Diagnostic procedures for assessing the severity of alloimmune fetal anemia

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On the origin of human amniotic fluid bilirubin

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Abstract

We studied the relationship between bilirubin concentrations in amniotic fluid and fetal blood in 68 non-hydropic rhesus D-alloimmunized anemic fetuses at first blood sampling. In these alloimmunized fetuses, the amniotic fluid / fetal blood ratio for bilirubin decreased from 0.09 at 28 weeks to 0.05 at 33 weeks. In normal fetuses, amniotic fluid / fetal blood ratios for bilirubin, and for albumin, are in the same range and show a similar decrease during gestation. We conclude that amniotic fluid bilirubin concentration is determined, firstly, by fetal blood bilirubin concentration and, secondly, by the amniotic fluid / fetal blood ratio of albumin. Among five possible pathways bilirubin could take to build up a concentration in amniotic fluid (fetal kidneys, lungs, skin, bowel, membranes), the intramembranous pathway is the only one that is compatible with the amniotic fluid / fetal blood ratios for bilirubin that we found and must therefore be the most important.

Introduction

Bilirubin is formed during the degradation of haem-containing compounds, mainly hemoglobin (Rosenthal, 1992). Bilirubin concentration is about four times higher in fetal than in maternal blood (Girling, Dow and Smith, 1997; Nava *et al.*, 1996) As a result of this concentration gradient, the unconjugated (liposoluble) bilirubin diffuses through trophoblastic layers from fetal to maternal blood (Odell, 1959). It is unclear whether active or passive carrier-mediated transport mechanisms play an additional role in placental transfer (Serrano *et al.*, 2002). Glucuronyl transferase activity in the fetal liver is minimal, less than 1% of its activity in neonatal and later life, and only a minor fraction of fetal bilirubin is conjugated (Kawade and Onishi, 1981; Nava *et al.*, 1996). In the fetal situation, this low glucuronyl transferase activity is probably beneficial because the clearance of conjugated (hydrophilic) bilirubin through the placental barrier is very slow (Bashore, Smith and Schenker, 1969). Unconjugated (hydrophobic) bilirubin in fetal and maternal blood is linked to albumin almost completely, and only a minute fraction is free (Brodersen, 1980).

Some of the fetal bilirubin is excreted into the amniotic fluid compartment, and less than 10% of this amniotic fluid bilirubin is conjugated (Halitsky and Krumholz, 1970). Each day, the fetus swallows about 75% of the amniotic fluid volume (Brace, 1999). Amniotic fluid bilirubin concentration is an important diagnostic tool in the management of blood group alloimmunization (Liley, 1961). Little is known, however, about how bilirubin reaches the amniotic fluid. Theoretically, there are five major possible pathways bilirubin can take to leave the fetal circulation and enter the amniotic fluid: via fetal kidneys, lungs, skin, bowel, or via placenta and membranes, which is called the intramembranous pathway. A first possible pathway would be via the kidneys. Fetal urine is, after all, the major constituent of amniotic fluid after 16-weeks´ gestation. A second pathway would be via the lungs. Fetal lung fluid contributes to approximately 10% of amniotic fluid (Brace, 1999). Many clinicians and investigators believe that the fetal lung pathway explains the clinically useful relation between amniotic fluid bilirubin concentration

and the degree of fetal anemia (American College of Obstetricians and Gynecologists, 1996). A third possible pathway, excretion of liposoluble substances through the fetal skin along a concentration gradient, probably occurs early in pregnancy, but is hampered during the second half of human gestation due to increasing keratinisation (Evans and Rutter, 1986; Parkin, Lind and Cheyne, 1969; Parmley and Seeds, 1970). Passage of meconium is a fourth possible pathway for bilirubin to enter the amniotic fluid. Fetuses regularly pass meconium into the amniotic fluid and small lumps of meconium have regularly been seen during fetoscopy (Hakguder *et al.*, 2002). A fifth possible pathway is the intramembranous pathway (Gilbert and Brace, 1989). The fetal surface of the placenta is well vascularized and probably plays an important role in the volume regulation and composition of amniotic fluid (Gilbert, Eby-Wilkens, and Tarantal, 1997). Under normal conditions, diffusion of fluid and solutes between amniotic fluid and fetal blood along this pathway is a fairly rapid process, one that has been shown to occur in both directions (Bashore *et al.*, 1969; Gilbert and Brace, 1989).

