Laboratory Identification of Erythroblastosis Fetalis

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ABSTRACT

Hemolytic disease of the newborn (HDN) is a disease of the fetus and newborn owing to fetal-maternal blood group incompatibility. It may present as a barely perceptible hemolytic process or manifest the severe symptoms of hydrops fetalis. The clinical laboratory assumes the role of supplying data needed for the diagnosis, treatment and prevention of the disease. Prenatal immunohematologic testing identifies those women whose fetus is at risk for Rh-HDN and may indicate those patients who should have amniocentesis. Analyses of amniotic fluid identifies those fetuses which need intrauterine transfusion. Postnatal studies can confirm suspected HDN, identify those neonates who are at risk of developing kernicterus, and determine which neonates may need exchange transfusion or phototherapy. Maternal prenatal and postnatal testing identifies those females eligible for Rh-immune globulin therapy to prevent HDN.

Introduction

Erythroblastosis fetalis or hemolytic disease of the newborn (HDN) is a disease of the fetus and neonate which is characterized by a hemolytic anemia with varying degrees of severity. The hemolytic anemia occurs as a result of maternal alloantibodies which are transmitted to the fetus via the placenta. The important clinical manifestations are anemia, jaundice, hepatosplenomegaly and irreversible damage to neurons of the central nervous system, especially those of the basal ganglia (kernicterus). The severity of the disease varies widely from a slight anemia and hyperbilirubinemia at birth to intrauterine death in the third trimester.46

Rh-Hemolytic Disease of the Newborn (Rh-HDN)

The syndrome of erythroblastosis had been recognized for many centuries.2 However, it was not until the discovery of the Rh blood group system in 1939 and 1940 with the detection of the antibody against the antigen D (also known as Rh0)
that the basic pathogenesis of erythroblastosis fetalis was established.\textsuperscript{28,31} Classically hemolytic disease of the newborn (HDN) owing to anti-D occurs in D negative women who have been previously sensitized to the D antigen. In the vast majority of cases, this sensitization to the D antigen with the production of anti-D antibody is the result of a previous pregnancy in which the fetal red cells contained the D antigen. The sensitizing dose of fetal red cells most commonly occurs at the time of delivery as a result of a fetal-maternal bleed. The ABO type of the mother and fetus is an ameliorating factor with regards to the development of maternal sensitization.\textsuperscript{29,30} Type ABO incompatible fetal red cells are often destroyed before maternal sensitization to the D antigen has time to take place. On rare occasions, maternal sensitization to the D antigen can occur during the first pregnancy and can result in HDN.\textsuperscript{39} Maternal sensitization to the D antigen can also result from a transfusion of D positive blood, spontaneous or induced abortions, and ectopic pregnancies.

IgG is the only immune globulin which is transported across the normal placenta.\textsuperscript{8,17,20} At 33 weeks of gestation, the maternal and fetal levels of IgG are approximately equal,\textsuperscript{18} and at term the fetal level may exceed the maternal level.\textsuperscript{17} Antibodies directed against antigens of the Rh system are of the IgG type. The subclasses IgG1 and IgG3 bind more efficiently than IgG2 and IgG4 to splenic macrophages, thereby readily inducing the destruction of immune globulin coated erythrocytes. Infants with red cells coated with IgG3 statistically have a much more severe form of hemolytic disease of the newborn while infants with IgG1 have a HDN of variable severity.\textsuperscript{11} Methods are available for differentiating the IgG subclasses.\textsuperscript{12,21,36,51}

The frequency of pregnancies in the general population which theoretically can result in Rh-HDN (Rh negative mothers and Rh positive fetus) is 10 percent in Caucasians, five percent in Negroes, and one percent in Orientals.\textsuperscript{4} Prior to the advent of Rh immune globulin, only about 12 percent of Rh negative mothers became sensitized.\textsuperscript{1,13} Type ABO incompatibility, poor antibody formation by the mother or an insufficient antigenic stimulus (small volume of fetal-maternal bleed) protect Rh negative mothers from sensitization.\textsuperscript{6,41,45} With the extensive use of Rh immune globulin to prevent maternal sensitization to the D antigen, the incidence of Rh sensitizations has fallen to one percent.\textsuperscript{15}

The most common cause of HDN is ABO incompatibility. However, the most common cause of severe fetal hemolytic anemia requiring exchange and possibly intrauterine transfusion is maternal anti-D. Maternal antibodies to Kell, C, and E can cause severe hemolytic disease, but are relatively rare.\textsuperscript{58} It is essential to establish the potential for fetal hemolytic disease as early as possible so that the pregnancy can be properly managed and appropriate therapy undertaken.

