Direct Antiglobulin Titer Strength and Hyperbilirubinemia

WHAT’S KNOWN ON THIS SUBJECT: Direct antiglobulin titer (DAT) positive, blood group A or B newborns born to group O mothers have a high incidence of hyperbilirubinemia, attributable to increased hemolysis.

WHAT THIS STUDY ADDS: DAT ++ readings were associated with a higher incidence of hyperbilirubinemia and a greater degree of hemolysis than DAT ± or DAT + counterparts. DAT strength should be taken into consideration when planning treatment strategies or follow-up of ABO-heterospecific newborns.

abstract

BACKGROUND AND OBJECTIVES: We recently demonstrated that direct antiglobulin titer (DAT) positive, blood group A or B newborns born to group O mothers had a high incidence of hyperbilirubinemia, attributable to increased hemolysis. We reanalyzed our data asking whether increasing DAT strength plays a modulating role in the pathophysiology of the hemolysis and hyperbilirubinemia.

METHODS: Data from previously published DAT-positive, ABO-heterospecific neonates were analyzed for hyperbilirubinemia and hemolysis according to strength of DAT. DAT was measured by using a gel agglutination technique and reported as values ranging from DAT + to DAT ++++. Hemolysis was evaluated by blood carboxyhemoglobin corrected for inspired, ambient CO (COHbc), and expressed as percent total hemoglobin (tHb). Hyperbilirubinemia was defined as any plasma total bilirubin value $>$95th percentile on the hour-specific nomogram.

RESULTS: Hyperbilirubinemia was more prevalent in those with DAT ++ readings (16 of 20, 80%) than those both DAT ± (37 of 87 [42.5%], relative risk: 1.88, 95% confidence interval: 1.35–2.61) and DAT + (32 of 56 [57.1%], relative risk: 1.40, 95% confidence interval: 1.02–1.92). COHbc values were higher for those with DAT ++ (1.45 ± 0.49% tHb [mean ± SD]) than those DAT ± (1.20 ± 0.37% tHb, $P = .01$) or DAT + (1.22 ± 0.37% tHb, $P = .02$).

CONCLUSIONS: DAT ++ readings were associated with a higher incidence of hyperbilirubinemia and higher COHbc values than DAT ± or DAT + counterparts. Increasing DAT strength may be a modulator of hemolysis and hyperbilirubinemia in ABO-heterospecific neonates. DAT strength, and not merely DAT presence or absence, should be taken into consideration in the management of ABO-heterospecific newborns. Pediatrics 2014;134:e1340–e1344

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KEY WORDS: ABO heterospecificity, direct antiglobulin titer, carboxyhemoglobin, hemolysis, hyperbilirubinemia

ABBREVIATIONS

AAP—American Academy of Pediatrics
ANOVA—analysis of variance
CI—confidence interval
COHb—blood carboxyhemoglobin
COHbc—carboxyhemoglobin corrected for inspired, ambient CO
DAT—direct antiglobulin titer
RR—relative risk
TB—total bilirubin
tHb—total hemoglobin

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Isoimmune hemolytic disease (blood group incompatibility with a positive direct antiglobulin titer [DAT], also known as the Coombs’ test) is listed by the American Academy of Pediatrics (AAP) in its 2004 Clinical Practice Guideline for the management of hyperbilirubinemia as a major risk factor for the development of severe hyperbilirubinemia. In a clarification to this guideline, DAT positivity is also implicated as a bilirubin neurotoxicity risk factor. However, a positive DAT is often regarded as only weakly predictive of hyperbilirubinemia. We recently determined that DAT-positive, ABO-heterospecific neonates (mother blood group O, newborn group A or B) are, in fact, at high risk for neonatal hyperbilirubinemia. Using blood carboxyhemoglobin determinations corrected for ambient, inspired CO (COHbcr) to index the rate of heme catabolism, we verified that the pathogenesis of the associated hyperbilirubinemia is increased hemolysis, increasing COHbcr values being directly correlated with the incidence of hyperbilirubinemia. In the absence of a specific point-of-care clinical bedside test for the determination of hemolysis, a positive DAT is often used in lieu of an explicit indicator of hemolysis. However, not all infants with a positive DAT are necessarily hemolyzing. For example, using corrected end tidal CO determinations >95th percentile as an indicator of hemolysis, Herschel et al determined presence of hemolysis in only 59% of DAT-positive cases. DAT, therefore, had a poor sensitivity for predicting hemolysis. The AAP guideline and subsequent clarification relates to the presence of a positive DAT, but not to its strength, as a risk factor for hyperbilirubinemia. In the current study, we performed a reanalysis of our previously published data to determine whether increasing DAT strength was associated with a greater degree of hemolysis and higher incidence of hyperbilirubinemia in ABO-heterospecific neonates than in those with weaker DAT strength.

