ARTICLE

Failure to Predict Hemolysis and Hyperbilirubinemia by IgG Subclass in Blood Group A or B Infants Born to Group O Mothers

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What's Known on This Subject

DAT-positive blood group A or B neonates who are born to group O mothers are at risk for hyperbilirubinemia. IgG1 and IgG3 antiglobulin subclasses may be associated with hemolysis to a greater extent than IgG2 or IgG4.

What This Study Adds

Identification of IgG1 antiglobulin subclass was not predictive of hemolysis or hyperbilirubinemia in ABO-heterospecific neonates.

ABSTRACT

OBJECTIVE. Direct antibody titer–positive, blood group A or B neonates who are born to group O mothers may be at risk for hemolysis and hyperbilirubinemia. Immunoglobulin G1 and immunoglobulin G3 subclasses are associated with increased hemolysis relative to immunoglobulin G2 and immunoglobulin G4. We investigated whether identification of immunoglobulin G subclass 1 or 3 may be predictive of hemolysis and hyperbilirubinemia.

METHODS. Direct antibody titer-positive, blood group A and B neonates born to group O mothers were tested for the presence of immunoglobulin G subclasses 1 and 3 in umbilical cord blood by using a commercially available gel testing technology. By inference, neonates in whom neither immunoglobulin G1 nor immunoglobulin G3 were detected were designated immunoglobulin G2 and/or 4. Mandatory plasma total bilirubin was measured at discharge, and additional measurements performed as clinically indicated. Hyperbilirubinemia was defined as any plasma total bilirubin value >95th percentile for hour of life. Blood carboxyhemoglobin and total hemoglobin concentrations were also measured on the predischarge sample. Measured carboxyhemoglobin, expressed as percentage of total hemoglobin, was corrected for ambient carbon monoxide to derive "corrected carboxyhemoglobin," a sensitive index of heme catabolism. The corrected carboxyhemoglobin/total hemoglobin ratio was calculated to correct for any differences in total hemoglobin mass between groups.

RESULTS. Eighty-two infants were studied, 18 of whom were designated as immunoglobulin G1, 0 as immunoglobulin G3, and 64 as immunoglobulin G2 and/or 4. The incidence of plasma total bilirubin >95th percentile was similar between the subgroupings. Corrected carboxyhemoglobin values and corrected carboxyhemoglobin/total hemoglobin ratio were also similar between the subgroupings.

CONCLUSIONS. Immunoglobulin G1 was found in 22% of direct antibody titer–positive, group A and B neonates who were born to group O mothers, whereas immunoglobulin G3 was rare. Hemolysis and hyperbilirubinemia could not be predicted by this gel technique that enabled identification of these immunoglobulin G subclasses. *Pediatrics* 2009; 123:e132–e137

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Key Words
hemolysis, carboxyhemoglobin, bilirubin, hyperbilirubinemia, hemolysis, gel technology, blood groups, ABO heterospecificity, direct antibody titer, prediction

Abbreviations
IgG—immunoglobulin G
DAT—direct antibody titer
COHb—carboxyhemoglobin
COHbc—COHb corrected for ambient (inspired) carbon monoxide
PTB—plasma total bilirubin
tHb—total hemoglobin
RBC—red blood cell

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ABO BLOOD GROUP heterospecificity, in which the mother has blood group O and the newborn has blood group A or B, may be associated with clinically significant neonatal hyperbilirubinemia. The jaundice results from immunoglobulin G (IgG) anti-A or anti-B antibodies' crossing the placenta and attaching to the appropriate antigen on the neonatal red cell. Resultant heme catabolism results in increased bilirubin production, thereby contributing to the body's bilirubin pool.[1] This process occurs to a much lesser extent in group A or B neonates who are born to heterospecific A or B mothers, because in this situation, the respective anti-A or anti-B globulin is predominantly immunoglobulin M and therefore unable to cross the placenta in appreciable quantities.

Presence of antibody on the red cell may be detected by determining the direct antibody titer (DAT), otherwise known as the Coombs' test. A positive DAT is regarded as a risk factor for hyperbilirubinemia[2]; however, not all neonates with a positive DAT necessarily develop hyperbilirubinemia.[3,4]

One factor that possibly contributes to the degree of hemolysis is an IgG subclass. IgG is the major immunoglobulin in normal human serum. Four human subclasses of the IgG molecule can be differentiated, IgG1, IgG2, IgG3 and IgG4, of which IgG1 is predominant. Although all 4 subclasses are able to cross the placenta, types 1 and 3 bind to the Fc receptor of phagocytic cells with greater affinity than do types 2 and 4. Hemolysis is therefore expected to occur to a greater extent in association with the former.[5–7]