The diagnosis of HDN begins with a complete history and physical examination at the first obstetric visit. Particular attention must be paid to the course and outcome of previous pregnancies (abortions, stillbirths) and to any prior transfusions. Immunohematologic testing should include ABO grouping of mother and father, maternal Rh typing, and an antibody screen. Rh negative women with negative antibody screens at
the initial visit should have repeat studies at 28, 32, and 36 weeks gestation to detect an early anamnestic (secondary immune) response. If these repeat studies are negative, no further testing during that pregnancy is indicated.

If the prenatal antibody screen is positive, the antibody must be identified. The antibody should be classified as to whether it is of IgG or IgM type (IgM does not cross the placenta) in order to determine if the antibody is clinically significant. If the antibody is clinically significant, the serum sample from the initial visit should be properly frozen and later titered in parallel with subsequent serum samples from the patient.

The antibody titer procedure consists of serial dilutions of maternal serum which are reacted with a suspension of group O RBC’s that possess the suspected antigen (usually D). The titer with the highest dilution at which hemagglutination occurs is reported. A two-tube antibody rise is regarded as a significant increase.

In a pregnancy in which a clinically significant antibody (usually anti-D) is demonstrated, rising serial antibody titers usually indicate the fetal erythrocytes possess the corresponding antigen and, therefore, the fetus is likely to suffer from hemolytic disease. The antibody titer, in general, does not correlate well with the severity of HDN. The primary value of an antibody titer is to identify patients who should have amniocentesis.

Amniocentesis provides the clinician with a means to evaluate the degree of intrauterine fetal hemolysis taking place. It is also useful to assess the overall condition of the fetus. Amniocentesis is indicated when the clinically significant maternal antibody titer is 1:32 or greater in the fourth or fifth month of pregnancy. If there is a history of a previous pregnancy complicated by HDN, amniocentesis is indicated regardless of the present maternal serum antibody titer. The critical antibody titer value that is associated with the need for amniocentesis must be determined by each laboratory. The initial amniocentesis is generally performed between the 28th and 32nd week of pregnancy. Amniocentesis may be repeated as often as necessary up to the time of delivery. Complications of amniocentesis include an increase in maternal Rh sensitization from fetal-maternal bleeding, infection, and premature labor. However, these complications are relatively infrequent.

Amniotic fluid is normally straw colored and slightly turbid. Inspection of amniotic fluid upon receipt in the laboratory can be quite helpful. Yellow, slightly turbid amniotic fluid may indicate an Rh positive fetus affected with HDN; red, opaque fluid indicates blood contamination; green, opaque fluid indicates meconium staining; and yellow-brown opaque fluid is often associated with intrauterine death of the fetus.

Bilirubin, a metabolic by-product of physiologic hemolysis, is present in very low concentrations in the amniotic fluid during the first trimester of pregnancy. Normally, as erythropoiesis accelerates, bilirubin levels in the amniotic fluid increase until the 20th to 26th weeks. Increased abnormal levels of amniotic fluid bilirubin give an indication as to the severity of hemolysis in the fetus.

Normal amniotic fluid shows a specific spectral absorbance pattern when scanned from 350 nm to 750 nm. A spectral scan of amniotic fluid containing bilirubin will show a characteristic change in absorbance or optical density (O.D.) characterized by a rise at 375 nm, a peak at 450 nm, and a return to the baseline at 525 nm (figure I). Transposing the spectral scan to a semilogarithmic scale (abscissa, wavelength in linear scale; ordinate, ΔO.D. in log scale) results in linearization of the scan results. The net difference between the peak absorbance at 450 nm and the
theoretic baseline taken between 375 nm and 525 nm is the absorbance contributed by bilirubin or the ΔO.D. 450.\textsuperscript{13,33} Since sunlight and artificial lighting destroy bilirubinoid pigment rapidly, amniotic fluid must be placed immediately in amber or foil wrapped test tubes. In the spectral scan of amniotic fluid, potential technical problems can arise from contamination owing to blood or meconium (figure 2). Hemolyzed blood gives the characteristic peaks of oxyhemoglobin at 415 nm, 540 nm, and 757 nm. Hemolysis tends to decrease the net absorbance at 450 nm; hence, the severity of hemolytic disease is underestimated. Meconium staining of amniotic fluid is indicative of fetal distress. Meconium typically peaks at about 405 nm and slowly recesses to baseline at about 550 nm.