METHODS
The clinical and laboratory aspects of this study were detailed in our original publication and will therefore be summarized here in brief only. The study was approved by the Institutional Review Board of the Shaare Zedek Medical Center, which, because of the routine nature of the clinical data collected and the minimally invasive blood collecting procedure performed at the time of routine metabolic screening, required oral parental consent only. Consecutive, healthy, DAT-positive, blood group A or B newborns born to blood group O mothers were enrolled. Newborns, who were ill in any way, had major congenital malformations, or had any condition with the potential of increasing hyperbilirubinemia, such as glucose-6-phosphate dehydrogenase deficiency or a positive DAT due to causes other than ABO heterospecificity, were excluded. One mandatory total plasma bilirubin (TB) test was performed at the time of routine metabolic screening with additional TB determinations as deemed necessary based on clinical judgment and visual assessment (routine transcutaneous bilirubinometry had not yet been introduced to our nurseries at the time of the study). All TB results were plotted on the hour-specific bilirubin nomogram. Phototherapy was administered according to the 2004 AAP guideline by using the graph for newborns with risk factors. Follow-up of neonates in whom jaundice had not yet resolved by the time of discharge was performed in our nursery by our medical staff and by using the identical laboratory facilities as during hospitalization. Blood sampling for COHb determination for the purpose of the study was performed at the time of routine metabolic screening. Within a few minutes of the COHb sampling, a sample of air for CO analysis from the nursery in which the infant was being cared for was collected into containers designed for gas sample collection, which had been supplied by Stanford University.

Laboratory Methods
DAT and Blood Type
DAT testing was performed routinely on umbilical cord blood with an agglutination technique and reported on a scale of 0 to ++++ (DiaMed-ID Micro Typing System, ID-Card “LISS/Coombs”; DiaMed AG, Cressier sur Morat, Switzerland). The aim of gel technology is to standardize red blood cell agglutination reactions by trapping the agglutinates in gel, thereby permitting simple and reliable reading. Red blood cells and serum are dispensed into microtubes containing a dextran gel and then centrifuged. The gel acts as a sieve, unagglutinated red blood cells forming a pellet at the bottom of the microtube, while agglutinated red blood cells are trapped in the gel. Reactions are easily visible and may be graded, those with intermediate agglutination reactions being scattered within the gel, and those with strong reactions being trapped at the top of the column.

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

Plasma TB
Routine TB testing was measured on heparinized, centrifuged, capillary tube samples by absorbance of bilirubin at 455 nm (NEO BIL Model A2; Digital and Analog Systems, Rome, Italy).

COHb
Blood for COHb determination (150 μL) was collected in custom-prepared capillary tubes containing heparin and saponin, supplied by Stanford University as described. COHb was determined at Stanford University by using a gas chromatographic method, and
expressed as a percentage of total hemoglobin (THb) as described.\textsuperscript{9} THb was measured from the same blood sample with a cyanmethemoglobin method, also at Stanford University, as described.\textsuperscript{9} 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, and measured COHbc values were corrected for inspired CO to derive COHbc, as described.\textsuperscript{9}

**Data Analysis and Definitions**

Hyperbilirubinemia was defined as any TB value >95th percentile on the hour-specific bilirubin nomogram.\textsuperscript{7} COHbc, TB, and the number of infants with any TB value >95th percentile were compared between those with varying DAT strengths. Categorical variables were compared with \( \chi^2 \) analysis. Continuous variables, presented as mean ± SD, with a normal distribution were compared by using analysis of variance (ANOVA). When significant, this was followed by all pairwise multiple comparison procedures (Holm-Sidak method) to isolate the group or groups that differed from the other. For those analyses in which the normality test failed, ANOVA was replaced by Kruskal-Wallis 1-way ANOVA on ranks followed, when appropriate, by all pairwise multiple comparison procedures (Dunn’s method). The incidence of hyperbilirubinemia and the need for phototherapy between the varying DAT strength subgroups were compared by calculating the relative risk (RR) and 95% confidence interval (CI), in which case significance was defined as a 95% CI that did not include the digit 1. For other comparisons, significance was defined as \( P < .05 \).