We wanted to study bilirubin concentrations in human amniotic fluid and fetal blood in cases with highly increased hemoglobin degradation, in order to gain more insight into the enigmatic relation between these concentrations and to possibly draw some conclusions regarding the origin of amniotic fluid bilirubin.

Methods

Leiden University Medical Center is the national referral center for the treatment of fetal anemia in the Netherlands. Our methods for diagnosis and treatment of severe fetal alloimmune anemia have been described previously (Kanhai *et al.*, 1990). We searched our database from January 1988 to October 2000 for contemporaneous amniotic fluid and fetal blood samples that were taken from singleton, Rhesus D-alloimmunized, non-hydropic, and not previously transfused fetuses. Amniotic fluid samples had to have been taken less than four days before fetal blood sampling.

Fetal blood samples were sent to our central laboratory for bilirubin and hematological measurements. Values were automatically entered into our database and checked by a specialized nurse. Amniotic fluid samples (5-10 ml), protected from light during transport, were centrifuged at 1000g for 10 minutes to remove vernix and erythrocytes. The absorption of the supernatant was measured at the wavelengths 365, 450 and 550 nm with an UltrospecPlus spectrophotometer (Amersham Pharmacia Biotech, UK). The bilirubin absorption, expressed as Δ OD 450, was calculated as the difference between the measured absorption at 450 nm and the background absorption at 450 nm, derived from the logarithmic function of the absorptions between 365 and 550 nm (Liley, 1961).

Normal total bilirubin concentrations in fetal blood increase during gestation. We used the reference values proposed by Nava *et al.*, 1996, which were derived from a large number of normal fetuses undergoing percutaneous umbilical blood sampling between 18 and 39 weeks (Nava *et al.*, 1996). Normal bilirubin concentrations in amniotic fluid decrease during gestation. We used the reference values proposed by Nicolaides *et al.*, 1986; these were derived from a large number of amniocenteses in normal pregnancies, equally distributed between 16 and 37 weeks (Nicolaides *et al.*, 1986). A factor of 1.585 was used to convert all Δ OD 450 values to bilirubin concentrations (mg/dl) (Egberts *et al.*, 2002, Egberts *et al.*, 2003). Normal concentrations of albumin in amniotic fluid and fetal blood were based on the literature (Legras *et al.*, 1978; Takagi*et al.*, 1989).

Results

We found 68 contemporaneous amniotic fluid and blood samples from untransfused non-hydropic D-alloimmunized fetuses. Mean gestational age was 29 weeks (range 21-35). Mean fetal hemoglobin concentration was 6.1 g/dl (range 3.1-10.1). Figure 1 shows the individual hemoglobin concentrations of fetuses in our study plotted against their gestational age. Eight fetuses were moderately anemic (hemoglobin concentration 2 to 5 standard deviations below the normal mean) and 60 were severely

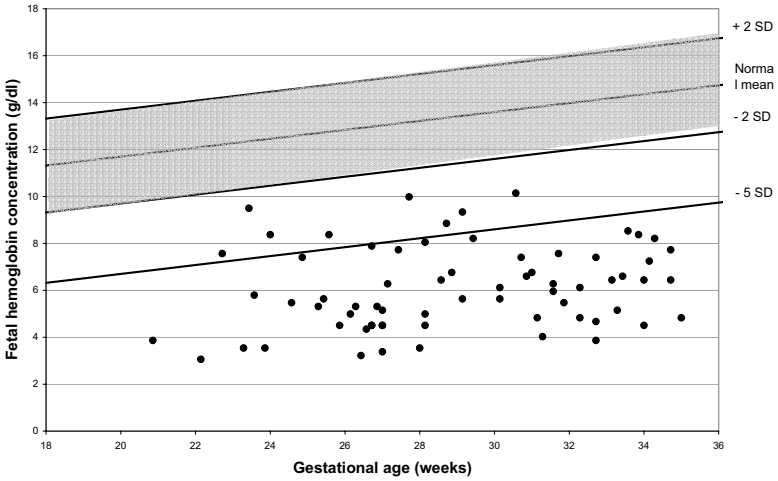

Figure 1 - Hemoglobin values of 68 non-hydropic rhesus D-alloimmunized fetuses at first blood sampling, plotted against their gestational age. The grey zone between the three upper ascending lines marks the limits of normal fetal hemoglobin concentrations (mean ± 2 standard deviations) (Nicolaides *et al.*, 1988). The lower line separates moderate (between –2 and –5 standard deviations) from severe (less than –5 standard deviations) fetal anemia.

