatellite polymorphism analysis and SSCP screening is an efficient strategy for detecting ankyrin-1 mutations in dominant hereditary spherocytosis. Br J Hematol 122:669-677, 2003.)

spectrin concentration in the red cell membrane can distinguish HS from AIHA, in which it is generally normal.[21]

Specific Tests of Membrane Proteins and Genes

Measuring membrane protein composition. Measuring the amount of spectrin (and/or ankyrin, band 3, or protein 4.2) in the red cell membrane can support the diagnosis of HS in atypical cases (when there is concomitant presence of red blood cell antibodies). However the measurements are difficult because small variations from normal must be accurately measured; some patients with HS have only a 10% to 15% decrement in the affected membrane protein. The simplest method is sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of red cell membranes, but to achieve the required precision multiple samples must be tested. Because band 3 is used as an internal standard to quantitate membrane proteins on SDS-PAGE and some band 3 is lost when membrane surface is shed and spherocytes form, small decreases in the other membrane proteins may be hard to measure in mild cases. The amount of spectrin and ankyrin is best determined by use of RIA[7,44] or ELISA.[45] Unfortunately these methods have not been developed to a robust routine level and they are not presently available. SDS-PAGE measurements are done in a few specialized laboratories but, in general, investigation of specific membrane proteins plays no practical part in the diagnosis or management of HS.

Defining specific molecular defects. Several approaches have been used to define specific molecular defects. In families with multiple affected members, lack of linkage between HS and polymorphic markers for α- or β-spectrin, ankyrin, band 3, or protein 4.2 can be used to genetically exclude specific membrane proteins from consideration. Frameshift and nonsense mutations are common in HS (see later section); they are often accompanied by absence of the mutant mRNA as well as the protein due to nonsense-mediated mRNA decay,[46] which can be detected by loss of heterozygosity of a polymorphic reticulocyte cDNA marker for one of the four HS genes compared to the same marker in genomic DNA (Fig 3 and Table 2).[47,48] This method also has been used to detect de novo dominant HS patients who lack a family history.[49] Finally, suspect proteins can be sequenced using genomic DNA and polymerase chain reaction (PCR) primers to amplify the exons.[50] Because most of the proteins that cause HS are large and contain

Table 2. Heterozygote Frequency of the Common Polymorphisms of Candidate Genes for Spherocytosis

Gene	Polymorphisms of cDNA*†	Proportion of Heterozygous Middle Europeans (%)
Ankyrin-1	Exon 4 (cd 105 AAC→AAT)/Exon 26 (cd 971 CTT→CTG)	39
	Exon 18 (cd 691 GGC→GGT)/Exon 21 (cd 783 ACC→ACT)	36
	Exon 39 (cd 1755 GTG→GTA)	33
	VNDR of 3′- UTR (AC-repeat)[47]	54
Band 3	Exon 11 (cd 417 CTG→TTG)	13
	Exon 12 (cd 441 CTG→CTA)	11
β-Spectrin	Exon 11 (N439S)	50
	Exon 14 (C→A at nucleotide 2249)	41
	Exon 16 (D1151N)	45

Abbreviation: cd, codon.

*With the exception of exon 11 and 14 in β-spectrin all other polymorphisms are silent.

†β-Spectrin polymorphisms are according to Hassoun et al.[101]; all others from Eber et al.[50]

many exons, this approach is arduous. Hence, a molecular diagnosis of HS is of scientific interest, for unclear heritage in patients with severe disease, or in patients with atypical HS associated with other clinical features (such as cardiomyopathy or spinocerebellar degeneration) that suggest the mutation may provide some new insight into the function of the defective protein. A rational procedure in these cases is to combine the use of polymorphic markers with a single-strand conformation polymorphism (SSCP) screen,[48] as will be discussed later.

Therapy

Splenectomy

Pros and cons of splenectomy. HS is the most common indication for splenectomy in childhood. Splenectomy cures almost all patients with HS. The spherocytes remain but the hemoglobin rises to normal and the reticulocyte count falls to 3% or less. Only in very rare patients with extremely severe HS is the response incomplete, and even they experience great improvement following splenectomy[7,23] (Lux S, personal communication).

However, the indications for splenectomy must be carefully weighed, as between 0.05 and 0.3 patients die of fulminant postsplenectomy sepsis for every 100 person-years of follow-up.[51-55] A recent, long-term 30-year evaluation up of more than 200 patients reported a frequency of 0.073 deaths per 100 person-years.[56] The actual incidence is lower because the risk of lethal sepsis declines with age and time postsplenectomy—although it never disappears completely.[51,56]

The spleen is important in controlling parasites like babesia and malaria, and patients who are splenectomized have an increased risk of fulminant infections from these organisms, an increasingly important consideration today, when travel to distant parts of the world is common.

Finally, the incidence of coronary heart disease and cerebral stroke is increased at least sixfold in splenectomized HS patients older than 40 years of age.[57] However the risk of coronary heart disease after splenectomy in patients is only slightly higher than in the normal population.[57,58] Much of the increased risk of atherosclerosis in splenectomized HS patients seems to due to the loss of protective factors (low hemoglobin and blood viscosity, low cholesterol level) associated with anemia rather than caused by the splenectomy.[58] The risk in nonsplenectomized (presumably anemic) HS patients appears to be much lower than among controls. These results and the increased risk of sepsis argue against splenectomy in all HS patients. Who should be splenectomized, when and what kind of operation should be performed, and how should the patient be treated postoperatively?

Who should have a splenectomy?. Splenectomy is indicated in moderate to severe or severe HS if recurrent transfusions are necessary beyond the neonatal period. Growth retardation (in severe transfusion-dependent HS) or significantly reduced physical ability due to anemia (in moderate or moderately severe HS) probably also justify the operation. Occasionally, marked, persistent visible jaundice, due to the combination of mild to moderate HS and homozygosity for the Gilbert's polymorphism, leads to consideration of a splenectomy for cosmetic reasons. More controversial justifications include fatigue with an attention deficit disorder or frequent absences from school plus consistently poor scholastic achievement. There is no proven relationship between severity of HS and school performance, but the improvement in energy and behavior that is often observed following splenectomy is suggestive. Splenectomy simply to remove a large spleen is not advised because there is very little risk of splenic rupture in HS. A transfusion-dependent aplastic crisis also should not require a splenectomy because the risk of a second aplastic crisis is low.

Patients with severe HS and frequent transfusions should have splenectomy around the age of 5. We prefer an earlier splenectomy rather than to initiate daily subcutaneous iron chelation with deferoxamine for transfusional iron overload, but we never undertake splenectomy before the patient is 3 years of age. Splenectomy is not recommended for mild HS during childhood and adolescence; exceptions may be adults with mild HS undergoing cholecystectomy or with marked jaundice, as noted earlier.

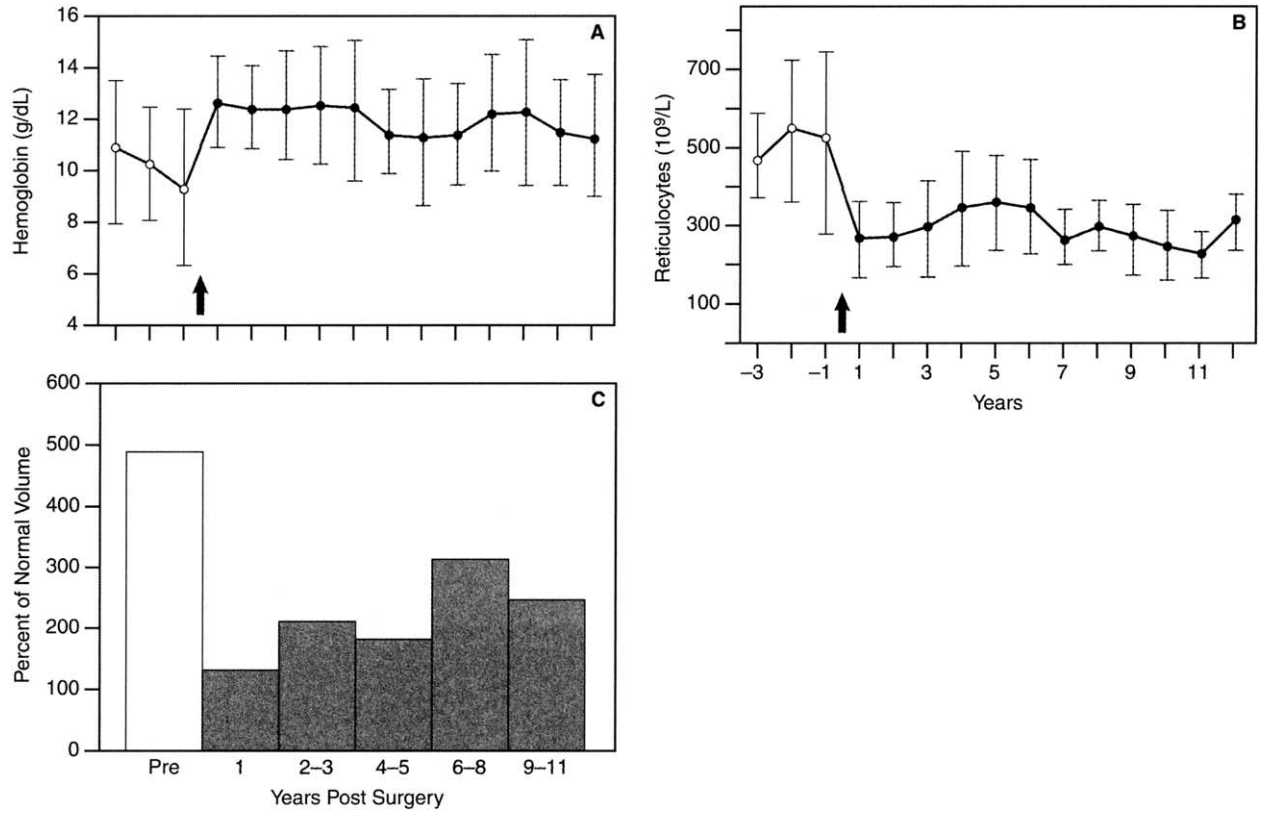

Figure 4. Long-term follow-up of subtotal splenectomy in hereditary HS. The data are the result of 12 years of experience with the procedure in 40 patients (1 to 25 years).[59] (A, B) Change in hemoglobin and reticulocyte count with time before and after the subtotal splenectomy (arrow). Mean and standard deviation are shown. Both the rise in hemoglobin and decline in reticulocytess have been sustained for more than a decade. (C) The size of the spleen during the same period; there is some regrowth of the splenic remnant, especially early. (Reprinted with permission from Walensky LD, et al: Disorders of the red blood cell membrane, in Handin RI et al (eds): Blood: Principles and Practice of Hematology (ed 2). © 2003 Lippincott Williams & Wilkins.)

Subtotal (partial) splenectomy. Because of the risk of postsplenectomy sepsis, subtotal (or partial) splenectomy has been advocated as an alternative.[59-63] Subtotal splenectomy has been done in more than 100 HS patients, who showed stable long-term improvement in hemoglobin and reticulocyte counts (Fig 4). Tchernia and coworkers[59] and Rice et al[62] remove about 80% to 90% of the enlarged spleen, leaving behind a remnant with about 25% of the volume of a normal spleen (≥30 mL). Eber et al[61] use a more radical approach, featuring near total splenectomy, and the remnant has a volume of only about 10 mL. Both approaches seem to ensure prolonged reduction, although not complete elimination, of hemolysis (Table 3).

In the studies of Tchernia et al and Eber et al, splenic phagocytosis, evaluated by postoperative nuclear scintigraphic scans and by counts of pitted red cells, showed that the splenic remnants retained function. It is not known whether subtotal splenectomy prevents postsplenectomy sepsis, although presumably the risk is decreased.