**RESULTS**

One hundred sixty-four eligible newborns were enrolled between January 2006 and April 2007. Demographic data were supplied in the original communication and will be summarized here in brief: mean (± SD) birth weight was 3401 ± 425 g, gestational age 39 ± 1 weeks, and 44% were boys. Ten percent were delivered by cesarean delivery, and 88% were either exclusively or partially breastfed. Overall, a TB value >95th percentile at any point was noted in 85 (51.8%). Age at first TB >95th percentile was 19 ± 11 hours, whereas early hyperbilirubinemia (TB >95th percentile during the first 24 hours) was noted in 56 (34.1% of the cohort).\textsuperscript{9} Further analysis in this communication will relate only to comparisons between infants with varying DAT strengths.

Subdivision into groups based on DAT strength was available for 163 neonates. No newborns were encountered with DAT results greater than ++. The distribution of the infants with varying DAT strengths and the subdivision within groups of those with blood groups A or B are shown in Table 1. The proportions of infants with blood groups A and B were not significantly different within each group based on DAT strength.

Data relating to hyperbilirubinemia and the need for phototherapy are displayed in Table 2. The number of newborns with hyperbilirubinemia was significantly higher in the DAT ++ group than in the DAT ± or DAT + groups, and the age at which the first TB value >95th percentile was noted was significantly earlier in the DAT ++ group compared with those DAT ±. Similarly, the need for phototherapy was higher in those DAT ++ than those DAT ± with a trend to being higher than those DAT -. The age at which phototherapy was started in the DAT ++ group was not significantly earlier than in the ± or + counterparts. COHbc was sampled at 55 ± 12 hours (range 36–120 hours), and the results are displayed graphically in Fig 1. As seen, COHbc values for the DAT ++ group were significantly higher than those of both the DAT ± and DAT + groups.

It is of note that even within the ++ group not all the newborns developed hyperbilirubinemia or met the requirements for phototherapy. No significant clinical differences for those who did not develop hyperbilirubinemia compared with those who did could be found. It is of interest, however, that the COHbc value of the former was somewhat lower than the latter (1.18 ± 0.41 vs 1.54 ± 0.50% THb) although this difference did not meet statistical significance, possibly

**TABLE 1** Subdivision of the Newborns Into Groups Based on DAT Strength, and Distribution of Newborns With Blood Groups A and B, Respectively, With the Various DAT Strength Groups

<table>
<thead>
<tr>
<th>Strength of DAT</th>
<th>DAT ±, n (%)</th>
<th>DAT +, n (%)</th>
<th>DAT ++, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total (n = 163)</td>
<td>87 (53.3)</td>
<td>56 (34.4)</td>
<td>20 (12.3)</td>
</tr>
<tr>
<td>Blood group A (n = 110)</td>
<td>56 (64.4)</td>
<td>41 (37.3)</td>
<td>13 (11.8)</td>
</tr>
<tr>
<td>Blood group B (n = 53)</td>
<td>31 (65.9)</td>
<td>15 (28.7)</td>
<td>7 (13.5)</td>
</tr>
</tbody>
</table>

Note that the A and B blood group distribution was similar within the varying DAT strength groups.

