Determination of Platelet Antigens and Glycoproteins in the Human Fetus

By Yves Gruel, Bernadette Boizard, Fernand Daffos, Francois Forestier, Jacques Caen, and Jean Luc Wautier

The autosomal recessive transmission of Glanzmann’s thrombasthenia (GT) and Bernard-Soulier syndrome (BSS), together with requests of families who already had children with these diseases, prompted us to investigate the feasibility of their antenatal diagnosis. The preliminary step leading to the early detection of GT or BSS was to characterize, in the normal human fetus, the platelet antigens and glycoproteins (GPs) and to define their normal amounts on the membrane surface. Blood samples from 32 fetuses between 18 to 26 weeks of gestation were collected by direct puncture of the umbilical vein using an ultrasound-guided needle. Polyclonal antibodies from human origin directed against PL, Lek, and the GPIb/IIa complex (IgGL), or murine monoclonal antibodies specific for GPIb (AN51, 6D1), GPIIa (AP-3), or GPIb/IIa (AP-2) were studied using platelet suspension immuno-fluorescence tests. The binding of each antibody was quantified using a cytofluorograph (Ortho 50H). PL, Lek, and GPIb/IIa antigens were expressed in normal amounts on fetal platelets as early as 16 weeks of intrauterine life. The GPIb/IIa complex quantified by polyclonal or monoclonal antibodies was in the same range in fetuses (IgGL = 427 ± 23 AUF, AP-2 = 459.5 ± 8.5; AP-3 = 536 ± 14) and in adults (IgGL = 420 ± 30; AP-2 = 498 ± 11; AP-3 = 515 ± 13). The platelet binding of antibodies that recognized GPIb was higher in fetuses (AN51 = 491.5 ± 14; 6D1 = 479 ± 15) than in adults (AN51 = 426.5 ± 9; 6D1 = 449 ± 8.7). These results suggest that immunological techniques can be applied as early as 18 weeks of gestation for the antenatal diagnosis of GT and BSS.

Subjects

Blood was obtained from 17 normal volunteers and 12 pregnant women, mothers of the fetuses that were studied. Blood sampling was performed after informed consent in accordance with the provisions of Helsinki.

Fetal blood was obtained in 32 fetuses between 18 and 26 weeks of gestation by direct puncture of the umbilical cord with a needle guided by ultrasound. In all cases, the blood sampling was done for antenatal diagnosis of either congenital toxoplasmosis, fetal rubella, or hemophilia. The blood not used for the diagnosis of the previously noted diseases was kept for platelet studies, and its volume varied between 0.5 and 1 mL. For each fetal sample, the absence of contamination by maternal blood was assessed by a Kleihauer Betke test, and isoelectrofocusing of hemoglobin. Cell size histograms of white and red blood cells estimated by Coulter S plus II were automatically recorded and compared to those from maternal blood. Fetal platelet count and volume were also systematically obtained (Coulter S plus II).

Antibodies

Three polyclonal antibodies from human origin were used: Anti-PL, anti-Lek, and IgGL. The anti-PL serum was obtained from the mother of a child with neonatal alloimmune thrombocytopenia. Its specificity was assessed by the study of platelets from PL-positive and PL-negative subjects and by immunoblot procedure. The anti-Lek serum, which was obtained from a patient with a posttransfusion purpura, permitted a definition of a new platelet-specific alloantigen, Lek, which is carried by membrane GPIIb, as demonstrated by immunoblotting. IgGL was isolated from the serum of a polytransfused patient with GT. IgGL reacted predominantly with GPIIb/IIIa complex, as demonstrated by immunoprecipitation. Platelet GPs were characterized using four monoclonal antibodies: AP-2 (supplied by T. Kunicki) is a murine monoclonal antibody that reacts specifically with the glycoprotein GPIIb/IIIa complex, as demonstrated by immunoprecipitation. AP-2 Platelet GPs were characterized using four monoclonal antibodies: AP-2 (supplied by T. Kunicki) is a murine monoclonal antibody that reacts specifically with the complex formed by human platelet membrane GPIIb and GPIIIa but not with the individual GPs. AP-3 (supplied by P. Newman), as recently demonstrated by indirect immunoprecipitation, reacts solely with GPIIIa. AN51 (supplied by G. Tobelem) is a monoclonal antibody directed against the glycoprotein region of GPIb; 6D1 (supplied by B. Coller) is also directed against the glycoprotein part of GPIb, but its specificity is probably different since it inhibits the binding of von Willebrand factor to platelets by 100% whereas inhibition obtained with AN51 is only 40% to 50%.