Predictive interpretations of fetal prognosis can be made based on the relative concentration of bilirubin at various stages of gestation.\textsuperscript{10,14,34} Liley, plotting the ΔO.D. at 450 nm versus gestational age, defined three major prognostic zones\textsuperscript{32} (figure 3). Zone III includes intrauterine deaths, neonatal deaths, and infants with low cord hemoglobin levels. Fetuses with values in Zone III, before the 32nd week, require intrauterine transfusion to prolong intrauterine survival until viability. After the 32nd week, Zone III values indicate the need for immediate delivery to prevent fetal death. Zone II values indicate the need for repeat analysis and possible early delivery. Zone I values indicate a good prognosis with regards to HDN in a near term fetus.

Serial amniotic fluid analyses will provide the clinician with an indication of any possible adverse trends. An increasing or stationary ΔO.D., depending on the gestational age and clinical circumstances, will indicate the type of intervention needed. This may be exchange transfusion or simply following the fetus until delivery.

It has been recommended that erythrocytes from amniotic fluid be ABO grouped, Rh typed,\textsuperscript{44} and that an acid elution test be performed to determine if
the cells are of fetal or maternal origin.24 This procedure is neither reproducible nor a standard technique and does not aid in the management of the patient.

Amniotic fluid creatinine (to assess fetal weight) and phospholipid determinations (to assess fetal lung maturity) will aid the clinician in the determination of the potential viability of the fetus that needs immediate delivery. Maternal estriol, human chorionic gonadotropin (HCG), and human placental lactogen (HPL) will aid in the assessment of placentation function. These tests provide an indication of fetal maturity or fetal distress in a pregnancy complicated by Rh-HDN.55

Intrauterine transfusions may be the only hope for a fetus with severe hemolytic disease. The timing of the procedure is crucial. With respect to gestational age, 91 percent of fetuses died in utero when the initial intrauterine transfusion (IUT) was given before the 25th week of gestation, 76 percent died when IUT was given between the 25th and 27th week, and 44 percent died when IUT was given at the 28th week or later.48,53 After the 32nd week, the chance of fetal survival following delivery is good and intrauterine transfusion is not generally indicated. Possible fetal complications of IUT include tissue trauma, hepatitis, and bacterial infection. Possible maternal complications include premature rupture of the membrane, infection, and bleeding.48,53

At birth, hemolytic disease can present as a barely perceptible hemolytic process identified only by immunohematologic testing. The most severe symptoms form a syndrome referred to as hydrops fetalis. This includes severe anemia with heart failure, hepatosplenomegaly, and marked generalized edema with epidermal exfoliation. The postpartum laboratory diagnosis of HDN is made on cord blood and should include:

(1) Direct antiglobulin test (D.A.T.), (2) Hemoglobin determination, (3) Differential cell count on a blood smear stained with Wright's stain, (4) Absolute reticulocyte count, (5) Indirect serum bilirubin, (6) Reserve albumin binding capacity, and (7) ABO group and Rh type.

The presence of maternal anti-D antibodies in an infant with a positive direct antiglobulin test (Coombs), low umbilical cord hemoglobin concentration, and an elevated indirect serum bilirubin is virtually diagnostic of HDN. In severely affected infants, the antiglobulin test almost always gives a strongly positive result. However, the strength of the direct antiglobulin test is not directly correlated with the severity of the disease.39

Cord blood hemoglobin concentration is an extremely valuable parameter in assessing the severity of the hemolytic anemia.39 Greater than 50 percent of affected infants have a cord hemoglobin level above 14 mg per dl, which is usually accepted as the lower limit of the reference range. Normally the hemoglobin level of the newborn falls within the first 72 hours of life, but in HDN the decrease in hemo-
globin concentration is precipitous and the signs and symptoms of anemia become apparent. Infants with the clinical syndrome of *hydrops fetalis* at birth may have umbilical cord hemoglobin levels as low as 3 g per dl. Such unusually low hemoglobin concentrations may be due to technical errors as a result of dilution of the cord blood by the marked tissue edema fluid. These hydropic infants, if they do not die in utero, usually do not survive the neonatal period.