anemic (hemoglobin concentration more than 5 standard deviations below the normal mean) at the time of first blood sampling. Mean total bilirubin concentration in fetal blood was 5.8 mg/dl (range 1.9-11.4). In all but three cases, the conjugated bilirubin concentration was less than 10% of the total bilirubin concentration. Figure 2 plots the concentrations of total bilirubin in fetal blood against gestational age. Values were above normal in all but one fetus. Figure 3 shows the amniotic fluid bilirubin concentrations against gestational age. Values were above normal in all but three fetuses. In our study, 50 amniotic fluid bilirubin values were in Liley's zone 3, 13 in the upper third of zone 2 and the remaining 5 in the lower two thirds of zone 2 (Liley, 1961; Sikkel*et al.*, 2002). Figure 4 shows the ratios between bilirubin concentrations in amniotic fluid and in blood of the fetuses in our study, plotted against their gestational age. Roughly, these ratios decreased from around 0.09 at 28 weeks to around 0.05 at 33 weeks. Thus, in our alloimmunized fetuses, these ratios were in the same range as bilirubin and albumin ratios in non-immunized fetuses (Nava *et al.*, 1996; Nicolaides *et al.*, 1986; Legras *et al.*, 1978; Takagi*et al.*, 1989), and showed a similar pattern of decrease as pregnancy progressed.

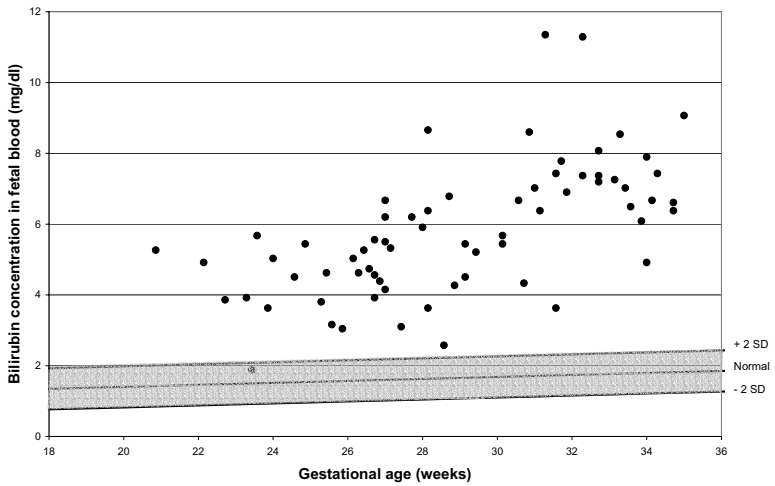

Figure 2 - Total bilirubin values in blood of 68 non-hydropic rhesus D-alloimmunized fetuses at first blood sampling, plotted against their gestational age. The grey zone between the three lines marks the limits of normal (mean ± 2 standard deviations) total bilirubin concentration in fetal blood (Nava *et al.*, 1996).

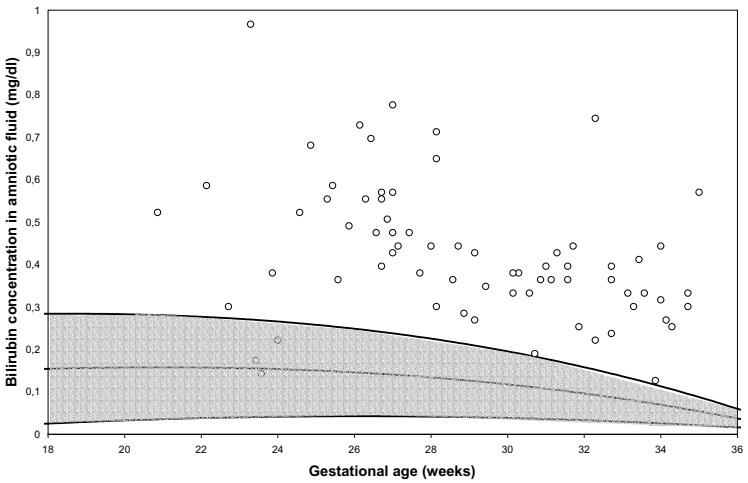

Figure 3 - Amniotic fluid bilirubin values of 68 non-hydropic rhesus D-alloimmunized fetuses at first blood sampling, plotted against their gestational age. The grey zone between the three lines marks the limits of normal (mean ± 2 standard deviations) bilirubin in amniotic fluid (Nicolaides *et al.*, 1986).