Rapid regrowth of the splenic remnant was noted during the first 2 years after surgery by both groups (Fig 4). After this initial spurt, the remnant spleen enlarged more slowly; regrowth has not ceased after 12 years of follow-up. Thus, it is possible that regrowth of the splenic remnant will eventually cause HS to recur.

In most patients the quality of life improves (92% of patients) and there is a gain in physical growth following subtotal splenectomy. Children may also profit by improved school performance (Eber SW, Riekhof J, unpublished data). In the French study, partial splenectomy did not fully prevent the formation of gallstones, particularly in patients with more severe disease.[59]

Indications for subtotal splenectomy. The major advantages of subtotal splenectomy are long-term normalization of hemoglobin value, improved physical ability and school performance, comparable to that obtained after full splenectomy, and avoidance of transfusions. The procedure appears to reduce the severity of HS by about one grade and thus can be

Table 3. Success of Subtotal Splenectomy in Hereditary Spherocytosis

	Bader-Meunier et al,[59] 2001	de Buys Roessingh et al,[60] 2002*	Rice et al,[62] 2003*	Eber et al,[61] 2001 + data by Stöhr et al, 2003*†
Operative technique	Subtotal (80-90%; ie, about 30 mL residual volume)	Partial; remnant 1/4 of the enlarged splenic volume	Subtotal (80-97%)	Near total: 10 mL residual volume
No. of patients (age)	40 (1-15 yr)	5 (1-7 yr)	16 (4-15 yr)	29 (3-21 yr)
Observation period	14 yr	6 yr	6 yr	9 yr
Postoperative hemoglobin (g/dL)	12.7 ± 1.2	8.3-12.8	11.5-14.7	12.2-15.9
Postoperative reticulocytes	≈50-66% of preop level	NA	1.4-11.5%	2.6-13.3%
Postoperately transfused patients	5 (aplastic crisis)	2	1	None
Postoperative splenic regrowth	≈2× normal size	Distinct	Stable at 15-30% of original size for 2 yr	To normal size
Secondary total splenectomy (years after surgery)	3 (3-6 yr)	2 (3-4 yr)	0	0
Loss of splenic remnant	None	None	None	2 (early period)†

Abbreviation: NA, data not available.

*In no case was postsplenectomy sepsis observed.

†Data of Stöhr G, Sobh H, Eber SW 2003 (unpublished).

‡In one patient the splenic remnant was lost due to an inappropriate surgical procedure (cauterization) in the first year after near total splenectomy; after changing procedure, no further loss was observed.

recommended for mild to moderate disease (see Table 1) but not for moderately severe or severe disease—these patients should have a total splenectomy. Patients with mild disease tend to have a supernormal hemoglobin concentration after a full splenectomy, which may predispose to coronary heart disease. Also, a second operation likely will not be necessary in patients with mild or moderate disease. Due to the increased risk of postsplenectomy infection, children between 3 and 5 years with severe HS who require a splenectomy should be considered for subtotal splenectomy, with the realization that a full splenectomy may be needed later. We do not recommend any kind of splenectomy before 3 years of age or prophylactic removal of gallbladder at the time of subtotal splenectomy.

Subtotal splenectomy is usually an open operation, but a laparoscopic procedure has been reported. The subtotal operation is more time-consuming than complete splenectomy and recovery is longer, since total splenectomy is frequently laparoscopic.[64] The authors are aware only of one local bleeding episode requiring conversion to a total splenectomy. Surgeons are strongly advised to seek the advice and follow the guidelines of one of the experienced international groups.

Prophylaxis after splenectomy. Virtually every patient without a spleen has a significantly increased risk of severe infection, mostly *Streptococcus pneumoniae*. Therefore, immunizations against *S pneumoniae, Haemophilus influenzae*, and, in cetain cases, *Neisseria meningitidis* are recommended.[65]

Penicillin should be administered after splenectomy but there is controversy regarding the duration of prophylaxis. There is no scientific justification for any specific regimen. One of us (S.W.E.) recommends treatment for at least 3 years followed by lifelong administration of broad-spectrum antibiotics early in any unclear infection or high fever.[65] In children with subtotal splenectomy, continuous penicillin prophylaxis may be stopped as early as 1 year after surgery if phagocytic function is normal, as assessed by the proportions of pocked red cells or by the uptake of radioactive colloid by the spleen. The other author (S.E.L.) prefers lifelong penicillin prophylaxis. The main argument for this approach is that late episodes of postsplenectomy sepsis, which are not uncommon,[51] almost always occur in individuals who have a poor response to pneumococcal immunization,[51] and who are not taking daily penicillin.[56] Unless pneumococcal antibody titers are be measured periodically following immunization and shown to be satisfactory, this author believes it is safest to continue penicillin prophylaxis.

The standard regimens of prophylaxis after splenectomy with proven efficiency are penicillin V doses of 200,000 IU (125 mg) twice per day until 5 years of age and 400,000 IU (250 mg) twice per day after 5

Table 4. Membrane Protein and Gene Defects in Spherocytosis

Affected Protein	Frequency (% of HS)	Heredity	Prevailing Mutations	Protein Reduction	Hemolytic Anemia	Abnormal Morphology
Ankyrin	US, Europe: 30-60%	AD, AR	AD: null mutations	Spectrin + ankyrin; 15-50%	Mild to moderately severe	Mostly typical spherocytes
	Japan: 5-10%		AR: missense; + promoter mutation (often −108T→C)			
Band 3	20-30%	AD	All rare, functionally null mutations	Band 3 + 4.2: 15-40%	Mild to moderate	Spherocytes, occasional mushroom-shaped or pincered cells
α-Spectrin	<5%	AR	α-Spectrin LEPRA (low expression, splicing defect) + null mutations	Spectrin: 50-75%	Severe, transfusion dependent	Spherocytes, contracted cells and other poikilocytes,
β-Spectrin	15-30%	AD	Null mutations	Spectrin: 15-40%	Mild to moderate	Spherocytes and 5-10% acanthocytes and echinocytes, or Spherocytic elliptocytes
Protein 4.2	US, Europe: <5% Japan: 45-50%	AR	Missense (esp. 4.2 Nippon)	Protein 4.2: 95-100%	Mild to moderate	Spherocytes, acanthocytes, ovalostomatocytes,

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; D, dominant spherocytosis; R, recessive spherocytosis; US, United States; EU, Europe.

years. In case of penicillin allergy an oral macrolide or cephalosporin may be given.

Molecular Defects and Etiology of HS

Meticulous work of several research groups has shown that HS is caused by defects in the red cell membrane proteins ankyrin, spectrin, band 3, and protein 4.2. There is no single frequent defect in European populations except in the rare patients with HS caused by a defect in α-spectrin (see below). Because most mutations are unique to a family, it is usually not worth determining the specific molecular defect. Molecular analysis should be reserved for severe cases needing prenatal diagnosis or for HS with a unique phenotype of special scientific interest. (For a full list of all defects, the reader is referred to excellent summaries by Walensky et al[66] and Yawata.[67])

Nature of Defects

A summary of the various molecular defects causing HS is given in Table 4 and Fig 5.

Ankyrin. Ankyrin defects are estimated to account for 30% to 60% of HS in northern European populations,[50,68,69] but only 5% to 10% of cases in Japan.[70] Patients with HS and ankyrin defects have prominent spherocytosis without other morphologies. Hemolysis and anemia vary from mild to moderately severe.[50,71,72] Ankyrin mutations cause both dominant and recessive HS and range from clinically mild to severe.

Because of its double linkage to β-spectrin and band 3, ankyrin plays a pivotal role in the stabilization of the membrane. Ankyrin is the high affinity binding site for spectrin heterodimers, which are stable only when bound to the membrane.[73] Since it is present in limiting amounts, deficiency of ankyrin leads to loss of both proteins.

Many spherocytes are deficient in both spectrin and ankyrin. A combined deficiency of spectrin and ankyrin was first described in two patients with atypical severe HS.[22] Later, two independent groups measured ankyrin and spectrin contents either by RIA[44] or ELISA[45] in 65 unrelated patients with typical HS: their combined data (Fig 6) show that about two-thirds have a diminution of both proteins to 40% to 80% of normal. The deficiency of one protein is strictly correlated with that of the other and is proportional to clinical severity.

Null mutations (frameshift, splicing, nonsense mutations) are frequent in dominant HS. Based on a comprehensive genomic screen of 46 unrelated German kindreds analyzed by SSCP and exon sequenc-

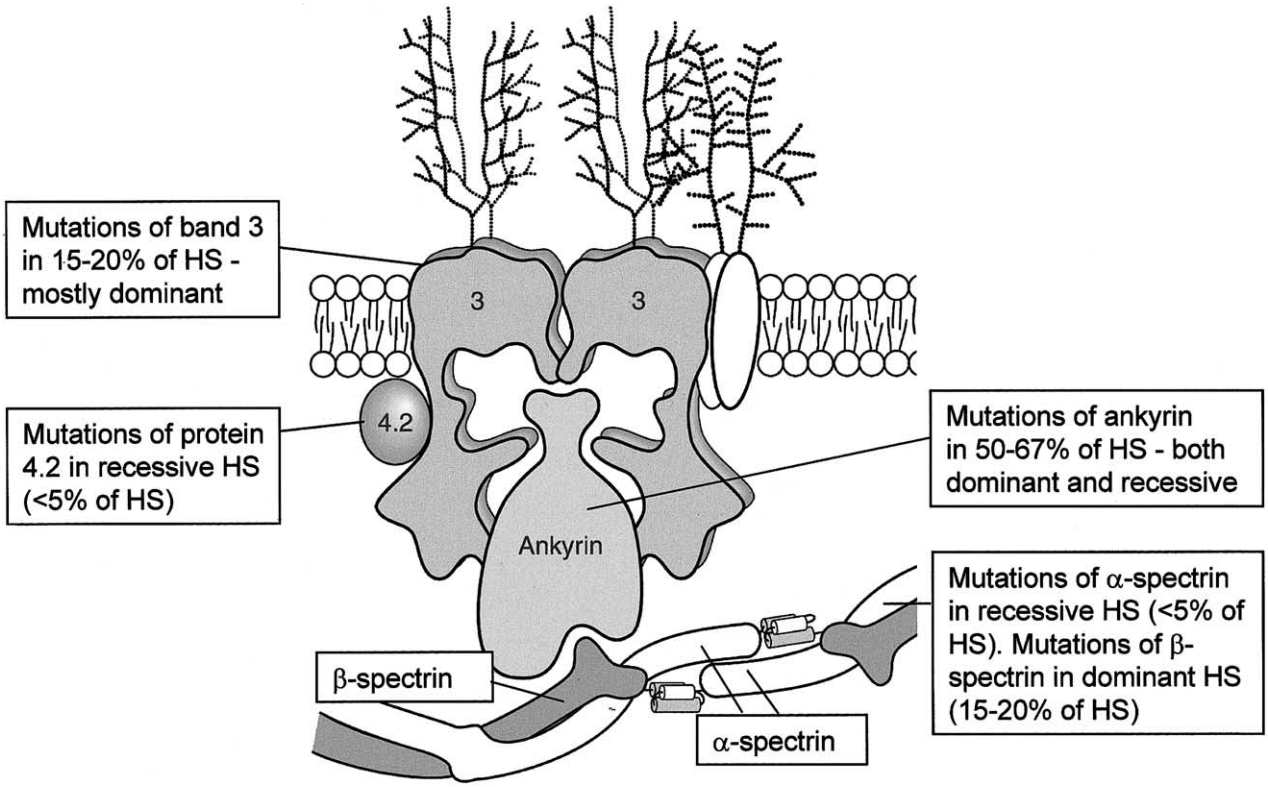

Figure 5. Membrane defects in HS affect the "vertical" interactions connecting the membrane skeleton and the lipid bilayer.

ing,[50] we concluded that ankyrin mutations account for 45% to 65% of HS in European populations; a similar high frequency of ankyrin mutations was found by others.[47,48] De novo mutations leading to decreased expression of one ankyrin allele are frequent in patients with HS who lack a positive family history[49]; frameshift or nonsense mutations prevail in dominant HS. These null mutations result in either unstable ankyrin transcripts or truncated peptides. In most cases the mutant mRNA is destroyed by nonsense-mediated mRNA decay[46] and no abnormal protein is detectable. Occasionally, if a truncation occurs near the C-terminus, the mutant mRNA is not destroyed and a stable, usually shortened protein results. Ankyrin Rakovnik, a nonsense mutation within the C-terminal domain, leads to selective deficiency of the major ankyrin isoform, band 2.1, but the minor isoform, band 2.2 is preserved.[74] Ankyrin mutations are located throughout the molecule, and nearly every family has its own mutation. The amount of residual "normal" ankyrin content is diminished in various degrees due to differing compensation by the normal allele.