The most characteristic findings on a Wright’s stained thin smear of the cord blood is the tremendous number of nucleated macrocystic erythrocytes (figure 4). These macrocystic red cells appear in all stages of maturation, hence the original name of the disease “erythroblastosis fetalis.” The erythrocytes may be normochromic or polychromatophilic. The reticulocyte count is often markedly elevated reflecting the bone marrow’s response to anemic stress. There may be a marked leukocytosis in the severely affected neonates. Thrombocytopenia with accompanying purpura may likewise occur in severely affected neonates. Disseminated intravascular coagulation may occur complicating the morphologic picture of the peripheral blood smear.56

The anemia should be treated by exchange transfusion. This will remove the antibody-coated erythrocytes from the neonate, incompatible antibody, and the intravascular indirect bilirubin. With improvement of the oxygen carrying capacity of the blood, symptoms of congestive heart failure are thereby relieved. There is clinical and pathologic evidence that the degree of neuronal damage of the basal ganglia of the brain by-products of bilirubin metabolisms is related to the severity of the anemia. An increase in the permeability of the blood brain barrier owing to hypoxia with resulting cerebral edema creates an environment for severe and permanent CNS damage.

A significant problem of hemolytic disease of the newborn is hyperbilirubinemia and potential bilirubin encephalopathy (kernicterus). Bilirubin in the fetus is normally transferred across the placenta to the maternal circulation, is conjugated, and excreted by the maternal liver. In normal neonates, serum bilirubin rises during the first few days of life, secondary to physiologic red cell destruction and also

![Figure 4. Immature red blood cells in peripheral blood in severe erythroblastosis. Note: rubriblast, prorubricyte, rubricyte, and metarubricytes.](image-url)
to a deficiency of hepatic glucuronyl transferase. This enzyme conjugates bilirubin to a water-soluble excretable form. In infants with HDN, bilirubin metabolism is further compromised by abnormally increased red cell destruction. The indirect bilirubin level that will produce kernicterus is not clearly defined. In full term infants with HDN, bilirubin above 20 mg per dl has been accepted as the level for significant risk of kernicterus.

Indirect bilirubin is bound to albumin. The fraction of indirect bilirubin not bound or loosely bound by albumin may be called the "free bilirubin" fraction. This "free bilirubin" fraction crosses the blood brain barrier and causes neuronal toxicity. The determination of the reserve albumin binding capacity (R.A.B.C.) has been used to develop indices at which kernicterus may develop and exchange transfusion is indicated.

No universal criteria have been established regarding when exchange transfusion should be done. However, an immediate exchange transfusion is usually indicated in a mature neonate with a cord hemoglobin less than 12.0 mg per ml and an indirect bilirubin concentration greater than 4.5 mg per dl. With complications such as prematurity, hypoxia, infection, and acidosis, the risk of developing kernicterus at lower levels of bilirubin concentration is significant.

For neonates not fulfilling the criteria for exchange transfusion, continuous phototherapy is utilized. Serum bilirubin levels are measured every eight to ten hours until the serum bilirubin level starts falling. This generally occurs between the third and sixth post-partum day.

**ABO—Hemolytic Disease of the Newborn (ABO-HDN)**

Type ABO incompatibility is the most common cause of hemolytic disease in the fetus and neonate. Most cases are not of clinical importance and do not require treatment. The hemolytic process due to ABO incompatibility often occurs in the first born, in contrast to the hemolytic process produced by fetal-maternal Rh incompatibility. Type ABO-HDN almost always involves mothers of group O, and neonates of groups A, B, or AB. The pathophysiology is essentially the same as that of HDN produced by antibodies to erythrocytic antigens of the Rh system.

