On fetomaternal hemorrhage

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Fetal cell survival in maternal blood after large fetomaternal hemorrhage

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Abstract

Objective: To investigate the clearance rates of alpha-fetoprotein (AFP), fetal red blood cells (RBCs), and fetal mononuclear cells (MNCs) from maternal blood postpartum following large fetomaternal hemorrhage (FMH).

Methods: Blood samples of a patient diagnosed with a large FMH at 36+2 weeks of gestation were drawn at several points of time during a period of 2 years. From each sample plasma was collected, whole blood smears were prepared and MNCs were isolated and stored. The AFP concentration was measured and fetal RBCs were quantified by the Kleihauer-Betke test. Fetal MNCs were detected by fluorescence microscopy by staining the paternally derived human leukocyte antigen (HLA)-A2, which was absent in the mother.

Results: Based on the Kleihauer-Betke test a red cell FMH of 8.1% was detected, corresponding to a volume of 284 ml fetal whole blood transfused into the mother. The AFP concentration decreased very rapidly, whereas fetal RBCs were undetectable in maternal blood after 2.5 months. Postpartum extremely small numbers of fetal MNCs were detected as opposed to the relatively high number of fetal RBCs. Only 13 fetal per $1x10^6$ maternal MNCs were found in the first postpartum sample. The number of fetal MNCs decreased further in time and disappeared completely from maternal blood after 2 years.

Conclusions: Following large FMH the AFP, fetal RBCs and fetal MNCs showed different clearance rates from maternal blood. The detection of fetal MNCs, based on HLA allelic differences between mother and child was enabled by a fluorochrome conjugated human monoclonal antibody directed against a paternally derived HLA alloantigen (HLA-A2) and is independent of the fetal sex. Our findings underline the low frequency of fetal MNCs circulating in maternal blood postpartum.

Introduction

It is well established that in normal pregnancy small amounts of fetal cells are present in the maternal circulation and that the frequency of these cells increases as pregnancy progresses.[1,2] Large fetomaternal hemorrhage (FMH) is a serious complication of pregnancy, which occurs in approximately 3 out of 1000 deliveries.[3] FMH may cause severe fetal anaemia, in some cases leading to fetal death due to exsanguination. Other serious consequences that may arise from fetal-maternal cell trafficking are red blood cell (RBC) and platelet immunization in case of blood group incompatibility between mother and fetus, potentially affecting present and future pregnancies.[4,5] Both under normal and pathological conditions, such as placental abruption or abdominal trauma, significant FMH may occur.[3,6-9] In addition, obstetrical interventions during pregnancy and delivery that cause breakdown of the placental barrier place patients at risk for FMH.[3,10-13] The acid elution test described in 1953 by Kleihauer and Betke has been the key method in the study of transplacental passage of fetal RBCs and the cause and prevention of RBC immunization.[14] The routine administration of anti-D immunoglobulin to Rh D negative women during pregnancy and after delivery of a Rh D positive child has decreased the incidence of RBC immunization significantly.[15,16]

Over the last decade the presence of fetal nucleated cells in maternal blood has been described and the possible role of microchimerism in the pathophysiology of autoimmune disease has been suggested.[17-19] It is currently believed that fetal cells in order to be able to persist in maternal blood and other organs must have stem-cell-like properties.[20] Many techniques are available to detect nucleated cells of fetal origin, but one of the limitations is the low frequency of these cells and restricted application of these techniques to women pregnant of a male fetus by the detection Y chromosome sequences. Our aim was to study the clearance rates of fetal RBCs, fetal mononuclear cells (MNCs) and the alpha-fetoprotein (AFP) after delivery in a patient with large FMH using the Kleihauer-Betke test (KBT) for fetal RBC detection and measurement of the AFP concentration. We present a new approach to detect MNCs from fetal origin using a staining with a monoclonal antibody (mAb) directed against a paternally derived human leukocyte antigen (HLA) alloantigen.