Promoter defects and compound heterozygosity for ankyrin defects are common in recessive HS. Several ankyrin defects have been identified in patients with recessive HS,[50] mostly missense or promoter mutations. Mutations in the promoter disrupts transcription factor binding sites or insulator function; examples include −108T→C, −153G→A, and del −72/73. In transgenic mice, these mutations reduce the expression of a reporter gene, confirming their relevance to the pathogenesis of HS.[75,76]

In a few families with recessive HS, patients have had a defect in the ankyrin promoter or 5′-untranslated region on one ankyrin allele and a missense or a null mutation in the other (Fig 7).[50] The mutation −108T→C[50] in the ankyrin promoter is particularly common and was found in four of eight families with recessive HS of different clinical severity (50%; allele frequency = 0.29), in none of 29 patients with dominant HS, and in 4% of 93 normal controls (allele frequency 0.02). In two of those families a further missense mutation was found on the other allele (ankyrin Walsrode [V463I] and ankyrin Bocholt [5619+16C→T]) (Fig 7).[50]

Ankyrin Walsrode contains a missense mutation (V463I) in the band 3 binding domain and has decreased affinity for band 3[77]; the affected patient has red blood cells that are more deficient in band 3 than in spectrin or ankyrin, opposite of the trend in other ankyrin defects. Ankyrin Bocholt bears a missense mutation in a rare alternate splice product, that may result in aberrant splicing. In each family one clini-

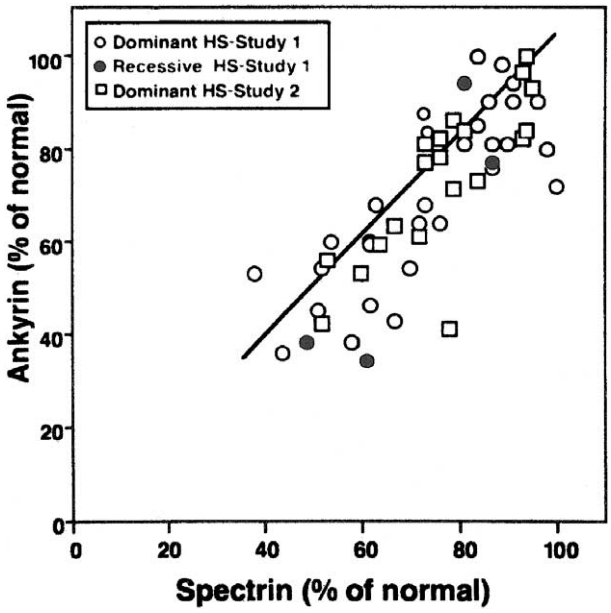

Figure 6. Spectrin and ankyrin diminution in HS. Combined data[44,45] show a good relationship between deficiency of ankyrin and spectrin in most patients with HS. Many of the patients were later proven to have specific ankyrin mutations.[50] Some patients with near normal concentrations of spectrin and ankyrin suffer from HS due to band 3 defects.

cally unaffected parent carried the promoter defect (Fig 7). The heterozygous carriers of ankyrin Walsrode and ankyrin Bocholt are healthy.

Band 3 (AE1). Fifty-five band 3 gene mutations, including 27 missense and 23 frameshift mutations, have been described; all are rare private mutations. Conserved arginine residues are frequent sites of mutations; examples include arginines R490C, R518C, R760Q, R808C, R808H, and R870W[50,78] (Fig 8). These highly conserved sites are positioned at the internal boundaries of transmembrane segments (Fig 8), and substitution probably interferes with cotranslational insertion of band 3 into the membranes of the endoplasmic reticulum during synthesis of the protein. In one case, mRNAs for both alleles were present but the mutant band 3 was not detected, demonstrating either a functional defect in incorporation of the protein into the membrane or instability of the mutant protein.[79] The effect of the mutation has been studied in band 3 Prague, a 10 nucleotide duplication near the C-terminus[80] that leads to a shift in the reading frame and an altered C-terminal sequence after amino acid 821. This mutation affects the last transmembrane helix and probably eliminates the carbonic anhydrase II binding site on band 3[81]; it may also impair insertion of band 3 into the membrane and abolish anion transport function.[80]

Mutations in the cytoplasmic domain of band 3 can interfere with its binding to other membrane skeleton proteins, resulting in a functional defect. An amino acid substitution (G130A) in the cytoplasmic domain in band 3 Fukuoka possibly affects protein 4.2 binding.[82] However, all patients with band 3 deficiency have a proportional decrease in protein 4.2 (as in band 3 Montefiore and Tuscaloosa) because band 3 apparently is needed for 4.2 stability or delivery to the membrane.[83] A mild decrement in protein 4.2 may simply reflect loss of band 3 rather than damage to 4.2 binding sites.

Band 3 deficiency causes HS in about 30% of European patients.[69,70,78,84] The protein is decreased by 15% to 40% in HS red cells. HS due to band 3 is inherited dominantly and is generally milder than HS caused by ankyrin or spectrin mutations. Most (~80%) band 3 deficient patients have a small number (1% to 2%) of mushroom-shaped cells in blood smears, sometimes called "pincered" cells, as though they had been pinched by a tweezers. These peculiar cells seem to occur only in band 3 mutants (there are no mushroom-shaped red blood cells in ankyrin Walsrode, a mutant ankyrin with predominant band 3 deficiency).

Band 3 mutations sometimes also cause distal renal tubular acidosis. The red cell form of band 3 is also present in the acid-secreting intercalated cells of the kidney cortical collecting ducts where it serves as the HCO_3-Cl^- exchanger. Patients who are homozygous for band 3 Coimbra (V488M)[9] and band 3 Pribram (1431+1G→A) have incomplete distal renal tubular acidosis.[85] The patient with homozygous band 3 Coimbra has chronic hyperchloremic metabolic acidosis (plasma HCO_3 = 15 mEq/L), a borderline low serum K^+ (3.5 mEq/L), nephrocalcinosis, and evidence of a distal urinary acidification defect.[9] In HS due to band 3 Campinas (694+1G→T), there is increased basal urinary bicarbonate excretion but efficient urinary acidification.[86] However, most patients with HS and band 3 deficiency do not have metabolic acidosis.[87] In contrast, band 3 missense mutations other than those identified in HS have been found in patients with dominant distal renal tubular acidosis without an accompanying hemolytic anemia.[87-90] Mutations affecting Arg 589 are particularly common in distal renal tubule acidosis (eight of 11 families). Apparently mutations in HS and distal renal tubule acidosis affect two different functional sites of band 3, and the kidney lesion is not caused by heterozygous loss of the anion transport activity of band 3.[87-89] Instead, the phenotype may result from faulty targeting of band 3 to the apical rather than the basolateral membrane of collecting tubule type A intercalated cells.

Protein 4.2. This form of HS is common in Japan but is rare in other populations (Table 4). Protein 4.2 is either completely or almost completely absent.[91] Only three mutations have been reported outside

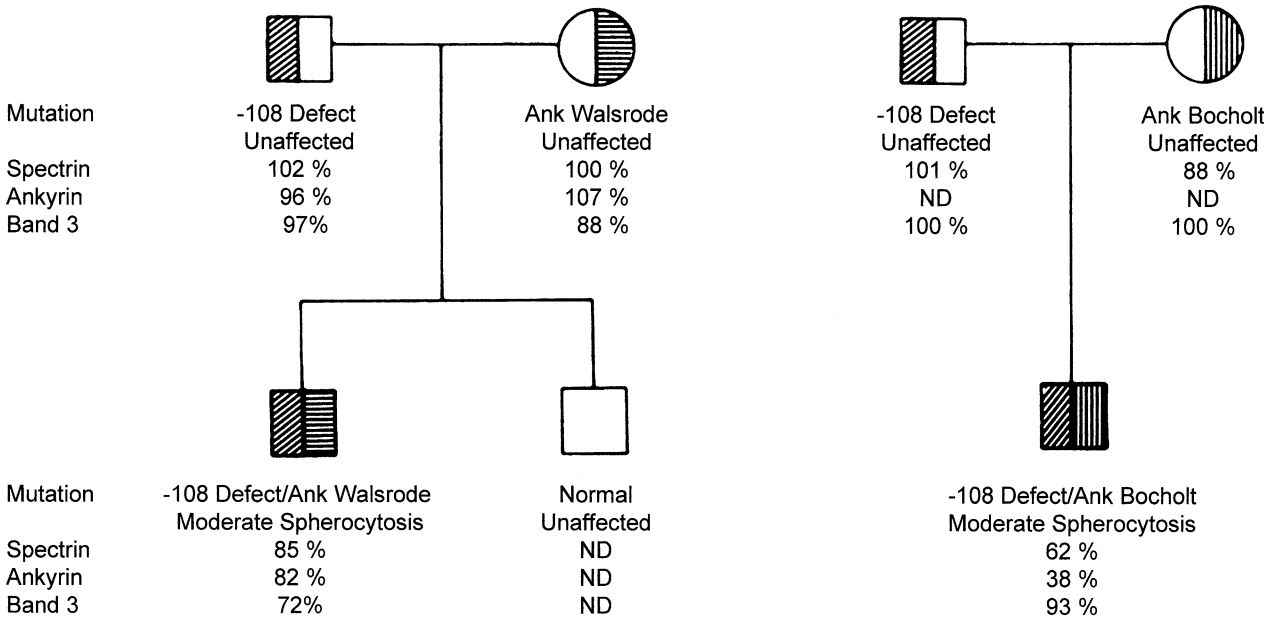

Figure 7. Compound heterozygosity for ankyrin defects in recessive HS. In two families with recessive HS, the patients had a defect in the ankyrin promoter (−108T→C) on one allele and a missense mutation (ankyrin Walsrode [V463I]) or a splice defect (ankyrin Bocholt ([619+16C→T]) on the other allele.[50] Note the greater reduction of band 3 than ankyrin in the patient with ankyrin Walsrode—this is due to the weakened binding of ankyrin Walsrode to band 3.[76] Both parents arere unaffected carriers of one of the two mutations.

Japan (protein 4.2 Tozeur, protein 4.2 Lisboa, and protein 4.2 Nancy). Protein 4.2 Nippon is the most frequent mutation (A142T)[92]: it affects the processing of 4.2 mRNA, so that red blood cells contain only traces of the 72/74-kd isoforms instead of the usually abundant 72-kd species. Most Japanese patients are either homozygous for 4.2 Nippon or compound heterozygous for 4.2 Nippon and another missense or splice mutation, such as 4.2 Fukuoka (W119X), Notame (a splice variant), or Shiga (R317C). The hemolytic anemia is moderate and red cell morphology is variable. Also the MCHC (34.8% ± 0.1) is not as high as in other HS patients.[91] In patients who totally lack protein 4.2, the red blood cells may be a mixture of ovalocytes and stomatocytes with only a few spherocytes. This confusing phenomenon raises the question whether protein 4.2 Nippon should be classified as HS.[91] In addition, splenectomy markedly improves but does not abrogate hemolysis in patients with HS due to 4.2 deficiency, also resembling hereditary stomatocytosis (see article by Delaunay in this issue). The disease is inherited as an autosomal recessive trait. Heterozygous parents are asymptomatic.