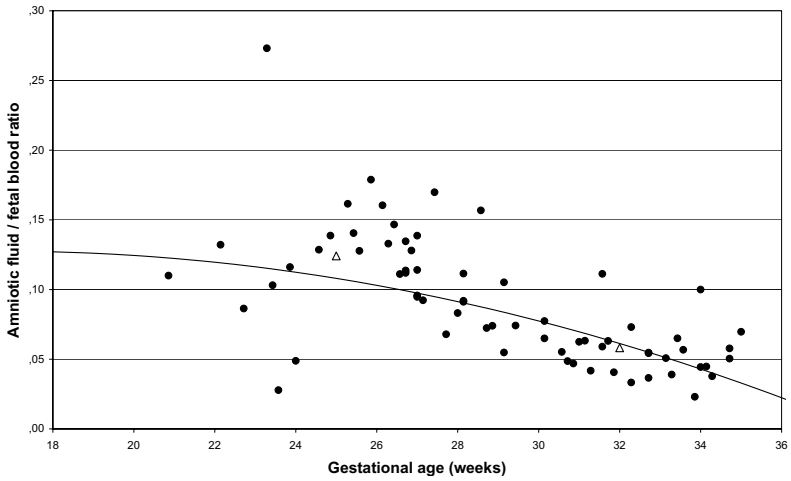

Figure 4 - Ratio between amniotic fluid and fetal blood concentrations of total bilirubin in 68 non-hydropic rhesus D-alloimmunized fetuses at first blood sampling, plotted against their gestational age. The black line marks the ratio between normal bilirubin concentrations in amniotic fluid and fetal blood (Nava *et al.*, 1996; Nicolaides *et al.*, 1986). The grey open triangles mark the ratio between normal albumin concentrations in amniotic fluid and fetal blood (Legras *et al.*, 1978; Takagi*et al.*, 1989).

Discussion

We studied bilirubin concentrations in amniotic fluid and blood in 68 alloimmunized fetuses and found that bilirubin values in blood were on average three times as high as in non-anemic fetuses. All values were, however, well below the threshold associated with a kernicterus risk (Poland, 2002). Amniotic fluid bilirubin values were also elevated, and most values were in Liley's zone 3, which warrants immediate treatment. We then calculated ratios of bilirubin in amniotic fluid to that in blood for these anemic fetuses and found these ratios to be very similar to ratios in normal fetuses. These ratios were also very similar to ratios of albumin in amniotic fluid to that in blood in normal fetuses. These ratios decreased with gestational age from around 0.09 at 28 weeks to 0.05 at 33 weeks.

The strength of the present study is that we measured bilirubin in a relatively large number of D-alloimmunized anemic fetuses. None of these fetuses were hydropic and this may be important because hydrops

is associated with an increase in the amniotic fluid / fetal blood ratio of albumin: it has been shown that in hydropic fetuses, the blood concentration of albumin decreases and the amniotic fluid concentration of albumin increases (Nicolaides, Warenski and Rodeck, 1985; Cherry, Rosenfield and Kochwa, 1970). A weakness of our study is that amniotic fluid samples were taken up to three days before fetal blood sampling (we called this contemporaneous) whereas one would prefer completely simultaneous samples. Prehydropic changes in some of our severely anemic fetuses may also have influenced our results. Finally, we did not measure bilirubin in non-anemic fetuses, and therefore we had to use normal mean values of bilirubin in amniotic fluid and in blood found in the literature (Nava *et al.*, 1996; Nicolaides *et al.*, 1986). Still, we think our results suggest rather convincingly that amniotic fluid / fetal blood bilirubin ratios in anemic and non-anemic fetuses are very similar.