Case report

The patient was a 40-year-old multipara referred to our hospital postpartum. She had a long-term history of infertility due to premature ovarian failure without further underlying cause of disease. The first pregnancy ended in a miscarriage. The second pregnancy, achieved by oocyte donation in combination with in vitro fertilization was uneventful and she delivered a boy. The third pregnancy was also achieved by oocyte donation. At 36+2 weeks of gestation she consulted her midwife because of decreased fetal movements and was referred to an affiliated hospital. Upon admission all vital signs were within normal range. The fundal height was according to the gestational age. The diagnostic work-up included fetal heart rate monitoring which showed a sinusoidal pattern typical for fetal anemia. An emergency Cesarean section was performed and a boy was delivered. His birth weight was 3085 g. The Apgar scores were 5 and 8 after 1 and 5 min, respectively. The arterial blood pH was 6.99 and the hemoglobin level at birth was 2.6 mmol/l. The placenta weighed 520 g without any macroscopic abnormalities. The physical examination showed no abnormalities. He was intubated because of respiratory insufficiency and was transported to the neonatal intensive care unit of our hospital. He was ventilated mechanically for another 12 h and received a blood transfusion. One day after CS the mother was transferred to the maternity ward of our hospital as well.

The diagnosis of a large FMH was confirmed in a postpartum blood sample of the mother. Both the Kleihauer-Betke test (KBT) and flow cytometry using antibodies against the fetal hemoglobin (HbF) were performed to quantify fetal RBCs. A FMH volume of 284 ml fetal whole blood was found. The blood groups of the mother and the child were both A positive. Pathological investigation of the placenta revealed no abnormalities.

The child recovered well and was discharged from the hospital after 2.5 weeks. At the age of two years his neurological development was normal. After informed consent several maternal blood samples and one neonatal blood sample were drawn starting the day after delivery.

Materials and methods

Patient samples

In the period from 13 May 2003 to 1 June 2005 maternal blood samples were drawn at several points of time postpartum. The samples were collected in vacutainer tubes containing ethylene diamine tetra-acetic acid (EDTA) and sodium heparin by venipuncture and were processed within 3 hours of collection. From each

maternal EDTA sample glass slides were prepared for fetal RBC quantification by the Kleihauer-Betke test (KBT) and plasma was collected for the measurement of the alpha-fetoprotein (AFP) concentration. One maternal and one neonatal sodium heparin blood sample was used for HLA typing. Low resolution molecular typing was performed on DNA obtained from samples by polymerase chain reaction (PCR) / sequence specific oligonucleotide using a reverse dot-blot method.[21] MNCs were isolated by ficoll-amidotrizoate density gradient centrifugation from other sodium heparin samples and cryopreserved for fetal MNC detection and quantification. Maternal sera taken at delivery and on days 1, 2, 15, 30 and 77 postpartum were examined for total HLA class I and II antibody content by ELISA (LAT, One Lambda Inc, Canoga Park, CA) followed by HLA specificity testing in the complement dependent cytotoxicity (CDC) test against a panel of peripheral blood MNCs from molecularly HLA typed individuals.

The patient gave written informed consent to collect blood for research purposes. The Medical Ethical Review Board of the Leiden University Medical Center approved the protocol (P01.016).

Measurement of the alpha-fetoprotein concentration in maternal blood

Plasma was collected and stored at -80° C until further processing. The AFP concentration of all samples was measured in a single run using a Chemiluminescent Microparticle Immuno Assay (Architect I-2000, Abbott, Hoofddorp, The Netherlands).

Quantification of fetal RBCs in maternal blood

Of each EDTA sample, 100 µl was mixed with 100 µl phosphate-buffered saline (PBS). Conventional blood smears were prepared on glass slides using 2.5 µl of this dilution. Fixation of the cells and elution of HbA were performed as described previously.[9,14] Fetal RBCs were counted in 400 subsequent microscopic fields using a 40x objective. The total number of maternal RBCs in 400 microscopic fields was estimated by counting the number of background cells in 5 different fields. All slides were evaluated in a blinded fashion. Negative and positive control samples consisted of spiked samples of fetal RBCs in adult RBCs. The FMH volume was calculated using the following formulas. The fetal RBC and whole blood volumes were calculated by assuming a maternal whole blood volume 5000 ml at term, and a fetal and maternal hematocrit of 0.50 and 0.35, respectively.[22]

(1) transfused fetal RBC (%) = fetal RBCs / number of maternal RBCs x 100
(2) transfused fetal RBC volume (ml) = fetal RBC % x 0.35 x 5000 ml
(3) transfused fetal whole blood volume (ml) = fetal RBC % x (0.35 / 0.50) x 5000 ml