As noted above, mild red cell protein 4.2 deficiencies are associated with primary loss of band 3, which contains its binding site.[93] Protein 4.2 probably also binds to ankyrin. Protein 4.2 Nippon–deficient membranes lose 70% of their ankyrin during low ionic strength extraction, where ankyrin is usually stable,[70,92] and the ankyrin loss is blocked by preincubation of the membranes with purified protein 4.2.

Spectrin. Spectrin deficiency is the most common protein alteration in HS. Red blood cells with mutations in the ankyrin or spectrin genes show various degrees of spectrin deficiency,[5,7,21,23] the extent of which is related to red cell spheroidicity,[21,23,40] the severity of hemolysis,[21,23] and the resistance of red cell membranes to shear stress.[40,94] The overall architecture of the membrane skeleton is preserved in spectrin-deficient red blood cells, but the number of junctional complexes interconnecting spectrin, actin, and protein 4.1 is reduced.

In general, HS caused by α-spectrin defects is a recessive trait and that due to β-spectrin mutations is dominant, because α-spectrin chains are produced in three- to fourfold excess compared with β-spectrin.[95] Hence, a moderate reduction of α-spectrin production, as would be seen in a heterozygote, would not decrease formation of the spectrin $\alpha-/\beta-$ dimer.

Patients with α-spectrin defects are often compound heterozygous for missense and low expression mutations. They have a marked reduction of spectrin dimer content (25% to 50% of normal). In many but not all of these families, one allele contains a missense mutation in the α-II domain (α-spectrin Bughill) that changes an alanine to an aspartic acid at residue 309 (A970D).[96] Peptide analysis of the α-spectrin peptides from the affected patients shows only

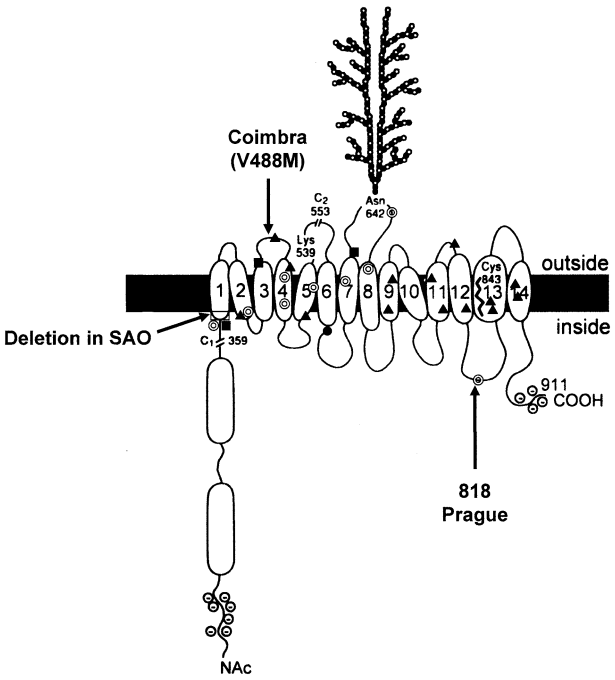

Figure 8. Defects in the transmembrane region of band 3 in patients with HS. The structure of band 3 is shown schematically and is not to scale or correctly folded. Missense mutations are mostly substitutions of critical arginine residues (closed triangles) at the internal boundaries of transmembrane segments of band 3. Frameshift mutations (open doughnuts) and nonsense mutations (closed rectangles) are also shown. Homozygous band 3 Coimbra causes severe hydrops fetalis. Band 3 Coimbra is associated with incomplete renal tubular acidosis. Band 3 Prague is a 10 nucleotide duplication near the C-terminal end. The deletion in South East Asian ovalocytosis (SAO) just at the transition from the cytoplasmic to the transmembrane portion of band 3 is marked because of its importance, although SAO is not a form of HS.

α-spectrin Bughill, but genomic DNA analysis reveals both an allele with α-spectrin Bughill and one without, indicating that the second α-spectrin allele probably contains a null mutation.[96] A candidate defect was discovered in a family with severe, nondominant HS.[8] One of the alleles, designated α-spectrin Prague (5187-2A→G), had a mutation in the penultimate position of intron 36, leading to skipping of exon 37 and premature termination of the α-spectrin peptide. The other α-spectrin allele had a partial splicing abnormality in intron 30 and produced only about one sixth of the normal amount of α-spectrin. This low-expression allele, named α-spectrin^{LEPRA} (low-expression allele Prague) (4339-99C→A), was linked to the α-spectrin Bughill variant in this patient and several others with severe, nondominant HS.[97] Homozygosity for α-spectrin^{LEPRA} alone does not appear to cause disease.

Patients with recessive HS and α-spectrin deficiency are rare. Clinically, only homozygous α-spectrin deficiency causes hemolytic anemia.[6-8,21] Blood smears contain numerous spherocytes and microspherocytes. Patients with very severe spectrin deficiency may also have misshapened spherocytes, spiculated red blood cells, and bizarre poikilocytes.[21,23]

Monoallelic expression of β-spectrin occurs frequently in HS patients with spectrin deficiency,[2,6,98] suggesting that null mutations of β-spectrin are common. About 15 null mutations have been described, including initiation codon disruption, frameshift and nonsense mutations, gene deletions and splicing defects. β-spectrin Kissimmee, from a missense mutation (W202R),[99] is both unstable and defective in its capacity to bind protein 4.1[100] (Fig 9). Patients with this spectrin–4.1 binding defect have only 80% of normal red blood cell spectrin, which may be the real explanation of their HS.

Overall, β-spectrin defects account for about 15% to 30% of HS in northern European populations. Patients with β-spectrin deficiency typically have mild to moderately severe HS. Where described, their blood smears contain moderate numbers (8% to 20%) of spiculated red blood cells (echinocytes and acanthocytes)[4,100] as well as spherocytes, a fairly reliable differentiating feature.

Spectrin defects that affect the self-association of spectrin α/β-heterodimers lead to hereditary elliptocytosis (HE) or hereditary pyropoikilocytosis (HPP), not HS (see article by Gallagher in this issue). Most of these defects involve the N-terminal end of the α-spectrin chain but some are missense mutations at the C-terminus of the β-chain (as in β-spectrins Cagliari, Providence, and Buffalo). C-terminal truncations of the β-spectrin chain that damage the spectrin self-association site and remove the more distal phosphorylation region (as in β-spectrins Prague, Tandil, Nice, Campinas, and Göttingen) cause spherocytic elliptocytosis—rounded elliptocytes with an increased osmotic fragility. The molecular explanation of this interesting intermediate phenotype is unknown.

Procedure for Molecular Screening Using Innocent Polymorphisms of Membrane Protein Genes

In addition to pathogenetically relevant molecular defects in HS, a wide variety of silent or innocent polymorphisms have been detected during screening for ankyrin, spectrin, and band 3 genes. These polymorphisms offer a rational approach to the study of ankyrin and other membrane proteins.[48] Frameshift or nonsense mutations are most common in dominant HS in most cases, and no mutant RNA or protein is present in reticulocytes and mature erythrocytes, respectively, reflecting either reduced transcription of one of the ankyrin alleles or instability of its mRNA. In either case, finding that one ankyrin,

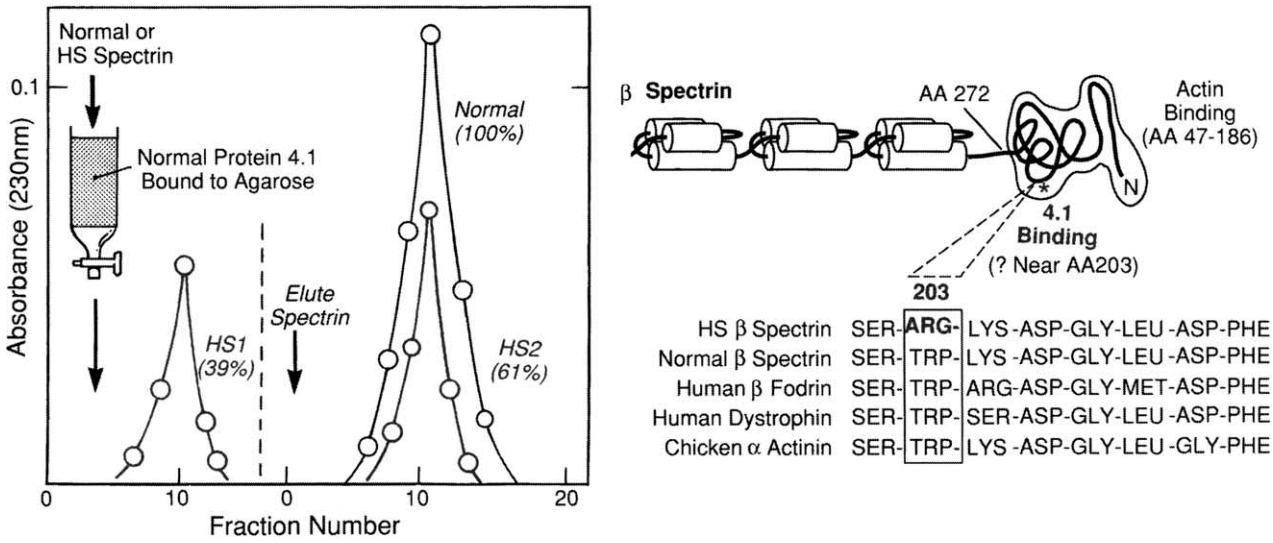

Figure 9. Defect in the binding site for protein 4.1 in β-spectrin Kissimmee. Spectrin from a normal individual or from a patient with β-spectrin Kissimmee was passed over a column containing immobilized normal protein 4.1. All normal spectrin and 61% of the HS spectrin bound and could be eluted under conditions unfavorable for spectrin-4.1 interactions. However, 39% of the HS spectrin failed to bind to the column and, in a separate assay, this fraction (Sp Kissimmee) lacked the ability to bind protein 4.1. Subsequent studies localized the defect to β-spectrin (W202R).[99] The mutation is in a conserved region in the N-terminal end of β-spectrin, just beyond the actin binding domain. The region is a likely location for the 4.1 binding site. (Reprinted with permission from Walensky LD, et al: Disorders of the red blood cell membrane, in Handin RI et al (eds): Blood: Principles and Practice of Hematology (ed 2). © 2003 Lippincott Williams & Wilkins.)

β-spectrin, or band 3 gene is not expressed, by comparing frequent polymorphisms in genomic DNA and cDNA (mRNA), is proof that a null mutation exists, and is an efficient procedure for identifying candidates for frameshift or nonsense mutations in one of the three genes.

Limited characterization of the underlying mutation may be useful in families with unclear heritage or severe or atypical disease. Because ankyrin mutation are most common, one option is to first compare genomic DNA and reticulocyte mRNA (as cDNA) for reduced expression of one cDNA allele using the $(AC)_n$ repeat of ankyrin[47-49] or other common ankyrin (ANK1) polymorphisms (Table 2).[50] If negative, reduced expression of one allele of band 3[50,84]or β-spectrin[2,101] can be sought. SSCP screening of all the exons in the candidate gene can then be done in patients who show absence of one allele in reticulocyte RNA. Alternatively, candidate genes may be identified by SDS-PAGE analysis of red cell membrane proteins and then characterized by SSCP and/or DNA sequencing.