Albumin contains one high affinity binding site for bilirubin and one or two secondary sites of lower affinity (Rosenthal, 1992). Unconjugated bilirubin is hydrophobic and in aqueous solutions linked to albumin almost completely (Rosenthal, 1992). Transfer of bilirubin between body compartments, however, is due to diffusion of albumin-free unconjugated bilirubin (Odell, 1959). The bilirubin gradient between compartments is a function of the concentration of albumin-free bilirubin and thus of the ratio between bilirubin and albumin in both compartments (Odell, 1959). As early as 1970, Cherry *et al.* proposed a strong experimental argument for this theory, measuring Δ OD 450 before and 12 hours after the injection of albumin in the amniotic fluid compartment in 3 alloimmunized pregnancies (Cherry, Rosenfield and Kochwa, 1970). They found a highly significant linear relationship between Δ OD 450 and albumin concentration. In 1967, Cherry and Rosenfield had already suggested that bilirubin / protein ratios in amniotic fluid could replace plotting Δ OD 450 in Liley's curve and suggested a bilirubin / protein ratio of 0.55 as the cut-off. In 1974, Bosch *et al*. found that this "Cherry-ratio" led to slightly more accurate predictions than the Liley chart (Bosch, Robinson and Fisher, 1974). Our study suggests the existence of a fixed amniotic fluid / fetal blood ratio for bilirubin. This ratio decreases between 26 and 34 weeks, probably concurrent with the decrease of the

amniotic fluid / fetal blood ratio for albumin. It is still unclear which factors contribute to the albumin concentration in amniotic fluid. In animal experiments, it has been shown that amniotic fluid albumin is, to a large extent, of maternal origin and that clearance occurs through fetal swallowing and digestion, as well as through absorption through fetal membranes (Gitlin *et al.*, 1972; Faber and Anderson, 2002). It seems clear that the origins and pathways of amniotic fluid albumin are distinct from those of bilirubin, but they are, at present, even more puzzling.

We conclude that the bilirubin concentration in amniotic fluid reflects the bilirubin concentration in fetal blood. This finding provides a logical explanation for the longstanding good performance of Liley's method in the diagnosis of severe fetal alloimmune hemolytic anemia. Further, we found that the amniotic fluid / fetal blood ratio for bilirubin mimicked that of albumin. Therefore, we suggest that the ratio between bilirubin and albumin in amniotic fluid equals the ratio between bilirubin and albumin in blood. The existence of a fixed ratio would shed some light on the origin of human amniotic fluid bilirubin: of the five possible pathways bilirubin could take, only one would agree with such a fixed ratio. To our knowledge, urinary or alveolar fluid concentrations of bilirubin have not been measured in the human fetus. It is very improbable, however, that urine or alveolar fluid contribute substantially to the bilirubin concentration in amniotic fluid because the protein concentrations in both fetal urine and alveolar fluid are 100 to 200 times lower than in fetal plasma (Awad *et al.*, 2002; Boston *et al.*, 1968; Muller *et al.*, 1996; Gitlin *et al.*, 1972). The protein concentration in amniotic fluid, on the other hand, is only 10 to 20 times lower than in fetal plasma (Legras *et al.*, 1978; Takagi *et al.*, 1989; Faber and Anderson, 2002; Nicolaides, Warenski and Rodeck, 1985). Because of the very low albumin concentrations in urine and alveolar fluid, these fluids act as a barrier for unconjugated bilirubin leaving the plasma and entering the amniotic fluid compartment. A meconial origin of amniotic fluid bilirubin is inconsistent with a clinically relevant correlation between amniotic fluid and fetal blood bilirubin concentration. The fetal skin probably serves as a major pathway for solute and water exchange between amniotic fluid and fetus in early gestation. Fetal skin keratinisation begins at approximately 17 weeks

and a complete stratum corneum is present by approximately 25 weeks (Hashimoto *et al.*, 1966). At 14 to 18 weeks, the skin has been shown to have similar permeability as chorion laeve and amnion. However, in fetuses of 24 weeks and older, the skin has become quite impermeable (Parmley and Seeds, 1970). The fetal membranes, on the other hand, retain a high permeability until term (Lloyd *et al.*, 1969). Therefore, bilirubin exchange between fetal blood and amniotic fluid most probably occurs through the intramembranous pathway, where both excretion and reabsorption of bilirubin take place throughout gestation.

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