In Rh hemolytic disease, infants who are sensitized primarily with IgG1 are more likely to be severely affected than their IgG3 predominant counterparts. IgG3, however, may also be associated with rapid postnatal rise of serum bilirubin concentrations. Combination of IgG1 and IgG3 seemed to result in the most severe form of hemolytic disease.[8] It has been suggested that identification of IgG subclasses may yield prognostic information regarding the development of hemolysis and hyperbilirubinemia.[9]

Few studies have assessed the role of IgG subclasses in the pathogenesis of hyperbilirubinemia in ABO heterospecificity. On the basis of data retrieved from Rh-isoimmunized newborns and 1 study of ABO-heterospecific neonates,[4] we hypothesized that the degree of hemolysis and hyperbilirubinemia would correlate with the anti-A or anti-B IgG subclass present and that the presence of subclass 1 or 3 would be predictive of hemolysis. Our objective was to determine whether identification of IgG1 or IgG3 subclass by a commercially available gel technology could be used as an adjunct to DAT in predicting whether a neonate would develop hyperbilirubinemia and whether presence of IgG1 or IgG3 would be associated with increased hemolysis, quantified by blood carboxyhemoglobin (COHb) determination and corrected for ambient CO (COHbc).[10]

METHODS

Clinical Protocol

Patient Population

The clinical wing of the study was conducted in the well-infant nurseries of the Shaare Zedek Medical Center from September 2006 to April 2007. Consecutive DAT-positive blood group A or B infants who were born at ≥37 weeks' gestation to mothers with blood group O were eligible for enrollment in the study. The study was approved by the institutional review board of the Shaare Zedek Medical Center.

Exclusions

Neonates with any known hemolytic condition other than ABO incompatibility were excluded. Such conditions included severe bruising, sepsis, Down syndrome, glucose-6-phosphate dehydrogenase deficiency, or a positive DAT from any cause other than ABO isoimmunization. Similarly, Rh-positive, DAT-positive newborns who were born to Rh-negative mothers were excluded because of the possibility that the positive DAT may have been caused by Rh isoimmunization.

Use of Umbilical Cord Blood in the Study

Blood type and DAT tests are routinely performed on umbilical cord blood on all infants who are born to blood group O mothers at this medical center. Umbilical cord blood is routinely collected after delivery of the infant. This blood is stored refrigerated in the hospital's blood bank and used for blood typing, Rh determination, and DAT testing as appropriate.

Routine Treatment

Neonates were treated routinely in the nursery, as previously described.[11] Newborns were assessed visually for jaundice at the time of admission to the nursery from the delivery room and at least once per nursing shift subsequently. When an infant at any stage appeared to have jaundice, a plasma total bilirubin (PTB) test was performed. All PTB results were plotted on the hour of life specific bilirubin nomogram, and the percentile was determined.[12] Phototherapy for DAT-positive infants was instituted in accordance with the 2004 American Academy of Pediatrics guidelines for neonates with risk factors.[2] At the time of routine metabolic screening, predischarge PTB determination was performed in DAT-positive infants. Follow-up PTB determinations, when necessary, were performed as outpatients at our hospital.

Study Protocol

For the purpose of the study and to avoid a nonroutine blood-taking procedure, a small amount of blood was collected for COHb determination at the time of the regular metabolic screening. This was the only deviation from routine treatment. Simultaneous with the COHb sampling, a sample of air from the nursery in which the infant was being cared for was collected for CO analysis. The timing of metabolic screening was suited to COHb sampling of neonates of smoking mothers, because, by 48 hours, there should no longer be any effect of the smoking on the newborns' COHb concentrations.[13]

Laboratory Methods

COHb

Blood for COHb determination (150 μL) was collected into custom-prepared capillary tubes that contained heparin and saponin (supplied by Stanford University, Stanford, CA). The filled tubes were sealed and after mixing of the contents stored at −18°C. The samples were thawed and sent on wet ice to Stanford University. COHb was determined by a gas chromatographic method, as previously described.[14,15] The results were expressed as a percentage of total hemoglobin (tHb), itself measured from the same blood sample by using a previously described cyanmethemoglobin method.[14,15]

CO content of the sampled ambient air was measured at Shaare Zedek Medical Center by using a CO analyzer supplied for this purpose by Stanford University. Measured COHb values were corrected for inspired CO by a previously determined formula to derive COHbc.[16] To correct for any differences in hemoglobin mass between the subgroups, thereby improving the sensitivity of COHbc as an indicator of heme catabolism, we calculated the ratio COHbc/tHb.