Because the volume of fetal blood available for our study was small (often ~500 μL), only 10 to 13 paired samples fetus/adult could be tested with each antibody.
Indirect Platelet Immunofluorescence Tests

Platelet immunofluorescence tests were done according to a technique previously described. In brief, platelets were prepared by differential centrifugation, washed three times in EDTA phosphate buffer, pH 7.4 (0.14 mol/L of NaCl, 0.0264 mol/L of Na2 HPO4-2H2O, 0.009 mol/L of EDTA-PBS), and then fixed with 1% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). PFA-fixed platelets (0.5 to 1.07/L in EDTA-PBS) were incubated with each antibody or antisera for 30 minutes at 37 °C. Monoclonal antibodies AP-2, AP-3, AN51, and 6D1 were tested under saturating conditions, using a dilution which yielded the highest clonal antibodies AP-2, AP-3, AN51, and 6D1 were tested under differential centrifugation, washed three times in EDTA phosphate technique previously described.8 In brief, platelets were prepared by incubation, platelets were washed three times in EDTA-PBS and intensity of fluorescence, after binding to normal platelets. After incubation, platelets were washed three times in EDTA-PBS and the binding of each antibody was revealed by a second specific antibody conjugated with fluorescein. The platelet suspension was then examined with a Zeiss vertical fluorescence microscope (Zeiss, Wetzlau, FRG), and the antibody binding was quantified using a cytofluorograph (Ortho SOH, Boulogne, France).

Flow Cytometry Analysis

Platelet-associated fluorescence was quantified with a cytofluorograph Ortho SOH linked to a computerized system (computer 21S0, mw, 488 nm). Each sample was introduced in a fluid capillary at a speed of 3,000 to 5,000 platelets per second, and three parameters were obtained: right-angle scatter, forward-angle scatter, and green fluorescence measurement. Integrated fluorescence signals were amplified using a logarithmic amplifier and were then digitized and displayed on a 1,000-channel scale. Results were expressed as arbitrary units of fluorescence (AUF). For each sample, mean fluorescence intensity was obtained by dividing the total fluorescence recorded by the number of platelets analyzed (10⁶ platelets).

Statistical Analysis

Student's t test was used to compare the mean values of fluorescence, and Wilcoxon test was used to analyze the results obtained for paired samples, fetus/adult or fetus/mother.

RESULTS

Fetal Platelet Parameters

The mean platelet count in the 32 fetuses was 246 ± 54.10⁹/L (mean ± SD), which is in the normal range for adults (150 to 400.10⁹/L). The fetal platelet volume, as estimated by Coulter S plus II, was 9.69 ± 0.8 μl, greater than that obtained in normal adults (8.4 ± 0.9 μl). However, the difference was not statistically significant and could be due to the presence of large platelets which were observed in fetuses but not in adults.

Fetal Platelet Antigens

Platelet antigen PL^A1 was present in all the fetal blood samples studied (N = 22). The apparent frequency of PL^A1 antigen was the same in fetuses between 18 and 26 weeks of gestation (100%) and in adults (98%).21 The quantity of PL^A1 antigen on platelets, as estimated by indirect immunofluorescence and cytofluorometry, was similar in fetuses (433 ± 30 AUF, mean ± SEM) and in adults (427 ± 13) (Fig 1).

In this study, the heterozygous subjects for PL^A1 antigen were not distinguished from homozygous individuals, since the relatives of the subjects were not tested.

The presence of Lek* antigen was demonstrated on platelets in 23 fetuses between 18 and 26 weeks. Six fetuses were found to be Lek*-negative. Therefore, the apparent frequency of Lek* (+) in fetal platelets (79.4%) is lower than that observed in adults (93.26%, N = 370), but the difference was not statistically significant. When Lek* was expressed on platelets, the mean fluorescence intensity measured by cytofluorometry in fetuses (437 ± 25 AUF) was similar to that recorded with adult platelets (459 ± 15 AUF, N = 23) (Table 1, Fig 2). Two of the Lek*-negative fetuses could be studied after their birth at the age of 6 months; they were still Lek*-negative.

Fetal Platelet Glycoproteins

**GPIIb/IIIa:** GPIIb/IIIa complex and GPIIIa were studied on platelets using three different antibodies: IgG, AP-2, and AP-3. (Table I, Figs 3 and 4).