Pathophysiology

Loss of Membrane Surface by Vesiculation

The primary membrane lesions described above all involve the "vertical interactions" between the skeleton and the bilayer, consistent with the prevailing theory that HS is caused by local disconnection of the skeleton and bilayer, followed by vesiculation of the unsupported surface components. These processes, in turn, lead to progressive reduction in membrane surface area and to a "spherocyte," actually a shape that ranges between a thickened discocyte and a spherostomatocyte. The phospholipid and cholesterol contents of isolated spherocytes are decreased by 15% to 20% due to the loss of surface area.[102]

Since budding red cells are rarely observed in typical blood smears of patients with HS, microscopic vesicles are probably lost; loss may occur preferentially in bywaters of the circulation, such as the reticuloendothelial system. When membrane vesicles are induced in normal red blood cells, they originate at the tips of spicules, where the lipid bilayer uncouples from the underlying skeleton.[103] The vesicles are small, about 100 nm, and devoid of hemoglobin and skeletal proteins, so that they are invisible on conventional examination of stained blood films. Tiny 50- to 80-nm bumps that may be vesicles have been detected in HS red cells on atomic force microscopy.[104] However, the hypothesis that spherocytes in HS originate by membrane vesiculation during shear stress in the circulation or in the metabolically inhospitable splenic cord and reticuloendothelial system has been recently questioned by Costa et al,[105] who found a similar decrease in the surface area of mature red cells and reticulocytes in HS. Their findings suggest in-

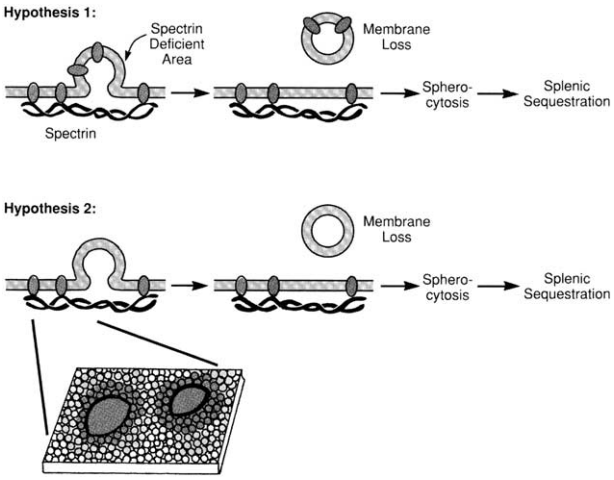

Figure 10. Two hypotheses concerning the mechanism of membrane loss in hereditary HS. Hypothesis 1 assumes that the "membrane" (the lipid bilayer and integral membrane proteins) is directly stabilized by interactions with spectrin or other elements of the membrane skeleton. Spectrin-deficient areas, lacking support, bud off, leading to HS. Hypothesis 2 assumes that the membrane is stabilized by interactions of band 3 with neighboring lipids. The influence of band 3 extends into the lipid milieu because the first layer of immobilized lipids slows the lipids in the next layer and so on. In band 3–deficient cells the area between lipid molecules increases and unsupported lipids are lost. Spectrin-ankyrin deficiency allows band 3 molecules to diffuse and transiently cluster, with the same consequences. (Reprinted with permission from Walensky LD, et al: Disorders of the red blood cell membrane, in Handin RI et al (eds): Blood: Principles and Practice of Hematology (ed 2). © 2003 Lippincott Williams & Wilkins.)

stead that surface area loss occurs in the bone marrow during the genesis of HS red blood cells.[105]

Is HS Caused by Disconnection of the Skeleton or a Lack of Band 3-Lipid Interactions?

The observation that spectrin or spectrin-ankyrin deficiencies are common in HS has led to the hypothesis that interactions of spectrin with bilayer lipids or proteins are required to stabilize the membrane (Fig 10, Hypothesis 1). Budding off of spectrin-deficient areas would lead to HS. Unexplained is how spherocytes develop in patients whose red blood cells are deficient in band 3 or protein 4.2 but have normal amounts of spectrin.[83,92]

The alternate hypothesis argues that the bilayer is stabilized by interactions between lipids and the abundant band 3 molecules (Fig10, Hypothesis 2). Each band 3 contains about 14 hydrophobic transmembrane helices, many of which must interact with lipids. In deficient red cells the area between band 3 molecules would increase, on average, and the stabilizing effect would diminish. Transient fluctuations in the local density of band 3 could aggravate this instability and allow unsupported lipids to be lost, resulting in HS. Spectrin- and ankyrin-deficient red blood cells could become spherocytic by a similar mechanism. Since spectrin filaments corral band 3 molecules and limit their lateral movement,[106] a decrease in spectrin would allow band 3s to diffuse and transiently cluster, fostering vesiculation. However, it is more likely that both mechanisms operate to variable degrees in different diseases: hypothesis 1 dominating in spectrin and ankyrin defects, and hypothesis 2 controlling in band 3 and protein 4.2 disorders.

Spectrin and ankyrin deficiency lead to secondary alterations of band 3, such as increased mobility, oligomerization, and aggregation, which could contribute to the loss of spectrin-free vesicles from the membrane. Red blood cells from HS patients with ankyrin mutations exhibit a marked increase in band 3 rotational diffusion.[107] The magnitude of the increase correlates inversely with the ankyrin/band 3 ratio and with the fraction of band 3 retained in the membrane skeleton following detergent extraction. These data suggest that ankyrin deficiency relaxes rotational constraints on the population of band 3 molecules. Increases in band 3 rotation could be due to release of band 3 from low-affinity binding sites on ankyrin, and there is evidence for increased band 3 density and aggregation in HS.[108]

Loss of Cellular Deformability

Hereditary spherocytes hemolyze because of the rheologic consequences of their decreased surface-to-volume ratio. The red cell membrane is very flexible but can only expand its surface area 2% to 3% before rupturing. Thus, the cell becomes increasingly less deformable as surface area is lost. For red blood cells in HS, poor deformability is a hindrance only in the spleen, since the cells have a nearly normal lifespan following splenectomy.

Sequestration of Hereditary Spherocytes in the Spleen

Figure 11 illustrates our current understanding of the pathophysiology of HS. The dominant role of the spleen is unquestioned, but the exact mechanism by which HS red cells are further damaged ("conditioned") and ultimately removed in the spleen is not well understood.

Spherocytes are selectively sequestered at the cordal-sinus junction. As a consequence, spleens from patients with HS have massively congested cords and relatively empty sinuses. In electron micrographs, few spherocytes are seen in transit through the sinus wall, in contrast to normal spleens where transiting cells are readily found.[109]

Spherocytes are "conditioned" in the metaboli-

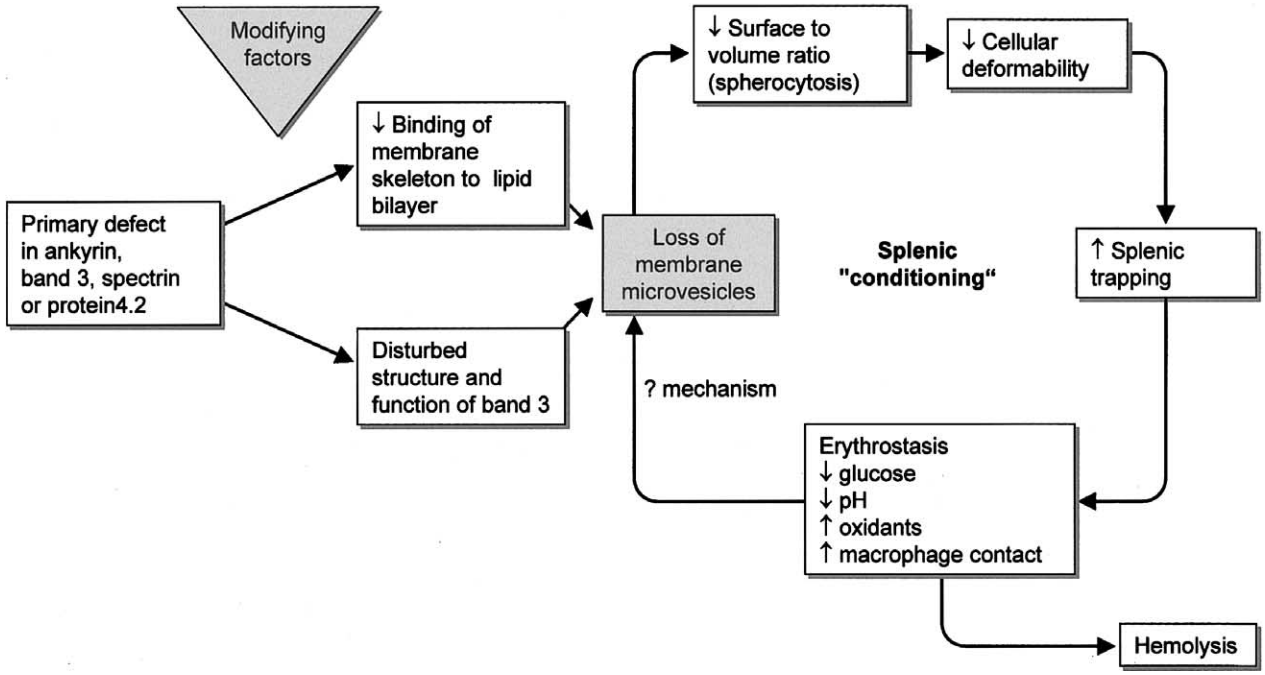

Figure 11. Pathophysiology of the splenic conditioning and destruction of hereditary spherocytes. The clinical symptoms vary distinctly with functionally similar defects (such as frameshift or nonsense mutations with no abnormal protein present). Thus modifying factors must exist that regulate the clinical expression of primary defects in the genes for ankyrin, band 3, spectrin, or protein 4.2. The primary defect leads to a weak "vertical" interaction between the membrane skeleton and the integral proteins in the plane of the lipid bilayer (such as band 3). Secondary events are a disturbed structure and function of band 3 that decreases the anchoring of the lipids and may contribute to the loss of spectrin-free lipid vesicles. The circle at the right represent the many factors of "splenic conditioning" that ultimately lead to premature hemolysis. (Modified with permission from Walensky LD, et al: Disorders of the red blood cell membrane, in Handin RI et al (eds): Blood: Principles and Practice of Hematology (ed 2). © 2003 Lippincott Williams & Wilkins.).

cally inhospitable splenic cords. There is also abundant evidence that red blood cells in HS suffer during detention in the spleen. The mechanism of this process, called "splenic conditioning," is less certain due to the lack of information about the cordal environment.[110] The average residence time of HS red cells in the splenic cords is 30- to 300-fold longer than that of normal red blood cells; still, this delay is far short of the time required for metabolic depletion.

Consequences of splenic trapping. The following consecutive damage in the spleen should lead to the premature hemolysis of spherocytes. (1) Oxidants may exacerbate membrane and water loss. Potassium loss and membrane instability may be increased by the high concentrations of acids and oxidants that must exist in a spleen filled with activated macrophages ingesting trapped HS red cells. (2) Splenic residence may activate membrane proteases. Oxidatively damaged membrane proteins are also subject to proteolysis in vitro[111]; proteolysis in vivo would contribute to skeletal weakness and membrane loss. (3) Macrophages may directly condition hereditary spherocytes. The involvement of macrophages is supported by the historic observations of Coleman and Finch,[112] who found that large doses of cortisone markedly ameliorated HS in nonsplenectomized patients. Similar doses of corticosteroids inhibit splenic processing and destruction of IgG-or C3b-coated red blood cells in patients with immunohemolytic anemias, probably by suppressing macrophage-induced red cell sphering and phagocytosis.

Binding of naturally occurring band 3 antibodies enhances the premature clearance of spherocytes with band 3 deficiency from the circulation.[113] In splenectomized patients with band 3 deficiency, red blood cell deformability inversely correlates with the number of red cell–bound IgG molecules (up to 140 per cell).

