ABSTRACT

BACKGROUND: Hemolytic disease of the fetus and newborn (HDFN) is a type of anemia in the fetus or newborn, characterized by anemia, jaundice, hyperbilirubinemia, and brain damage. IgG is the only antibody that can cross the placenta. The IgG subtypes have a different ability to destroy red blood cells (RBCs). IgG1 and IgG3 can bind to Fc-phagocyte cell receptors and cause hemolysis, while IgG3 has more ability than IgG1. This study aimed to identify the antibody IgG subtype contributing to clinical manifestation differences in HDFN.

METHODS: This study used blood and umbilical cord blood samples from 30 pairs of mother-baby. The samples were grouped into control (not jaundice/normal bilirubin levels) and jaundice/hyperbilirubinemia groups. A self-developed IgG subtype enzyme-linked immunosorbent assay protocol was performed on maternal samples, resulting in optical density. Statistical analysis was performed using SPSS software version 23.

RESULTS: Blood type was associated with total bilirubin expression ($p = 0.005$). IgG1 anti-A, IgG3 anti-A, IgG4 anti-A, IgG1 anti-B, IgG3 anti-B, and IgG4 anti-B significantly affected hyperbilirubinemia in newborns ($p = 0.041$, 0.013, 0.017, 0.028, 0.001, and 0.007, respectively).

CONCLUSIONS: IgG1 and IgG3 were more significant in causing clinical problems. IgG4 suppressed IgG activation, resulting in no destruction of the infant’s RBCs.

KEYWORDS: ABO incompatibility, hemolytic disease of newborn, immunoglobulin G
occur in the first pregnancy. However, premature babies with ABO incompatibility experience more severe symptoms than full-term babies. Previous studies have shown increased abortion numbers due to ABO incompatibility. Bandyopadhyay et al. conducted a study on 124 spontaneous abortions that occurred during the first 16 weeks of gestation and found that ABO incompatibility between the father and mother was likely to be a risk factor for early spontaneous abortions and heterozygote selection of ABO blood group genotypes. Soni and Mukherjee conducted a study in India involving 104 couples with ABO incompatibility that found 21 abortions occurred and 18 newborn babies from the total pregnancy. Infants born to mothers who were incompatible with the ABO blood group of the fetus but did not experience abortion may have hyperbilirubinemia. Moreover, babies born with ABO incompatibility tended to experience hemolysis and hyperbilirubinemia. Bhat and Kumar found that of 878 births, 151 babies were born with ABO incompatibility, of whom 46 had hyperbilirubinemia and 25 had hemolysis. Yahya et al. conducted a study at the Dr. Hasan Sadikin Hospital, Bandung, involving 95 newborns, and hyperbilirubinemia occurred in 11.6% of the infants. Differences in the clinical manifestations of HDFN were due to several factors, including differences in antibody titers and IgG subtypes.

IgG is the only antibody that crosses the placenta. This class of antibodies is divided into four subtypes: IgG1, IgG2, IgG3, and IgG4 with different abilities to destroy RBCs. IgG1 and IgG3 can bind to the Fc-phagocyte cell receptors and cause hemolysis, but IgG3 has more ability than IgG1. Various studies have been conducted on IgG subtypes in various countries; however, it is not yet known which IgG subtypes affect HDFN in Indonesia. This study aimed to determine which IgG subtype plays a role in HDFN and can cause clinical symptoms.

METHODS

Subjects
This study included 30 pairs of mothers and newborns with different blood types from the Division of Perinatology, Department of Obstetrics and Gynecology, Cipto Mangunkusumo Hospital and Budi Kemuliaan Hospital in Jakarta. The gestational age ranged from 36 to 40 weeks. The cut-off bilirubin level in the baby was 5–13 mg/dl (0–48 hours). This study was approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia (No: KET-311/UN2.F1/ETIK/PPM.00.02/2020).

Reagents and equipment
Blood type A bovine serum albumin (BSA) and blood type B BSA were purchased from Dextra Laboratories, UK. Mouse anti-human IgG1, IgG3, and IgG4 antibodies (MH1715, MH1732, and MH174, respectively) were purchased from Invitrogen (Thermo Fisher Scientific, USA).

Collection of blood samples
Blood (5 ml) and umbilical cord blood (3 ml) was drawn from the mother on delivery day and were mixed with anticoagulant ethylenediaminetetraacetic acid. Blood samples were divided into two equal portions for blood antibody and blood group test.