DAT Testing

DAT testing was performed routinely in the blood bank of the Shaare Zedek Medical Center on umbilical cord blood by using a commercially available kit (DiaMed-ID MicroTyping System, ID-Card "LISS/Coombs" [DiaMed AG, Cressier s/Morat, Switzerland]).

IgG Subclasses

IgG subclasses were determined in the same laboratory as the DAT testing, on umbilical cord blood, by using a commercially available kit that used gel testing technology (DiaMed-ID MicroTyping System, ID-Card "DAT IgG1/IgG3" [DiaMed AG]). The principle of the test is that anti-IgG1 and anti-IgG3 globulins are added to the gel, in which they agglutinate red blood cells (RBCs) should these cells have specific corresponding antibody subclass adhered. A positive reaction is recognized by agglutinated RBCs' forming a red line on the surface of the gel or agglutinates dispersed within the gel. Whereas IgG subclassing of sensitized RBCs is not feasible by using standard agglutination techniques,[17] this test is able to identify IgG1 and IgG3 subclasses. Neonates in whom neither IgG1 or IgG3 was detected were assumed to have IgG2 and/or IgG4 and were designated as such.

Blood Type

Blood group typing was performed routinely on umbilical cord blood by using standard blood bank techniques.

Plasma Total Bilirubin

Routine PTB testing was measured on spun, heparinized capillary tube samples by a direct spectrophotometric method by using absorbance of bilirubin at 455 nm (NEO BIL Model A2 [Digital and Analog Systems, Rome, Italy]).

Definition

Hyperbilirubinemia was defined as any PTB value ≥95th percentile on the hour of life specific nomogram.[12]

Data Analysis

Study and Control Groups

For the purpose of analysis, those with IgG subclass 1 or 3 were regarded as the study group and those with presumed IgG subclass 2 or 4 the control groups.

Estimation of Sample Size

Presuming equal distribution between neonates with either IgG1 or IgG3 subclass and those IgG2/4, a 20% incidence of hyperbilirubinemia in DAT-positive neonates with either IgG1 or IgG3 subclass, and a 5% baseline incidence of hyperbilirubinemia in the IgG2 or IgG4 subclass groups, with an α value of .05 and power of 0.8, it was estimated that 72 patients would be required in the study and control groups, respectively. An interim analysis was performed after sampling 82 neonates, at which point it became apparent not only that the IgG subclass distribution was unequal with a dearth of IgG3 subclass neonates but also that the results of COHbc and the incidence of hyperbilirubinemia were virtually identical between the study and control subgroups. Additional enrollment was therefore ceased.

Statistical Analysis

COHbc values, tHb and COHbc/tHb ratio, and the incidence of hyperbilirubinemia were compared between study and control subgroups. Categorical variables were compared by using χ^2 analysis. Because all continuous variables included in the analysis had a normal distribution, comparisons were made using Student's t test. The incidence of hyperbilirubinemia was evaluated by using relative risk (95% confidence interval). Significance was defined as $P < .05$ or, in the case of relative risk, a 95% confidence interval that did not straddle the digit 1.

RESULTS

Of a representative sample of the 1121 neonates who were delivered at the Shaare Zedek Medical Center and selected midway through the study, 240 (21%) composed blood group A or B newborns who were born to group O, Rh-positive mothers. Of these, 37 (15%) were DAT positive. Eighty-two neonates were enrolled in the study. Mean ± SD birth weight was 3407 ± 437 g, and gestational age was 39 ± 1 weeks. Forty-four (54%) were male, 5 were delivered by cesarean section, and 60 (73%) were exclusively breast fed. Three were born to smoking mothers. In accordance with study protocol, all were born to blood group O mothers, and all were DAT positive. The blood group A to B ratio of the newborns was 61:21 (74%:26%).

IgG1 was found in 18 neonates. No infant with IgG3 subclass was identified. The remaining 64 newborns were therefore assigned to the IgG2/4 subgroups. Of the IgG1 subclass, 17 (94%) of 18 neonates were blood group A, whereas of the assumed IgG2/4 subclass, 45 (70%) of 64 were blood group A ($P = .06$).