The diagnosis of ABO-HDN is almost always made after delivery and is often only made after excluding all other possible causes of early post-partum hyperbilirubinemia. Type ABO-HDN cannot be predicted by any antenatal testing since the demonstration of immune anti-A or anti-B in the maternal serum has little predictive value as to the presence or severity of the hemolytic process.

The ABO group and Rh type should be determined on any neonate who develops hyperbilirubinemia shortly after birth. A direct antiglobulin test performed on the neonate's erythrocytes should also be done. If the direct antiglobulin test is positive, the mother's serum should be checked against a red cell panel and the specificity of her antibody determined. If the reaction of the direct antiglobulin test is weakly positive, the mother and infant are usually ABO incompatible. However, antibodies other than anti-A and anti-B may also be present and, as stated previously, should be identified and their clinical significance determined.

The most satisfactory test to detect fetal-maternal ABO incompatibility is an elution of the ABO antibody from the affected infant's red cells. The eluate is then tested against adult A and B red cells. The detection of free antibody in the serum of ABO incompatible infants can be demonstrated and may substantiate the diagnosis of ABO incompatibility.

Hematologic findings in fetal-maternal ABO incompatibility usually include a mild degree of anemia associated with slight reticulocytosis and spherocytosis.
The most common manifestation of ABO disease is jaundice. It appears during the first 24 hours of life. It rarely, if ever, is severe enough to be associated with the development of kernicterus. Treatment, when necessary, consists of phototherapy. Rarely an exchange transfusion may be necessary. Serial indirect serum bilirubin concentrations and reserve albumin binding capacity are used to determine the necessity and type of therapy. Fresh, saline washed, packed erythrocytes which are group O are used if an exchange transfusion is indicated.

Other Blood Antigen Induced HDN

Although anti-D and the ABO system antibodies are the most frequent causative antibodies of HDN, other antibodies in the Rh system, Duffy, Kidd, Kell and other systems can cause a hemolytic process of varying severity if the antibody can cross the placenta in sufficient concentration and gain access to the fetal circulation. Severe HDN has been reported by antibodies directed against low incident antigens. Antibodies directed against high incident antigens also cause HDN. The same principles normally involved in prenatal testing and neonatal testing apply with these antibodies to identify those cases at risk and to provide therapy as are used in Rh-HDN.

Prevention of Rh-HDN

In women who are already alloimmunized, no practical means are available to suppress clinically the concentration of circulating maternal alloantibody. The methods used to control antibody formation in organ transplant cases and autoimmune disease states are not applicable. Plasmapheresis can reduce a circulating maternal alloantibody on a short-term basis only.

The usual candidates for Rh-immune globulin prophylaxis are women who are not alloimmunized to the D-antigen and who are D and D⁺ negative and who have delivered a D or D⁺ positive infant. If the direct antiglobulin test of the cord blood is positive, an elution test should be performed and the antibody identified. Rh-immune globulin should be given in such cases unless the antibody is identified as anti-D.

An important dosage consideration in the prophylactic administration of Rh-immune globulin is the volume of the fetal-maternal bleed. The detection and quantitation of the fetal-maternal hemorrhage can be determined by an acid-elution technique. Even though the technique is relatively simple, the test should be performed by experienced personnel in order to obtain reliable results. Rh-immune globulin is supplied as one ml of sterile, clear fluid. In the United States, a single dose of Rh-immune globulin, comprising 300 µg IgG anti-D, is used to prevent immunization in cases where no more than 30 cc of fetal blood, or the equivalent of 15 cc of packed fetal cells, have entered the maternal circulation. The dose is generally administered as an intramuscular injection within 72 hours after delivery. The failure rate in preventing alloimmunization is approximately two percent.

Ante partum administration of Rh-immune globulin has been advocated in Rh-negative women at risk who have amniocentesis, ectopic pregnancies, and spontaneous or induced abortions. The Rh-immune globulin reduces the incidence of fetal cells in the maternal circulation and causes no adverse reaction in the D positive fetus. In Rh-negative females who have received a transfusion with Rh positive blood, it is advisable to attempt to prevent alloimmunization by the appropriate use of Rh-immune globulin.

Summary

The clinical laboratory assumes the paramount role of supplying accurate data
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