Quantification of fetal MNCs

Preparation of the anti-HLA-A2 mAb SN230G6

A hybridoma was derived by EBV transformation of lymphocytes from an HLA antibody seropositive multiparous woman, followed by electrofusion of an antibody-secreting EBV line and rigorous subcloning. The specificity of the human HLA mAb SN230G6 was determined by CDC and defined as HLA-A2/B17. The mAb SN230G6 (isotype IgG, λ) was purified from hybridoma supernatant by protein A chromatography. A $F(ab')_2$ fragment was prepared by pepsin digestion followed by protein A chromatography to remove Fc fragments and undigested mAb.[23] The isolated F(ab')2 fragment was conjugated to the fluorochrome Alexa Fluor 546 (Molecular Probes Inc, Eugene, OR) according to the manufacturer's instructions. The Alexa Fluor 546-dye labeled mAb has an absorption and fluorescence emission maximum of approximately 558 nm and 573 nm, allowing detection of red fluorescent cells.

Lymphocyte staining protocol

Upon thawing, 1 x 10^6 MNCs were incubated with 1.25 µg Alexa Fluor conjugated SN230G6 F(ab')2 fragment in a final volume of 20 µl at 4 C° for 30 minutes in the dark. After incubation, cells were washed once and resuspended in 1 ml PBS supplemented with 10% fetal calf serum (FCS).

Microscopic evaluation

Approximately 150,000 mAb-stained cells were spun on glass slides using a Cytofuge centrifuge (Nordic Immunological Laboratories, Tilburg, The Netherlands) and air-dried for 1 hour. Slides were mounted in 0.01 µg/ml 4'-6-Diamidino-2-phenylindole (DAPI) in Vectashield (Vector Laboratories, Burlingame, CA).

Of each blood sample drawn at different points of time postpartum, 10 glass slides (equivalent to approximately 1.5 x 10^6 MNCs) were evaluated in a blinded fashion. The slides were manually analyzed using a Leica DMRXA fluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with a 40, 63 and 100x oil immersion objective and 4 different filters: a DAPI (blue excitation), a HQ-TRITC (red excitation), a HQ-FITC (green excitation) and a triple band pass filter (red, green and blue excitation). All slides were analyzed for their cell content, morphology and staining intensity. Cells with a brightly positive HLA-A2 membrane staining (red) were classified as fetal. Photographs were recorded using the same microscope and a CCD camera (CH250, Photometrics, Tucson, AZ).

Validation study of mAb SN230G6 anti-HLA-A2
To investigate sensitivity and linearity of detection of mAb SN230G6 stained MNCs, spiked samples of HLA-A2 positive MNCs derived from term umbilical cord blood in HLA-A2 negative, non-pregnant adult MNCs were prepared. Negative control samples stained with mAb SN230G6 consisted of: 1) HLA-A2 negative MNCs of a non-pregnant healthy adult, 2) HLA-A2 negative MNCs of a pregnant woman near term carrying a HLA-A2 negative child and 3) mixtures of HLA-A2 negative MNCs from term umbilical cord blood in HLA-A2 negative MNCs of a pregnant woman near term carrying a HLA-A2 negative child. Additionally, HLA-A2 positive and negative MNCs without staining for mAb SN230G6 were included. Positive controls consisted of HLA-A2 positive MNCs from term umbilical cord blood and blood from non-pregnant adults. Spiked samples consisted of 0.1%, 0.01% and 0.001% concentrations. Of each mixture and control sample 6 glass slides were available. All slides were evaluated in a blinded fashion by two investigators, resulting in 12 replicate measurements per mixture or control sample.

Statistical analysis
Validation study of the mAb SN230G6 was performed by using Pearson's correlation coefficient (Excel2000, Microsoft Inc., Redmond, WA) and calculation of confidence intervals (SPSS 10.0 for windows, SPSS Inc., Chicago, IL). The clearance rates of AFP, fetal RBCs and fetal MNCs are displayed as scatterplots (Excel2000).

Results

AFP and fetal RBC quantification
The AFP concentration and fetal RBC percentage at different points of time after delivery are shown in table 1. The AFP concentration decreased very rapidly, whereas fetal RBCs were undetectable in maternal blood after 2.5 months (figure 1a and 1b). The $t_{½}$ of AFP and fetal RBCs in maternal blood were 3 and 15 days, respectively.