Data regarding the incidence of hyperbilirubinemia, information concerning PTB values, and the need for phototherapy, for the entire group as well as for the subgroups on the basis of IgG subclass, are summarized in Table 1. No significant differences were observed between the 2 subgroups for any of the measured parameters. Table 2 provides data for COHbc and tHb measurements and the COHbc/tHb ratio, again for the entire group as well as the subclasses. For reference and comparison, COHbc values for a previously reported cohort[18] sampled in the same nursery as this study and processed in the identical Stanford University laboratory are supplied in Table 2. Overall, COHbc values were signifi-

TABLE 1 **Data Relating to Neonates With Any PTB Value ≥95th Percentile for Hour of Life and the Need for Phototherapy, for the Entire Group as Well as for the Subgroups, on the Basis of IgG Subclass**

Category	Entire Group	IgG1 Subgroup	IgG2/4 Subgroup	*P* (IgG1 vs IgG2/4)
No. of neonates	82	18	64	
Any PTB ≥95th percentile, *n* (%)	39 (47.6)	10 (55.5)	31 (48.4)	.57[a]
Age at first PTB ≥95th percentile, mean ± SD, h	19.0 ± 11.0	20.0 ± 10.0	18.0 ± 11.0	.50
First PTB ≥95th percentile, mean ± SD, mg/dL	9.6 ± 2.2	9.8 ± 2.7	9.6 ± 2.1	.74
Phototherapy, *n* (%)	37 (45.0)	7 (39.0)	30 (47.0)	.60
Age at start of phototherapy, mean ± SD, h	20.0 ± 12.0	22.0 ± 13.0	19.0 ± 12.0	.50
PTB at start of phototherapy, mean ± SD, mg/dL	10.1 ± 2.3	10.5 ± 2.9	10.0 ± 2.2	.60

No cases of IgG3 were identified.

[a] Relative risk: 1.14 (95% confidence interval: 0.71[en]1.86).

TABLE 2 **Special Investigations: Data Relating to COHbc and tHb Values**

Category	Entire Group	IgG1 Subgroup	IgG2/4 Subgroup	*P* (IgG1 vs IgG2/4)
n	82	18	64	
COHbc, mean ± SD, % tHb[a]	1.26 ± 0.36	1.30 ± 0.21	1.25 ± 0.40	.60
tHb, mean ± SD, g/dL	17.40 ± 3.00	17.40 ± 2.60	17.40 ± 3.10	.90
COHbc/tHb, mean ± SD,	0.08 ± 0.03	0.08 ± 0.02	0.08 ± 0.04	.82
Age at sample, mean ± SD, h	56.00 ± 12.00	55.00 ± 10.00	56.00 ± 13.00	.76
PTB at sampling, mean ± SD, mg/dL[b]	10.00 ± 2.00	11.00 ± 1.40	9.70 ± 2.10	.04

[a] COHbc values from a previous cohort of 131 neonates are supplied for reference: 0.77 ± 0.19. Values in this study were significantly higher ($P < .001$).

[b] PTB data do not necessarily represent the natural PTB, because 30 neonates (5 and 29 in the IgG1 and IgG2/4 subgroups, respectively) were being treated or had recently been treated with phototherapy at time of sampling.

cantly higher than those of the previously reported cohort. No significant differences existed between the IgG1 and IgG2 and/or IgG4 subclass for COHbc and tHb values or the COHbc/tHb ratio. Although there were slight, albeit statistically significant, differences in PTB values at the time of sampling, phototherapy, which was being or had recently been administered to 30 neonates (5 and 29 in the IgG1 and IgG2/4 subgroups, respectively), may have altered these values, and reported PTB values in Table 2 therefore may not represent the natural concentrations.

DISCUSSION

Most group A or B neonates, heterospecific with their group O mothers, are affected only minimally by this blood group incompatibility; however, some may develop severe hemolysis and hyperbilirubinemia. For example, of a recently reported series of 258 neonates with serum total bilirubin level >25.0 mg/dL, the cause of the hyperbilirubinemia was determined for 93. Thirty-two (34%) of these 93 were blood group A or B newborns who were born to group O mothers, making this the most commonly encountered etiologic category in these newborns.[19] In another series, ABO incompatibility was the reason for exchange transfusion for 21% of 56 neonates[20]; therefore, in the era of early discharge from birth hospitalization, it would be useful to predict which of these newborns may be potentially endangered by hyperbilirubinemia.

Many factors participate in the hemolytic process and interact to determine the degree of hemolysis in the ABO setup.[21] For example, in group A or B neonates who are born to group O mothers, in contrast to Rh isoimmunization, the IgG2 subclass constitutes a significant component of the anti-A or anti-B antibody. IgG2 may be less prone to cause hemolysis, because it crosses the placenta less efficiently than IgG1 or IgG3 and is a less efficient mediator of macrophage-induced RBC clearance than the other subclasses. Also, in contrast to Rh isoimmunization, there is a low density of A or B antigen sites on the fetal RBC, and anti-A or anti-B antibody can be adsorbed onto other tissues that bear these surface antigens. The titer of maternal anti-A or anti-B antibody is another mediator of the immune response; therefore, the mere presence of a positive DAT test does not necessarily mean that a given newborn will inevitably develop hyperbilirubinemia.