**TABLE 2** Data Relating to Hyperbilirubinemia and Phototherapy, Stratified According to DAT Strength

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DAT ±, n = 87</th>
<th>DAT +, n = 56</th>
<th>DAT ++, n = 20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any TB &gt;95th percentile, n (%)</td>
<td>37 (42.5)</td>
<td>32 (57.1)</td>
<td>16 (80)%</td>
</tr>
<tr>
<td>Age at first TB &gt;95th percentile, mean ± SD, h</td>
<td>22 ± 14</td>
<td>18 ± 11</td>
<td>15 ± 11%^</td>
</tr>
<tr>
<td>Phototherapy, n (%)</td>
<td>35 (40)</td>
<td>31 (55)</td>
<td>15 (75)\textsuperscript{+}</td>
</tr>
<tr>
<td>TB at initiation of phototherapy, mean ± SD, mg/dL</td>
<td>10.9 ± 2.5</td>
<td>10.7 ± 3.0</td>
<td>9.4 ± 5.3</td>
</tr>
<tr>
<td>Age at initiation of phototherapy, mean ± SD, h</td>
<td>22 ± 15</td>
<td>21 ± 12</td>
<td>14 ± 12</td>
</tr>
</tbody>
</table>

TB at initiation of phototherapy: ANOVA testing, \( P = .26 \); Age at initiation of phototherapy: ANOVA, \( P = .13 \).

\* DAT ++ versus DAT ±: RR: 1.88, 95% CI: 1.35–2.61; DAT ++ versus DAT +: RR: 1.40, 95% CI: 1.02–1.89.
\textsuperscript{a} Kruskal-Wallis 1-way ANOVA on ranks, \( P < .05 \). Multiple comparison revealed DAT ++ versus DAT ± significant (\( P < .05 \)); DAT ++ versus DAT + and DAT ++ versus DAT ± were not significant.
\textsuperscript{b} DAT ++ versus DAT ±: \( P = .01 \); DAT + versus DAT ±: \( P = .08 \).
Despite globally increased COHbc values attributable to increased hemolysis, a high incidence of hyperbilirubinemia, and phototherapy in DAT-positive, ABO-incompatible newborns, strength of DAT ++ may therefore be an important risk factor for the development of hyperbilirubinemia and should be taken into account when planning the duration of birth hospitalization, phototherapy, as well as postdischarge follow-up.

In the current AAP guidelines and subsequent clarification do not consider the strength of the DAT when making recommendations for the treatment of hyperbilirubinemia. In the current reanalysis, newborns with a DAT ++ strength had an increased risk of hyperbilirubinemia and increased need for phototherapy, compared with those with positive, but weaker DAT strength. These increased risks were higher than the overall, already high risk for hyperbilirubinemia and phototherapy in DAT-positive, ABO-incompatible newborns. Strength of DAT > + may therefore be an important risk factor for the increased incidence of hyperbilirubinemia. However, even in the DAT ++ newborns, despite the increased hemolysis, not all developed hyperbilirubinemia. The reason we suggest is variation in the conjugative and excretory ability of bilirubin from individual to individual. The TB at any point in time can be represented as bilirubin production minus bilirubin elimination. Thus, increased bilirubin production, but a mature and efficient conjugating and excretory ability, may not necessarily lead to hyperbilirubinemia. On the other hand, only moderately increased heme catabolism, together with an immature bilirubin conjugation or excretion, or coexpression with UDP-glucuronosyltransferase 1A1 (TA), promoter polymorphism associated with Gilbert syndrome, may be sufficient to upset the bilirubin production-elimination equilibrium and result in hyperbilirubinemia.

In parallel with our previous COHbc data in this patient database, the increased risk of hyperbilirubinemia in the DAT ++ group is attributable to increased hemolysis. Increased hemolysis is not only a risk factor for developing hyperbilirubinemia, but may be associated with increased risk of bilirubin neurotoxicity at given levels of TB. For these reasons, the AAP guideline recommends instituting phototherapy and performing exchange transfusion at lower levels of TB in newborns with isoimmune hemolytic disease compared with newborns without any obvious hemolytic etiology.

The current AAP guideline and subsequent clarification do not consider the strength of the DAT when making recommendations for the treatment of hyperbilirubinemia. The results of the current reanalysis suggest that increasing DAT strength may be an important risk factor in the pathogenesis of neonatal hyperbilirubinemia. Increased hemolysis may exacerbate the potential for bilirubin neurotoxicity in those with high concentrations of TB. We encourage hospital blood banks to report DAT results according to strength of the reaction. Our results suggest that newborns with a DAT strength of ++ (or more) should be regarded as at especially high risk and this factor taken into account when managing a newborn with hemolytic disease due to ABO heterospecificity.

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