Detection of maternal IgG antibody subtypes
The IgG antibody subtypes were detected using a self-developed enzyme-linked immunosorbent assay protocol. The IgG subtypes detected were IgG1, IgG3, and IgG4. IgG2 was omitted because it is predominantly responsible for anti-carbohydrate IgG responses against bacterial capsular polysaccharides.

We added 50 μl of blood type A and B to wells A and B, respectively, containing 0.01% BSA solution in 0.1 M bicarbonate buffer (pH 9.6). The plates were covered and incubated for 24 hours at 4°C. After incubation, each well was aspirated and washed four times with 200 μl of phosphate-buffered saline (PBS), followed by removal of any remaining PBS by aspirating or decanting. The plate was then inverted and blotted against clean paper towels. Then, 200 μl of 0.5% Tween 20 and 2% BSA was added to each well, covered, and incubated for 2 hours at 37°C. The aspiration process was repeated once, followed by the addition of 100 μl serum (dilution 1:1 in PBS) to the samples in well A and B; IgG1, IgG3, and IgG4 (each sample had six replicas), 100 μl aquadest as a blank (six replicas), and 100 μl serum type AB to negative control well (six replicas). The plate was covered and incubated for 3 hours at 37°C, followed by aspirating and washing five times. Then, 50 μl of mouse anti-human IgG1, IgG3, and IgG4 (horseradish peroxidase-conjugated) was added to the wells, covered, and incubated for 2 hours at 37°C. After incubation, each
well was aspirated and washed seven times with 200 μl of PBS, followed by removal of any remaining PBS. Finally, 100 μl of tetrathymethylbenzidine (TMB) peroxidase and peroxidase substrate solution (1:1) was added to each well and incubated for 15 min at room temperature. After incubation, 50 μl of TMB stop solution was added to each well, and the plate was gently tapped to ensure thorough mixing. The optical density (OD) was measured at 450 nm within 15 min using a microtiter plate reader.

Data analysis

Data on subject characteristics and subtype IgG1, IgG3, and IgG4 anti-A and anti-B were compared between subjects with hyperbilirubinemia and normal bilirubin level (control) using the independent \( t \)-test or Mann–Whitney \( U \) test, as appropriate. SPSS software version 23 (IBM Corp., USA) for Windows 10 was used for the statistical analysis. A \( p \)-value of 0.05 was considered significant.

RESULTS

Baseline characteristics of the subject

This current study examined blood types A, B, O, and AB in fathers and newborns. The mothers solely with blood types A, B, and O were included in the study (Table 1).

The total bilirubin level is shown in Figure 1. Newborns with blood types A and B had higher total bilirubin levels than blood type O \( (p = 0.05) \).

Subtype IgG antibodies

Owing to the varying bilirubin levels across different blood types, we investigated the potential roles of IgG1, IgG3, and IgG4. Approximately 30–50% of mothers had elevated OD of IgG1 and IgG3, leading to the induced total bilirubin levels in newborns. Low OD values of both IgG1 anti-A and IgG3 anti-A resulted in significantly lower bilirubin levels than those with low OD levels of IgG1 anti-A and high OD levels of IgG3 anti-A \( (p = 0.041) \) or high OD levels of IgG1 anti-A and low OD levels of IgG3 anti-A \( (p = 0.013) \). Additionally, a significantly lower rate of total bilirubin was observed when the OD of anti-A IgG1 was low \( (p = 0.017) \). A similar trend was observed for anti-B antibodies. However, total bilirubin levels were significantly lower both in the low-OD IgG1 anti-B and IgG3 anti-B groups than in the low-OD IgG1 anti-B and high IgG3 anti-B \( (p = 0.028) \) or high OD levels of IgG1 anti-B and low anti-B IgG3 \( (p = 0.001) \) groups. Newborns with low bilirubin levels were predominant in region 1, whereas those with high bilirubin levels were predominant in region 4 (Figure 2). When the OD of IgG1 anti-B and IgG4 anti-B were low, total bilirubin levels were significantly lower than those in samples with high OD of IgG1 anti-B and low IgG4 anti-B \( (p = 0.007) \). Based on the roles of IgG1, IgG3, and IgG4 in total bilirubin levels, we ranked the subjects using scores of 1, 2, 3, and 4.