One factor that possibly moderates the degree of hemolysis is the subclasses of IgG. We are aware of only 1 report of ABO-incompatible neonates in which IgG subclasses were evaluated as to their ability to cause hemolysis. In a report by Ukita et al,[5] of 138 blood group A or B neonates who were born to group O mothers and had antibody present on their RBCs determined either by DAT or elution techniques, 7 developed hemolytic disease. This was defined as hyperbilirubinemia (no specific PTB mentioned) within the first 24 hours, in association with hemolysis, defined as anemia and/or reticulocytosis. IgG1 was found in all 7 infants, and weak IgG3 was found in the eluates of 2 of 3 cases with a negative DAT but positive eluate. The case with the most severe hemolysis had both IgG1 and IgG3 present. In addition to the former, IgG2 was present in the eluates of all 7 newborns. Of the remaining 131 infants who did not have hemolytic disease, IgG2 only was found in 43 infants in whom antibody was detected, whereas no IgG subclass could be identified in 59. IgG3 and IgG4 were not found in any infant. IgG1 or IgG1 with IgG2 was detected in 29, but because these infants did not have clinical evidence of hemolysis, the authors postulated that there was insufficient antibody present to cause RBC destruction. IgG1, in all 29 cases, was detected only by elution techniques, not by DAT.[5] These authors concluded that in ABO-incompatible neonates, hemolytic disease did not occur in those with IgG2 subclass, even with a positive DAT. Rather, hemolysis was associated primarily with the IgG1 subclass and depended on the titer of antibody present; however, because IgG2 was present along with IgG1 or IgG3 in those with severe

hemolysis, a possible role of IgG2's contributing to the hemolysis cannot be definitively excluded.

In this study, in which all enrolled neonates were DAT positive, the degree of hemolysis was universally higher than for non–DAT-positive neonates from the same nursery as our patient sample, sampled at or approximately the same postnatal time, and processed in the same Stanford University laboratory; however, COHbc values were not significantly different between the IgG1 and presumed IgG2/4 subgroups. Similarly, the incidence of hyperbilirubinemia, PTB concentrations and the need for phototherapy were similar between the 2 IgG subgroupings. Within the bounds of hemolytic disease encountered, there was no measurable effect of IgG subclass on the development of hemolysis or hyperbilirubinemia.

This study contrasts to that of Ukita et al[5] in both its design and its results. Rather than defining hemolysis as the presence of severe clinical disease manifest by early hyperbilirubinemia in association with anemia and reticulocytosis, we evaluated the effect of IgG subclasses on hemolysis by using blood COHbc as determined by a sensitive gas chromatographic assay.[14,15] This is an accurate and reliable index of hemolysis in newborns. Hematologic indices of hemolysis that are used for older children or adults may frequently demonstrate overlap between hemolytic and nonhemolytic states in newborns.[22] Furthermore, we assessed the effect of IgG subclass on the development of neonatal jaundice by plotting the PTB concentrations on the hour of life specific bilirubin nomogram, thereby taking into account the changes that take place in neonatal PTB concentrations in the first days of life. We used a modern definition of hyperbilirubinemia, any PTB ≥95th percentile for hour of life, in line with other studies.[23] Only blood group A or B newborns who were born to group O mothers and had a positive DAT in cord blood were eligible for inclusion. In these respects, we believe our study to be more precise and comprehensive than that of Ukita et al.[5]

A limitation of the study is that the gel technique that was used for IgG subclass determination did not actually measure IgG2/4; rather, these were assumed in cases in which neither IgG1 nor IgG3 was detected. Also, the technique did not allow for identification of infants with a combination of IgG1 and either IgG2 or IgG4. Another drawback is that the test did not allow for quantification of the IgG subclasses; however, this test is suitable for rapid identification of IgG1 and IgG3 and can be performed with relative ease in a clinical laboratory and was therefore chosen for evaluation. Because no neonate in this study had severe hyperbilirubinemia that required exchange transfusion, the role of IgG1 in such a situation can not be extrapolated from our results.

CONCLUSIONS

Within the range of hemolysis and clinical disease encountered in the ABO blood group–heterospecific infants who were included in our study, the presence of IgG subclass 1, identified by the gel technique used, was not predictive of hemolysis or hyperbilirubinemia. Other factors will have to be sought for the prediction of hyperbilirubinemia in these neonates.

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