Fetal MNC quantification
HLA class I and II typing of the mother and child
The HLA types of the mother and child were:

Mother:	class I:	A*01, A*29, B*37, B*44, Cw*06, Cw*16
	class II:	DRB1*07, DRB1*11, DRB3, DRB4, DQB1*02, DQB1*03
Child:	class I:	A*01, A*02, B*08, B*40, Cw*03, Cw*07
	class II:	DRB1*13, DRB1*14, DRB3, DQB1*05, DQB1*06

Table 1 - Different clearance rates of AFP, fetal RBCs and fetal MNCs from maternal blood after delivery

Follow-up postpartum	AFP concentration	Fetal RBCs		Fetal MNCs	
	(µg/l)	per $1x10^6$	(%)	per $1x10^6$	(%)
1 day	3953	81000	8.1	ND*	ND
2 days	2753	68000	6.8	13.3	0.0013
4 days	656	54000	5.4	10.7	0.0011
15 days	95	39000	2.9	10.7	0.0011
30 days (1 month)	9	23000	2.3	5.3	0.0005
52 days (1.5 month)	5	2000	0.2	6.7	0.0007
77 days (2.5 months)	ND	0	0	2.0	0.0002
102 days (3.5 months)	ND	0	0	ND	ND
574 days (1.5 year)	ND	0	0	2.6	0.0003
726 days (2.0 years)	ND	0	0	0	0

ND: not determined (sample volume too small)