DISCUSSION

We found that newborns with blood types A, B, and AB had high bilirubin levels due to blood type incompatibility, where maternal antibodies

Table 1. Blood types of the participants

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Figure 1. Correlation of newborn’s blood type with total bilirubin expression. *\( p < 0.05 \)
attacked fetal RBCs. Fetal antigens entering maternal circulation would trigger the production of IgM antibodies, followed by IgG antibodies response. IgG-coated fetal RBCs are destroyed by fetal spleen macrophages, leading to hemolysis. This process releases hemoglobin, which is indirectly metabolized into bilirubin, causing jaundice in newborns. ABO HDFN may result in early hyperbilirubinemia with a rapid increase in serum bilirubin levels. Maternal IgG antibodies persist after birth, and antibody binding and hemolysis persist from days to weeks because IgG has a half-life of 25 days and is distributed in both the extravascular and intravascular compartments. ABO HDFN causes early hyperbilirubinemia and may present with a rapid increase in serum bilirubin levels.12-15

The mother produces anti-A and anti-B IgG due to the presence of fetal erythrocytes transplacentally pass using neonatal Fc receptors (FcRn). FcRn transports immunoglobulins from syncytiotrophoblasts, protecting IgG from normal serum protein catabolism. In the first trimester, the transfer was minimal and significantly increased in the second and third trimesters. The average fetal bilirubin concentration at 24 weeks of gestation was 1.8 g/dl. At term, the IgG antibody levels were higher in the fetus than in the mother. Although all four IgG subtypes can cross the placenta, IgG1 and IgG3 are more efficient in the hemolysis of RBCs than IgG2 and IgG4.14,15

In the present study, IgG1 and IgG3 amounts significantly correlated with bilirubin levels. When IgG1 or IgG3 levels increase, the infants may develop hyperbilirubinemia. However, if both IgG1 and IgG3 increased, the developed hyperbilirubinemia can progress into jaundice. The results are similar to a study in India that found mothers’ titers of more than 1:64 and concentration of IgG1 and IgG3 subtypes, particularly IgG1, significantly affected ABO HDFN.16 Moreover, Kaplan et al17 in California, USA, found that determining the IgG subtypes in cord blood could not predict hemolysis in ABO-incompatible infants.

The IgG1 subtype is the most abundant subtype in serum and can effectively bind to complement component 1q, causing complement-dependent

Figure 2. Scatter plot of OD IgG1 anti-A against IgG3 anti-A (a), OD IgG1 anti-B against IgG3 anti-B (b), OD IgG1 anti-A against IgG4 anti-A (c), and OD IgG1 anti-B against IgG4 anti-B (d) for subjects with normal bilirubin level (green dots) and hyperbilirubinemia (red dots). OD=optical density.
cytotoxicity. Additionally, it can bind to different Fc receptors, resulting in antibody-dependent cell-mediated cytotoxicity. However, although IgG3 is present in small amounts, it has a very robust effector mechanism. IgG subtype concentration is highly dependent on the age of the fetus and on active transport across the placenta. The concentration of IgG1 increases earlier than IgG3 at gestational age, and there is an increase in the ratio of IgG1/IgG3, and IgG4 in the third trimester. The concentration of IgG2 in umbilical cord blood is deficient because Fc receptors for syncytiotrophoblasts in placental tissue have a weaker affinity to IgG2 than IgG1.

We observed a significant difference in the increase in bilirubin levels between the OD IgG1 and IgG3 groups. The group high in IgG1 anti-A and IgG3 anti-A had a similar increase in bilirubin levels as in patients with relatively low IgG1 anti-B and IgG3 anti-B. This result aligns with Haque and Rahman, who found a more severe HDFN in patients with anti-B antibodies than in patients with anti-A antibodies.

The current study has several limitations. It was conducted over one year during the coronavirus disease 2019 pandemic, resulting in a limited number of participants. Additionally, the exclusion of mothers and babies with the same blood type and the limited number of mothers and babies with different blood types contributed to the small number of study samples.

In conclusion, the infant’s blood type is a risk factor for HDFN. Newborns with blood types A and B are at higher risk of developing HDFN. IgG1 and IgG3 play a significant role in developing clinical problems with less severe symptoms. In cases where mothers had infants without clinical problems, IgG4 was identified as the subtype contributing to this effect. So, besides blood group analysis, we suggest adding an assay for obstetrics and gynecology patients, which is subtype IgG antibody anti-A and anti-B analysis.

Conflict of Interest
The authors affirm no conflict of interest in this study.

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