**IgG.** IgG, which is specific for GPIIb/IIIa complex, reacted with all the fetal platelet samples studied (N = 23). The binding of IgG to fetal platelets quantified in 11 cases was identical (427 ± 23 AUF) to that obtained with adult platelets (420 ± 30 AUF).

**AP-2.** AP-2 recognizes an epitope located on the GPIIb/IIIa complex and was found to react with the 16 fetal samples tested. The mean fluorescence intensity recorded in 12 cases appeared to be lower for the fetal platelets (459 ± 30

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**Table 1. Mean Fluorescence Values (± SEM)**

<table>
<thead>
<tr>
<th>Glycoproteins</th>
<th>Platelets</th>
<th>Immunofluorescence Intensity (AUF)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fetuses (Mean ± SD)</td>
<td>Adults (Mean ± SD)</td>
</tr>
<tr>
<td>Antigens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL^A1</td>
<td>433.0 ± 30.0</td>
<td>427 ± 13.5</td>
</tr>
<tr>
<td>Lek*</td>
<td>441.5 ± 25.0</td>
<td>459 ± 15.0</td>
</tr>
<tr>
<td>Glycoproteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPIIb, IgG</td>
<td>427.0 ± 23.0</td>
<td>420.0 ± 30.0</td>
</tr>
<tr>
<td>GPIIb, AP-2</td>
<td>459.5 ± 8.5</td>
<td>498.0 ± 11.0</td>
</tr>
<tr>
<td>GPIIa, AP-3</td>
<td>536 ± 14.0</td>
<td>515.0 ± 13.0</td>
</tr>
<tr>
<td>GPIb, AN51</td>
<td>491.5 ± 14.0</td>
<td>426.5 ± 9.0</td>
</tr>
<tr>
<td>GPIb, 6D1</td>
<td>479 ± 15.0</td>
<td>443.0 ± 8.7</td>
</tr>
</tbody>
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Values measured with the specific antibodies directed against platelet antigens PL^A1, Lek* and platelet glycoproteins (GPIIb, GPIa, AP-3, GPIb, AN51) in 10 to 13 fetuses and 23 adults. Only three fetuses were tested with 6D1.
Fig 2. Fetal platelet antigens. Histograms of fluorescence recorded after binding of anti-PLA1 or anti-LekA antibodies to the platelets of a fetus and his mother. Fluorescence signals were amplified using a logarithmic amplifier which was then digitized and displayed on a 1,000-channel scale.

8.5 AUF) as compared with that obtained with adult platelets (498 ± 11 AUF, N = 23, P < .02, Student’s t test). However, when the values of fluorescence recorded in the same experiment for paired samples fetus/adult or fetus/mother were analyzed by Wilcoxon test, the difference did not remain statistically significant.

AP-3. Fetal platelet binding of AP-3, specific for a GPllla epitope, was demonstrated in the 22 fetuses studied with this antibody. At variance with that obtained with AP-2, the fluorescence intensities with AP-3 were identical in fetal and adult populations: fetuses = 523 ± 18.5 AUF, N = 12; adults = 515 ± 15 AUF, N = 23.

GPllb. (Table I, Figs 3 through 5) GPllb was studied on human fetal platelets using two monoclonal antibodies, AN51 and 6D1, which are both directed against determinants located on glycoprotein. The binding of AN51 was demonstrated in all fetuses studied, and the mean fluorescence intensity was higher (492 ± 13.5 AUF, N = 12) than in adults (426 ± 9 AUF, N = 23, P < .01). The platelet binding of 6D1 was also higher for the three fetuses tested (479 ± 15 AUF) as compared with that of adults (449 ± 8.7 AUF). In an attempt to standardize the results, we have calculated the ratio of the values of fluorescence intensity
and AN51/AP3. The ratio of ANSi to AP2 in fetuses was higher than in adults; 1.108 ± 0.062 and 0.869 ± 0.0029, respectively (P < 0.01). The ratio of AN51 to AP3 was also higher in fetuses (1.013 ± 0.058) than in adults (0.838 ± 0.020). The difference was statistically significant (P < 0.01) and demonstrated a relative increase in GPIb reactivity with AN51 in the human fetus.