Summary of Pathophysiology

Red cells in HS are selectively detained by the spleen and this custody is detrimental, leading to a loss of membrane surface that fosters further splenic trapping and eventual destruction (Fig 11). The primary membrane defects involve deficiencies or defects of spectrin, ankyrin, protein 4.2, or band 3, but the etiologic relationship of these defects to surface loss

is less clear. Current speculation is that the membrane skeleton (including band 3) may not adequately support all regions of the lipid bilayer in HS, leading to loss of small areas of untethered lipids and integral membrane proteins. Uncertain is whether the effect is directly due to deficiency of spectrin and ankyrin, or spectrin-ankyrin deficiency indirectly increases the lateral mobility of band 3 molecules and decreases their stabilization of the lipid bilayer, or both (Fig 11). In addition, the loss of band 3 due to band 3 or protein 4.2 defects may directly diminish lipid anchoring in HS. The mechanisms of splenic conditioning and red blood cell destruction also remain uncertain.

Animal and Fish Models of HS

The availability of well-characterized mouse and zebrafish models has contributed to our understanding of the pathophysiology of HS. Four types of spherocytic hemolytic anemia have been identified in the common house mouse, *Mus musculus*[114]: *ja/ja* (jaundice); *sph/sph* (spherocytosis) and its alleles [sph^{1J}/sph^{1J} (hemolytic anemia), sph^{2J}/sph^{2J} (now lost), sph^{2BC}/sph^{2BC}, and sph^{Dem}/sph^{Dem}]; *nb/nb* (normoblastosis); and *wan/wan*. The nomenclature indicates that anemia is observed only in the homozygous state and that the mutants represent four loci: *ja*, *sph*, *nb*, and *wan*. All of the mutants have severe hemolysis.

Spectrin Mutants

The *ja/ja* mutant has no detectable spectrin. The mice carry a nonsense mutation in the β-spectrin gene (*R1160X*).

The *sph/sph* variants lack α-spectrin but have small amounts of β-spectrin; they have defects in α-spectrin synthesis, function, and/or stability. The *sph* and sph^{2BC} alleles are frameshift mutations and null alleles. These mice have both spherocytes and elliptocytes, and some poikilocytes, and are a cross between HS and hereditary pyropoikilocytosis. The sph^{1J} allele, previously called *ha* and sph^{ha}, lacks the last 13 amino acids of α-spectrin and exhibits marked spherocytosis.[115] The mutant protein is produced in nearly normal amount, proving that the C-terminus of α-spectrin has some critical although still mysterious function in supporting the lipid bilayer.

Cardiac thrombi, fibrotic lesions and renal hemochromatosis are found in *ja/ja* and *sph/sph* mice in adulthood.[116] Transplantation of hematopoietic cells from *sph/sph* mice are sufficient to induce thrombotic events in the recipients.[117]

Ankyrin Mutants

Nb/nb mice have 50% to 70% of the normal quantity of spectrin and no normal ankyrin. They have normal spectrin synthesis[118] but are moderately spectrin-deficient because their ankyrin is very unstable (in contrast with human ankyrin deficiency where ankyrin and spectrin levels are comparably depressed). The *nb* mutation causes premature termination of the ankyrin protein in exon 36 at the beginning of the C-terminal domain. A small amount of the 157-kd remnant is found in mature *nb/nb* red blood cells and, along with some expression of an Ank2-related peptide, may explain why fetal *nb/nb* mice have normal reticulocyte counts and no anemia at birth.[119] Humans may also be protected in utero, at least partially, since hydrops fetalis has not been reported in patients with ankyrin defects or probable ankyrin defects (such as combined spectrin-ankyrin deficiency).

The *nb/nb* mice develop ataxia when they reach maturity, due to loss of cerebellar Purkinje cells[120]; ank-1 protein is markedly reduced in the Purkinje cells, which may explain their fragility. Spinocerebellar degeneration and related syndromes have also been reported in a few adults with HS, although it is not yet known whether ankyrin is affected.

Clinical Variability of Band 3 Mutations Indicates the Presence of Genetic Modifiers of Disease Severity

Complete absence of band 3 was first described in a recessive form of HS in cattle , due to a nonsense mutation at codon 646.[89] The cattle, like band 3–deficient mice and humans, have deficient anion transport, lack protein 4.2, and have a reduced number of intramembranous particles by electron microscopy. However, they have a relatively mild hemolytic phenotype compared to other band 3–deficient organisms.

New mouse mutants with defects in membrane skeleton proteins have been generated by targeted mutagenesis in embryonic stem cells. Mice completely deficient in band 3 survive gestation but tend to die in the neonatal period,[83] often from thrombotic complications.[121] Survivors have a profound spherocytic hemolytic anemia, closely resembling the most severe forms of HS in humans. The mice have undetectable protein 4.2 and glycophorin A but normal amounts of spectrin, actin, and protein 4.1 in their red cell membrane skeletons, and normal membrane skeleton architecture by electron microscopy. Despite their normal skeletons, red blood cells lacking band 3 shed astonishing amounts of membrane surface in small vesicles and long tubules (Fig 12).[83] These observations indicate that band 3 is, surprisingly, *not* required for membrane skeleton assembly but has a critical function in stabilizing membrane lipids. Loss of this function may be fundamental to the pathogenesis of HS.

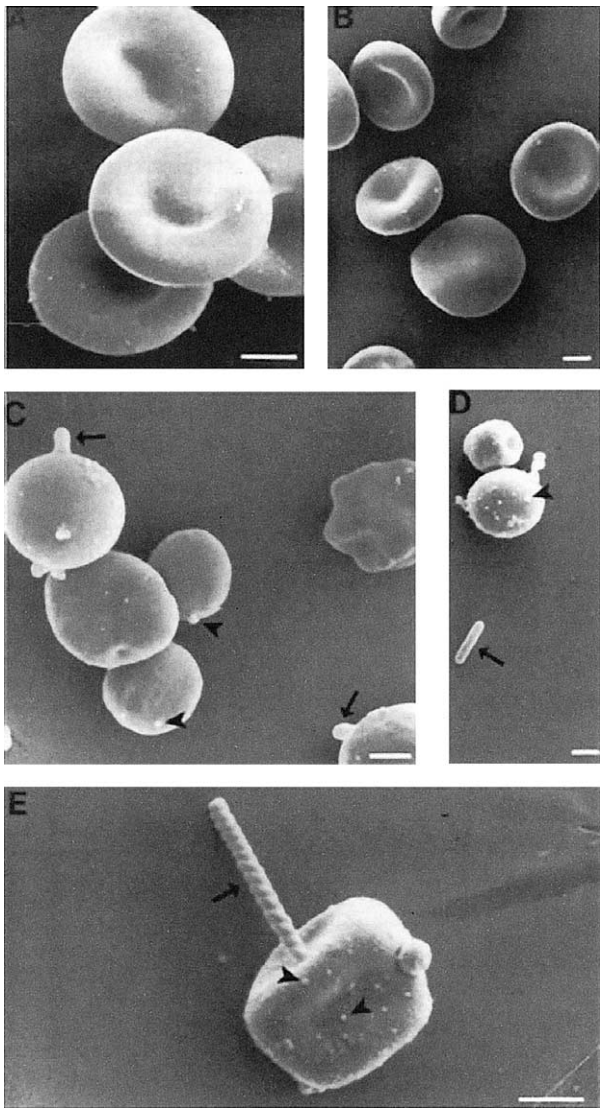

Figure 12. Marked membrane fragility of mice lacking band 3. Scanning electron micrographs of red cells from wild-type (A), heterozygous (B), and homozygous band 3–deficient (C-E) mice.[83]. Note the markedly spherocytic shape of the band 3 (-/-) red cells. Many are shedding multiple tiny membrane vesicles (arrowheads). Others extrude rod-like membrane extensions (arrows), which are frequently detached (D, arrow). The membrane extensions often reach considerable length and are highly coiled (E). Bar, 1 μm. (Reprinted with permission from Walensky LD, et al: Disorders of the red blood cell membrane, in Handin RI et al (eds): Blood: Principles and Practice of Hematology (ed 2). © 2003 Lippincott Williams & Wilkins.)

A newly discovered mouse mutation (*wan*), in a C3H/HeJ background, is a null defect in the band 3 gene. *wan/wan* mice also have a very severe anemia (100% lethal in the neonatal period). But when *wan/wan* mice are crossed to wild-type *Mus castaneus* mice, the F_2 generation shows a wide variation in severity, from lethal anemia to normal hematologic values (hemoglobin, hematocrit, and mean corpuscular volume). These observations show that a strong genetic modifier is segregating in the *M castaneus* background. A genome-wide scan for the modifier (termed a "quantitative trait locus" or QTL), using mean corpuscular volume as the variable trait, identified a single QTL on chromosome 12, centered over the β-spectrin gene,[122] an obvious candidate for a modifier gene.

Band 3–*wan/wan* and band 3 knockout mice also contain excess binucleate erythroblasts, which suggests that absence of band 3 is associated with a defect in cytokinesis. Indeed, in homozygous *retsina* (*ret/ret*) zebrafish with HS the mitotic spindles of late erythroblasts are grossly disrupted and the masses of DNA separate poorly and interfere with cell cleavage. This behavior explains the high frequency of binucleate cells in zebrafish and mouse erythroblasts lacking band 3, but not in other forms of severe HS.[123] Since band 3 is only expressed in erythroblasts and one cell type in the kidney collecting duct, the data imply that vertebrates have developed a special kind of cytokinesis for the last one or two cell divisions, a cytokinesis that depends on band 3—perhaps to help attach the mitotic spindle to the poles of the cell.

Zebrafish with spherocytosis and null mutations in β-spectrin (*reisling*) and protein 4.1 (*merlot/chablis*) also have been identified.[124,125] These fish should prove useful for in vivo structure-function studies, since it is relatively easy to test whether a derivative of the missing protein can rescue the HS phenotype. For example, normal band 3 cDNA injected into a one- or two-cell embryo rescues the anemia of band 3–deficient *ret/ret* zebrafish, but band 3 lacking both 4.1 binding sites is unable to rescue these deficient animals[124]; band 3 with only one of the two 4.1 sites has intermediate rescue function. The 4.1-band 3 interaction appears functionally important, at least in fish, and likely band 3–skeleton as well as band 3–lipid interactions have pathogenic roles in HS.

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References

1. Vanlair CF, Masius JB: De la microcythémie. Bull R Acad Med Belg 5:515-613, 1871
2. Miraglia del Giudice E, Lombardi C, Francese M, et al: Frequent de novo monoallelic expression of β-spectrin gene (SPTB) in children with hereditary spherocytosis and isolated spectrin deficiency. Br J Haematol 101:251-254, 1998
3. Özcan R, Kugler W, Feuring-Buske M, et al: Parental mosaicism for ankyrin-1 mutations in two families with hereditary spherocytosis. Blood 90:4a, 1997 (suppl, abstr)

4. Basseres DS, Duarte AS, Hassoun H, et al: β-Spectrin S(ta) Barbara: A novel frameshift mutation in hereditary spherocytosis associated with detectable levels of mRNA and a germ cell line mosaicism. Br J Haematol 115:347-353, 2001
5. Eber SW, Armbrust R, Schroter W: Variable clinical severity of hereditary spherocytosis: Relation to erythrocytic spectrin concentration, osmotic fragility, and autohemolysis. J Pediatr 117:409-416, 1990
6. Miraglia del Giudice E, Nobili B, Francese M, et al: Clinical and molecular evaluation of non-dominant hereditary spherocytosis. Br J Haematol 112:42-47, 2001
7. Agre P, Orringer EP, Bennett V: Deficient red-cell spectrin in severe, recessively inherited spherocytosis. N Engl J Med 306:1155-1161, 1982
8. Wichterle H, Hanspal M, Palek J, et al: Combination of two mutant α-spectrin alleles underlies a severe spherocytic hemolytic anemia. J Clin Invest 98:2300-2307, 1996
9. Ribeiro ML, Alloisio N, Almeida H, et al: Severe hereditary spherocytosis and distal renal tubular acidosis associated with the total absence of band 3. Blood 96:1602-1604, 2000
10. Perrotta S, Nigro V, Iolascon A, et al: Dominant hereditary spherocytosis due to band 3 Neapolis produces a life-threatening anemia at the homozygous state. Blood 92:9, 1998 (abstr)
11. Chilcote RR, Le Beau MM, Dampier C, et al: Association of red cell spherocytosis with deletion of the short arm of chromosome 8. Blood 69:156-159, 1987
12. Lux S, Tse W, Menninger J, et al: Hereditary spherocytosis associated with deletion of the human erythrocyte ankyrin gene on chromosome 8. Nature 345:736-739, 1990
13. Fukushima Y, Byers MG, Watkins PC, et al: Assignment of the gene for β spectrin (SPTB) to chromosome 14q23-q24.2 by situhybridization. Cytogenet Cell Genet 53:232-233, 1990
14. Palek J, Lambert S: Genetics of the red cell membrane skeleton. Semin Hematol 27:290-332, 1990
15. Friedman EW, Williams JC, Van Hook L: Hereditary spherocytosis in the elderly. Am J Med 84:513-516, 1988
16. Krueger HC, Burgert EOJ: Hereditary spherocytosis in 100 children. Mayo Clin Proc 41:554-567, 1966
17. Jensson O, Jonasson JL, Magnusson S: Studies on hereditary spherocytosis in Iceland. Acta Med Scand 201:187-195, 1977
18. Koepke JF, Koepke JA: Reticulocytes. Clin Lab Haematol 8:169-179, 1986
19. Guarnone R, Centenara E, Zappa M, et al: Erythropoietin production and erythropoiesis in compensated and anaemic states of hereditary spherocytosis. Br J Haematol 92:150-154, 1996
20. Pajor A, Lehoczky D, Szakacs Z: Pregnancy and hereditary spherocytosis. Report of 8 patients and a review. Arch Gynecol Obstet 253:37-42, 1993
21. Agre P, Casella JF, Zinkham WH, et al: Partial deficiency of erythrocyte spectrin in hereditary spherocytosis. Nature 314:308-383, 1985
22. Coetzer TL, Lawler J, Liu SC, et al: Partial ankyrin and spectrin deficiency in severe, atypical hereditary spherocytosis. N Engl J Med 318:230-234, 1988
23. Agre P, Asimos A, Casella JF, et al: Inheritance pattern and clinical response to splenectomy as a reflection of erythrocyte spectrin deficiency in hereditary spherocytosis. N Engl J Med 315:1579-1583, 1986
24. Godal HC, Heist H: High prevalence of increased osmotic fragility of red blood cells among Norwegian donors. Scand J Haematol 27:30-34, 1981
25. Eber SW, Pekrun A, Neufeldt A, et al: Prevalence of increased osmotic fragility of erythrocytes in German blood donors: Screening using a modified glycerol lysis test. Ann Hematol 64:88-92, 1992
26. Maberry MC, Mason RA, Cunningham FG, et al: Pregnancy complicated by hereditary spherocytosis. Obstet Gynecol 79:735-738, 1992
27. Iolascon A, Faienza MF, Moretti A, et al: UGT1 promoter polymorphism accounts for increased neonatal appearance of hereditary spherocytosis. Blood 91:1093, 1998 (letter)
28. Delhommeau F, Cynober T, Schischmanoff PO, et al: Natural history of hereditary spherocytosis during the first year of life. Blood 95:393-397, 2000
29. Tchernia G, Delhommeau F, Perrotta S, et al: Recombinant erythropoietin therapy as an alternative to blood transfusions in infants with hereditary spherocytosis. BJH 1:146-152, 2000
30. Young N: Hematologic and hematopoietic consequences of B19 parvovirus infection. Semin Hematol 25:159-172, 1988
31. Pekrun A, Eiffert H, Eber SW, et al: Aplastische Krisen bei hereditärer Sphärozytose. Monatsschr Kinderheilkd 136: 173-175, 1988
32. Peter G: Parvovirus B19, in Red Book: Report of the Committee on Infectious Diseases (ed 24). Elk Grove Village, IL, American Academy of Pediatrics, 1997, p 383
33. Malinow M, Duell PB, Hess DL, et al: Reduction of plasma homocyst(e)ine levels by breakfast cereal fortified with folic acid in patients with coronary heart disease. N Engl J Med 338:1009-1015, 1998
34. Bosma PJ, Chowdhury JR, Bakker C, et al: The genetic basis of the reduced expression of bilirubin UDP-glucuronosyltransferase 1 in Gilbert's syndrome. N Engl J Med 333:1171-1175, 1995
35. Miraglia del Giudice E, Perrotta S, Nobili B, et al: Coinheritance of Gilbert syndrome increases the risk for developing gallstones in patients with hereditary spherocytosis. Blood 92:470a, 1998 (suppl, abstr)
36. Marchetti M, Quaglini S, Barosi G: Prophylactic splenectomy and cholecystectomy in mild hereditary spherocytosis: Analyzing the decision in different clinical scenarios. J Intern Med 244:217-226, 1998
37. Michaels LA, Cohen AR, Zhao H, et al: Screening for hereditary spherocytosis by use of automated erythrocyte indexes. J Pediatr 130:957-960, 1997
38. Mohandas N, Kim YR, Tycko DH, et al: Accurate and independent measurement of volume and hemoglobin concentration of individual red cells by laser light scattering. Blood 68:506-513, 1986
39. Cynober T, Mohandas N, Tchernia G: Red cell abnormalities in hereditary spherocytosis: relevance to diagnosis and understanding of the variable expression of clinical severity. J Lab Clin Med 128:259-269, 1996
40. Chasis JA, Agre P, Mohandas N: Decreased membrane mechanical stability and in vivo loss of surface area reflect spectrin deficiencies in hereditary spherocytosis. J Clin Invest 82:617-623, 1988
41. Clark MR, Mohandas N, Shohet SB: Osmotic gradient ektacytometry: Comprehensive characterization of red cell volume and surface maintenance. Blood 61:899-910, 1983
42. Schröter W, Kahsnitz E: Diagnosis of hereditary spherocytosis in newborn infants. J Pediatr 103:460-463, 1983
43. Zanella A, Izzo C, Rebulla P, et al: Acidified glycerol lysis test: A screening test for spherocytosis. Br J Haematol 45: 481-486, 1980
44. Savvides P, Shalev O, John KM, et al: Combined spectrin and ankyrin deficiency is common in autosomal dominant hereditary spherocytosis. Blood 82:2953-2960, 1993

45. Pekrun A, Eber SW, Kuhlmey A, et al: Combined amkyrin and spectrin deficiency in hereditary spherocytosis. 67:89-93, 1993
46. Byers PH: Killing the messenger: New insights into nonsense-mediated mRNA decay. J Clin Invest 109:3-6, 2002
47. Jarolim P, Rubin HL, Brabec V, et al: Comparison of the ankyrin (AC)n microsatellites in genomic DNA and mRNA reveals absence of one ankyrin mRNA allele in 20% of patients with hereditary spherocytosis. Blood 85:3278-3282, 1995
48. Özcan R, Jarolim P, Lux SE, et al: Simultaneous (AC)n microsatellite polymorphism analysis and SSCP screening is an efficient strategy for detecting ankyrin-1 mutations in dominant hereditary spherocytosis. Br J Haematol 122:669-677, 2003
49. Miraglia del Giudice E, Francese M, Nobili B, et al: High frequency of de novo mutations in ankyrin gene (ANK1) in children with hereditary spherocytosis. J Pediatr 132:117-120, 1998
50. Eber SW, Gonzalez JM, Lux ML, et al: Ankyrin-1 mutations are a major cause of dominant and recessive hereditary spherocytosis. Nature Genet 13:214-218, 1996
51. Eber SW, Langendorfer CM, Ditzig M, et al: Frequency of very late fatal sepsis after splenectomy for hereditary spherocytosis: Impact of insufficient antibody response to pneumococcal infection. Ann Hematol 78:524-528, 1999
52. Green JB, Shackford SR, Sise MJ, et al: Late septic complications in adults following splenectomy for trauma: A prospective analysis in 144 patients. J Trauma 26:999-1004, 1986
53. Hansen K, Singer DB: Asplenic-hyposplenic overwhelming sepsis: Postsplenectomy sepsis revisited. Pediatr Dev Pathol 4:105-121, 2001
54. Holdsworth RJ, Irving AD, Cuschieri A: Postsplenectomy sepsis and its mortality rate: Actual versus perceived risks. Br J Surg 78:1031-1038, 1991
55. Schwartz PE, Sterioff S, Mucha P, et al: Postsplenectomy sepsis and mortality in adults. JAMA 248:2279-2283, 1982
56. Schilling RF: Estimating the risk for sepsis after splenectomy in hereditary spherocytosis. Ann Intern Med 122:187-188, 1995
57. Schilling RF: Spherocytosis, splenectomy, strokes, and heat attacks. Lancet 350:1677-1678, 1997
58. Robinette CD, Fraumeni JFJ: Splenectomy and subsequent mortality in veterans of the 1939-45 war. Lancet 2:127, 1977
59. Bader-Meunier B, Gauthier F, Archambaud F, et al: Long-term evaluation of the beneficial effect of subtotal splenectomy for management of hereditary spherocytosis. Blood 97:399-403, 2001
60. de Buys Roessingh AS, de Lagausie P, Rohrlich P, et al: Follow-up of partial splenectomy in children with hereditary spherocytosis. J Pediatr Surg 37:1459-1463, 2002
61. Eber SW, Bolkenius M, Heidemann P, et al: Subtotale Splenektomie bei hereditärer Sphärozytose im Kindesalter. Chir Gastroenterol 17:12-17, 2001 (suppl)
62. Rice HE, Oldham KT, Hillery CA, et al: Clinical and hematologic benefits of partial splenectomy for congenital hemolytic anemias in children. Ann Surg 237:281-288, 2003
63. Tchernia G, Gauthier F, Mielot F, et al: Initial assessment of the beneficial effect of partial splenectomy in hereditary spherocytosis. Blood 81:2014-2020, 1993
64. Katkhouda N, Hurwitz MB, Rivera RT, et al: Laparoscopic splenectomy: Outcome and efficacy in 103 consecutive patients. Ann Surg 228:568-578, 1998
65. Eber SW, Belohradsky BH, Weiss M: Infektionsprophylaxe bei Asplenie. Klin Pädiatrie 213 Sonderheft 1:1-4, 2001
66. Walensky LD, Narla M, Lux SE: Disorders of the red blood cell membrane, in Handin RI, Lux SE, Stossel TP (eds): Blood: Principles and Practice of Hematology (ed 2). Philadelphia, PA, Lippincott Williams & Wilkins, 2003, pp 1709-1858
67. Yawata Y: Hereditary spherocythosis, in Cell Membrane. Weinheim, Germany, Wiley-VCH, 2003, pp 173-212
68. Lanciotti M, Perutelli P, Valetto A, et al: Ankyrin deficiency is the most common defect in dominant and non dominant hereditary spherocytosis. Haematologica 82:460-462, 1997
69. Saad ST, Costa FF, Vicentim DL, et al: Red cell membrane protein abnormalities in hereditary spherocytosis in Brazil. Br J Haematol 88:295-299, 1994
70. Yawata Y, Kanzaki A, Yawata A, et al: Characteristic features of the genotype and phenotype of hereditary spherocytosis in the Japanese population. Int J Hematol 71:118-135, 2000
71. Miraglia del Giudice EM, Hayette S, Bozon M, et al: Ankyrin Napoli: A de novo deletional frameshift mutation in exon 16 of ankyrin gene (ANK1) associated with spherocytosis. Br J Haematol 93:828-834, 1996
72. Randon J, Miraglia del Giudice E, Bozon M, et al: Frequent de novo mutations of the ANK1 gene mimic a recessive mode of transmission in hereditary spherocytosis: Three new ANK1 variants: Ankyrins Bari, Napoli II and Anzio. Br J Haematol 96:500-506, 1997
73. Woods CM, Lazarides E: Spectrin assembly in avian erythroid development is determined by competing reactions of subunit homo- and hetero-oligomerization. Nature 321:85-89, 1986
74. Jarolim P, Rubin HL, Brabec V, et al: A nonsense mutation 1669Glu→Ter within the regulatory domain of human erythroid ankyrin leads to a selective deficiency of the major ankyrin isoform (band 2.1) and a phenotype of autosomal dominant hereditary spherocytosis. J Clin Invest 95:941-947, 1995
75. Gallagher PG, Nilson DG, Wong C, et al: A two base pair deletion at position -72/-73 of the ankyrin-1 promoter associated with hereditary spherocytosis disrupts TFIID complex binding and decreases transcription of a linked reporter gene. Blood 100:77, 2002 (suppl, abstr)
76. Weisbein JL, Wong C, Cline AP, et al: Mutations at positions −108 and −153 of the erythroid ankyrin promoter decrease expression in transgenic mice by disrupting insular function. Blood 100:78, 2002 (suppl, abstr)
77. Eber SW, Pekrun A, Reinhardt D, et al: Hereditary spherocytosis with ankyrin Walsrode, a variant ankyrin with decreased affinity for band 3. Blood 84:362a, 1994 (suppl, abstr)
78. Dhermy D, Galand C, Bournier O, et al: Heterogenous band 3 deficiency in hereditary spherocytosis related to different band 3 gene defects. Br J Haematol 98:32-40, 1997
79. Jarolim P, Rubin HL, Brabec V, et al: Mutations of conserved arginines in the membrane domain of erythroid band 3 lead to a decrease in membrane-associated band 3 and to the phenotype of hereditary spherocytosis. Blood 85:634-640, 1995
80. Jarolim P, Rubin HL, Liu SC, et al: Duplication of 10 nucleotides in the erythroid band 3 (AE1) gene in a kindred with hereditary spherocytosis and band 3 protein deficiency (band 3 PRAGUE). J Clin Invest 93:121-130, 1994
81. Vince JW, Reithmeier RA: Carbonic anhydrase II binds to the carboxyl terminus of human band 3, the erythrocyte C1-/HCO3- exchanger. J Biol Chem 273:28430-28437, 1998
82. Kanzaki A, Hayette S, Morle L, et al: Total absence of protein 4.2 and partial deficiency of band 3 in hereditary spherocytosis. Br J Haematol 99:522-530, 1997