DISCUSSION

The platelet counts of the 32 fetuses that were sampled in our study were similar to those of adults. This result is in agreement with previous reports, which found no significant variation of the fetal platelet number from 18 weeks to the end of pregnancy. As estimated by Coulter S plus II, the mean platelet volume appeared slightly greater in fetuses as compared with that in normal adults. Bleyer et al reported that circulating platelets existed in 11-week-old fetuses, and some cells, resembling mature platelets, were slightly larger and contained fewer granules. In contrast, Holmberg et al found no significant difference between fetal and adult mean platelet volume and used the platelet volume as a criterion to exclude the diagnosis of Wiskott-Aldrich syndrome in an 18-week-old fetus.

We have studied fetal platelet antigens and GPs using an indirect immunofluorescence technique associated with flow cytometry, since this method can be applied to the low blood volumes (1 to 2 mL) that can be obtained from human fetuses during the second trimester of pregnancy.

Our results showed that PL^A1 and Lek^A antigens are expressed on fetal platelets as early as 16 weeks of gestational age. The frequency of PL^A1 antigen in the group of fetuses was similar to that obtained in adults.

After the binding of anti PL^A1, cytometry analysis showed identical values in fetal and adult groups. The expression of PL^A1 antigen in 16-week-old fetuses is complete and may explain the high frequency of immunization in PL^A1-negative women during their first pregnancy, estimated at 59%. The early expression of PL^A1 could play a role in the early occurrence of the fetal thrombocytopenia due to anti-PL^A1 antibodies that has been observed after 20 weeks of gestation.

The expression of Lek^A does not seem to be different in fetuses and adults. Whatever the gestational age between 18 and 26 weeks, the amount of Lek^A antigen was similar. The study of membrane GPs showed that GPIIb/IIIa, as demonstrated by the binding of monoclonal or polyclonal antibody, appeared to be normally expressed on fetal platelets as early as 18 weeks of gestational age. The difference in the AP-2 binding was not significant, and the fluorescence intensities recorded with AP3 and IgG1 were identical in adults and fetuses. In these conditions, the amount of GPIIb/IIIa complex appeared to be similar in fetuses and adults. However, AP-2 recognizes a conformational structure of the complex, and we cannot exclude a slight difference between fetuses and adults. When radiolabeled monoclonal antibodies were used in adults, the number of specific sites per platelet for AP-2 was 57,400 ± 9,700 and 40,200 for AP-3. Our technique did not allow a precise estimation of the number of specific sites per platelet; however, because the results with AP-2 and AP-3 were similar in adults and in fetuses between 18 and 26 weeks of gestation, we can postulate that the number of GPIIb/IIIa is similar in the two groups. In addition, a strong correlation between fluorescence values obtained by cytofluorometry and the number of antigens sites expressed on cell surface has been reported.

GPIb was characterized on all fetal platelet samples studied, and the binding of AN51 appeared significantly higher as compared with that in adults. Moreover, binding of 6D1, which may react with a different GPIb epitope, was higher in fetuses. The number of specific GPIib sites recognized by AN51 and 6D1 on adult platelets is similar: 26,000 to 29,000 per platelet. The reactivity of GPIb was higher in fetal platelets. This did not seem to be due to the slightly increased platelet volume, since the individual fluorescence ratios—AN51/AP-2 or AN51/AP-3—were significantly higher in fetuses. The normal expression of GPIb and GPIIb/IIIa in fetuses indicates that antenatal diagnosis of congenital platelet disorders such as GT or BSS is feasible. In GT type I, GPIIb/IIIa complex is undetectable, whereas GPIIb/IIIa appears to be normally expressed on fetal platelets as early as 18 weeks of gestational age. As a result, antenatal diagnosis of GT could be performed at this term. In a similar manner, diagnosis appears to be possible for GT type II subjects who have ~15% of the normal level of GPIIb/IIIa. Recently, new patients with thrombasthenia variants presenting all the functional abnormalities observed in type I but a normal or subnormal level of membrane GPIIb/IIIa were described. Therefore, immunological techniques cannot be applied to the diagnosis of these variants, emphasizing the importance of the family study using several different techniques as a prerequisite step in the approach of the antenatal diagnosis of GT. The antenatal diagnosis can only be done after a complete study of the family members by means of functional, immunological, and biochemical techniques associated with appropriate genetic information. The results obtained in this study indicate that the antenatal diagnosis is feasible at 18 weeks of gestation. This appears to be progress but is still unsatisfactory from an ethical point of view. We hope that the technology using DNA analysis that has been developed for hemoglobinopathies or hemophilia can be applied at an early stage of pregnancy to the detection of congenital thrombopathies.

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REFERENCES


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