83. Peters LL, Shivdasani RA, Liu SC, et al: Anion exchanger 1 (band 3) is required to prevent erythrocyte membrane surface loss but not to form the membrane skeleton. Cell 86:917-927, 1996
84. Jarolim P, Murray JL, Rubin HL, et al: Characterization of 13 novel band 3 gene defects in hereditary spherocytosis with band 3 deficiency. Blood 88:4366-4374, 1996
85. Rysava R, Tesar V, Jirsa MJ, et al: Incomplete distal renal tubular acidosis coinherited with a mutation in the band 3 (AE1) gene. Nephrol Dial Transplant 12:1869-1873, 1997
86. Lima PR, Gontijo JA, Lopes de Faria JB, et al: Band 3 CampinasA novel splicing mutation in the band 3 gene (AE1) associated with hereditary spherocytosis, hyperactivity of Na^+/Li^+ countertransport and an abnormal renal bicarbonate handling. Blood 90:2810-2818, 1997
87. Jarolim P, Shayakul C, Prabakaran D, et al: Autosomal dominant distal renal tubular acidosis is associated in three families with heterozygosity for the R589H mutation in the AE1 (band 3) Cl-/HCO3- exchanger. J Biol Chem 273:6380, 1998
88. Bruce LJ, Cope DL, Jones GK, et al: Familial distal renal tubular acidosis is associated with mutations in the red cell anion exchanger (Band 3, AE1) gene. J Clin Invest 100: 1693-1707, 1997
89. Inaba M, Yawata A, Koshino I, et al: Defective anion transport and marked spherocytosis with membrane instability caused by hereditary total deficiency of red cell band 3 in cattle due to a nonsense mutation. J Clin Invest 97:1804-1817, 1996
90. Karet FE, Gainza FJ, Gyory AZ, et al: Mutations in the chloride-bicarbonate exchanger gene AE1 cause autosomal dominant but not autosomal recessive distal renal tubular acidosis. Proc Natl Acad Sci USA 95:6337-6342, 1998
91. Palek J, Jarolim P: Clinical expression and laboratory detection of red blood cell membrane protein mutations. Semin Hematol 30:249-283, 1993
92. Rybicki AC, Heath R, Wolf JL, et al: Deficiency of protein 4.2 in erythrocytes from a patient with a Coombs negative hemolytic anemia. Evidence for a role of protein 4.2 in stabilizing ankyrin on the membrane. J Clin Invest 81:893-901, 1988
93. Lombardo CR, Willardson BM, Low PS: Localization of the protein 4.1-binding site on the cytoplasmic domain of erythrocyte membrane band 3. J Biol Chem 267:9540-9546, 1992
94. Waugh RE, Agre P: Reductions of erythrocyte membrane viscoelastic coefficients reflect spectrin deficiencies in hereditary spherocytosis. J Clin Invest 81:133-141, 1988
95. Hanspal M, Yoon SH, Yu H, et al: Molecular basis of spectrin and ankyrin deficiencies in severe hereditary spherocytosis: Evidence implicating a primary defect of ankyrin. Blood 77:165-173, 1991
96. Tse WT, Gallagher PG, Jenkins PB, et al: Amino-acid substitution in α-spectrin commonly coinherited with nondominant hereditary spherocytosis. Am J Hematol 54:233-241, 1997
97. Jarolim P, Wichterle H, Palek J, et al: The low expression α-spectrin LEPRA is frequently associated with autosomal recessive/nondominant hereditary spherocytosis. Blood 88: 4a, 1996 (suppl, abstr)
98. Hassoun H, Vassiliadis JN, Murray J, et al: Molecular basis of spectrin deficiency in β-spectrin Durham. A deletion within β-spectrin adjacent to the ankyrin-binding site precludes spectrin attachment to the membrane in hereditary spherocytosis. J Clin Invest 96:2623-2629, 1995
99. Becker PS, Tse WT, Lux SE, et al: β-Spectrin Kissimmee: A spectrin variant associated with autosomal dominant hereditary spherocytosis and defective binding to protein 4.1. J Clin Invest 92:612-616, 1993
100. Wolfe LC, John KM, Falcone JC, et al: A genetic defect in the binding of protein 4.1 to spectrin in a kindred with hereditary spherocytosis. N Engl J Med 307:1367-1374, 1982
101. Hassoun H, Vassiliadis JN, Murray J, et al: Characterization of the underlying molecular defect in hereditary spherocytosis associated with spectrin deficiency. Blood 90:398-406, 1997
102. Cooper RA, Jandl JH: The role of membrane lipids in the survival of red cells in hereditary spherocytosis. J Clin Invest 48:736-744, 1969
103. Liu SC, Derick LH, Duquette MA, et al: Separation of the lipid bilayer from the membrane skeleton during discocyte-echinocyte transformation of human erythrocyte ghosts. Eur J Cell Biol 49:358-365, 1989
104. Zachee P, Boogaerts MA, Hellamans L, et al: Adverse role of the spleen in hereditary spherocytosis: Evidence by the use of the atomic force microscope. Br J Haematol 80:264-265, 1992
105. Da Costa L, Mohandas N, Sorette M, et al: Temporal differences in membrane loss lead to distinct reticulocyte features in hereditary spherocytosis and in immune hemolytic anemia. Blood 98:2894-2899, 2001
106. Corbett JD, Agre P, Palek J, et al: Differential control of band 3 lateral and rotational mobility in intact red cells. J Clin Invest 94:683-688, 1994
107. Cho MR, Eber SW, Liu SC, et al: Regulation of band 3 rotational mobility by ankyrin in intact human red cells. Biochemistry 37:17828-17835, 1998
108. Reinhardt D, Witt O, Miosge N, et al: Increase in band 3 density and aggregation in hereditary spherocytosis. Blood Cells Mol Dis 27:399-406, 2001
109. Weiss L: A scanning electron microscopic study of the spleen. Blood 43:665-691, 1974
110. Groom AC: Microcirculation of the spleen: New concepts, new challenges. Microvasc Res 34:269-289, 1987
111. Fujino T, Ishikawa T, Inoue M, et al: Characterization of membrane-bound serine protease related to degradation of oxidatively damaged erythrocyte membrane proteins. Biochim Biophys Acta 1374:47-55, 1998
112. Coleman DH, Finch CA: Effect of adrenal steroids in hereditary spherocytic anemia. J Lab Clin Med 47:602-610, 1956
113. Reliene R, Mariani M, Zanella A, et al: Splenectomy prolongs in vivo survival of erythrocytes differently in spectrin/ankyrin- and band 3-deficient hereditary spherocytosis. Blood 100:2208-2215, 2002
114. Peters LL, Barker JE: Spontaneous and targeted mutations in erythrocyte membrane skeleton genes: Mouse models of hereditary spherocytosis, in Zon LI (ed): Hematopoiesis, a Developmental Approach. Oxford, UK, Oxford University Press, 2001, pp 582-609
115. Wandersee N, Birkenmeier C, Bodine D, et al: Mutations in the murine erythroid α-spectrin gene alter spectrin mRNA and protein levels and spectrin incorporation into the red blood cell membrane skeleton. Blood 101:325-330, 2003
116. Kaysser TM, Wandersee NJ, Bronson RT, et al: Thrombosis and secondary hemochromatosis play major roles in the pathogenesis of jaundiced and spherocytic mice, murine models for hereditary spherocytosis. Blood 90:4610-4619, 1997
117. Wandersee NJ, Lee JC, Kaysser TM, et al: Hematopoietic cells from α-spectrin-deficient mice are sufficient to induce thrombotic events in hematopoietically ablated recipients. Blood 92:4856-4863, 1998

118. Bodine DM, Birkenmeier CS, Barker JE: Spectrin deficient inherited hemolytic anemias in the mouse: Characterization by spectrin synthesis and mRNA activity in reticulocytes. Cell 40:959-969, 1984
119. Peters LL, Turtzo LC, Birkenmeier CS, et al: Distinct fetal Ank-1 and Ank-2 related proteins and mRNAs in normal and nb/nb mice. Blood 81:2144-2149, 1993
120. Peters LL, Birkenmeier CS, Bronson RT, et al: Purkinje cell degeneration associated with erythroid ankyrin deficiency in nb/nb mice. J Cell Biol 114:1233-1241, 1991
121. Hassoun H, Wang Y, Vassiliadis J, et al: Targeted inactivation of murine band 3 (AE1) gene produces a hypercoagulable state causing widespread thrombosis in vivo. Blood 92:1785, 1998
122. Peters LL, Andersen SG, Gwynn B, et al: A QTL on mouse chromosome 12 modifies the band 3 null phenotype: β spectrin is a candidate gene. Blood 98:437a, 2001 (suppl, abstr)
123. Paw BH, Zhou Y, Li R, et al: Cloning of the zebrafish retsina blood mutation: Mutations in erythroid band 3 in dyserythropoiesis and cytokinesis defects. Blood 96:440a, 2000 (suppl, abstr)
124. Liao EC, Paw BH, Peters LL, et al: Hereditary spherocytosis in zebrafish riesling illustrates evolution of erythroid β-spectrin structure, and function in red cell morphogenesis and membrane stability. Development 127:5123-5132, 2000
125. Shafizadeh E, Paw B, Foott H, et al: Characterization of zebrafish merlot/chablis as non-mammalian vertebrate models for severe congenital anemia due to protein 4.1 deficiency. Development 129:4359-4370, 2002