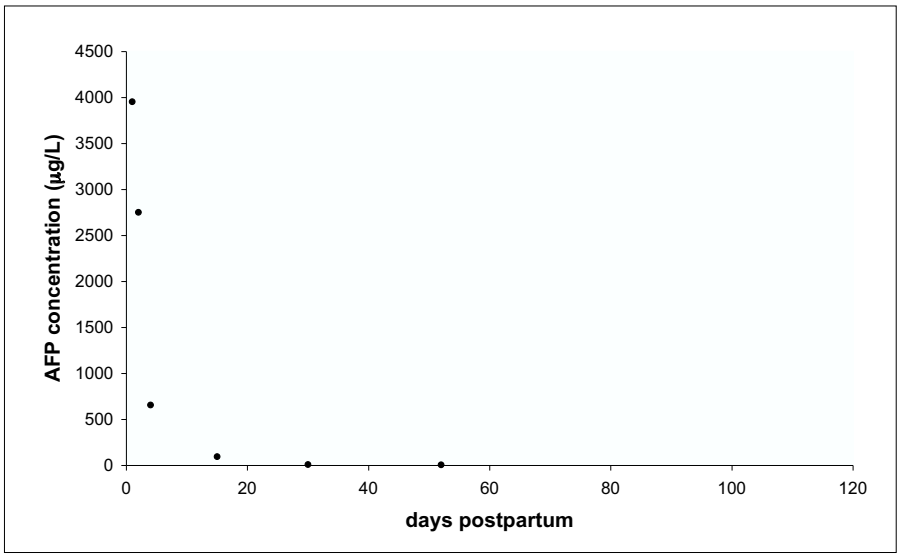

Figure 1a - The clearance rate of AFP from maternal blood after delivery

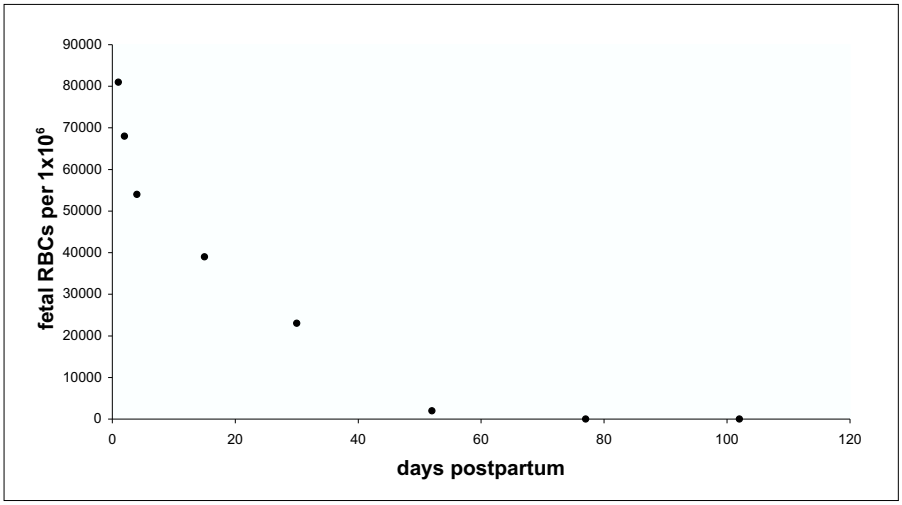

Figure 1b - The clearance rate of fetal RBCs from maternal blood after delivery

Table 2 - Quantitative assessment of HLA-A2 positive fetal MNCs in spiked samples: expected versus measured cell numbers per 1x10^6 adult MNCs

Theoretical number of HLA-A2 positive MNCs		Detected mean number of HLA-A2 positive MNCs		
%	per 1x10^6	%	per 1x10^6	95% CI
0	0	0	0	[0 – 0]
0.001	10	0.0013	13	[3 – 23]
0.01	100	0.0077	77	[50 – 103]
0.1	1,000	0.0682	682	[620 – 743]

Based on the HLA type differences between mother and child, it was decided to apply mAb SN230G6, which was reactive with the fetal HLA-A2 antigen.

Validation study of mAb SN230G6 anti-HLA-A2

No systematic differences between both investigators were found, therefore the data were pooled. No fetal MNCs were found in negative control samples. Samples consisting of HLA-A2 positive MNCs derived from adult and umbilical cord blood were

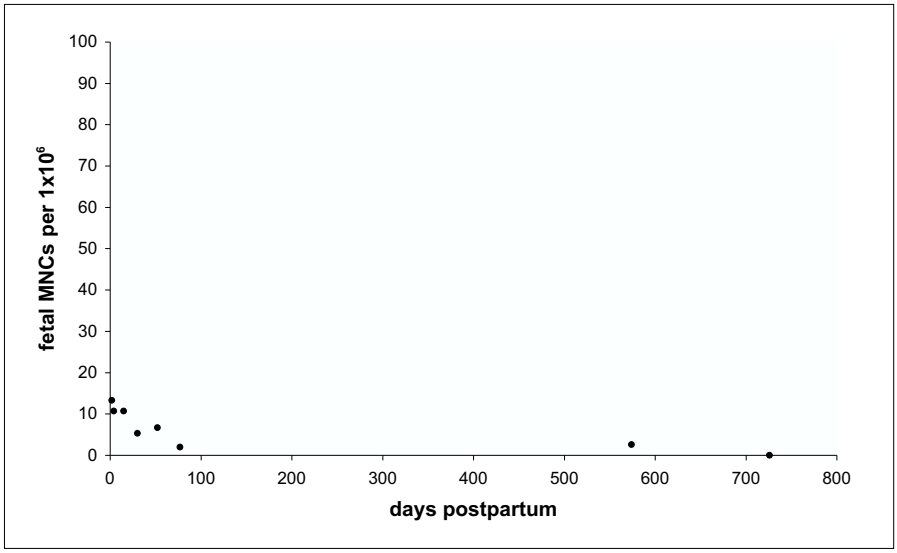

Figure 1c - The clearance rate of fetal MNCs from maternal blood after delivery

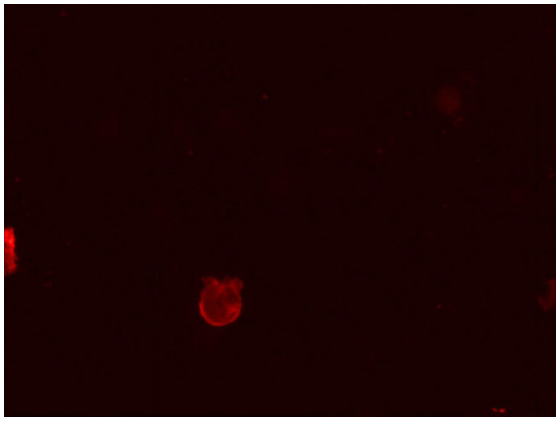

Figure 2a
Patient sample stained with mAb SN230G6 anti-HLA-A2: 1 fetal MNC positive for HLA-A2 (red membrane staining) against a background of HLA-A2 negative maternal MNCs.

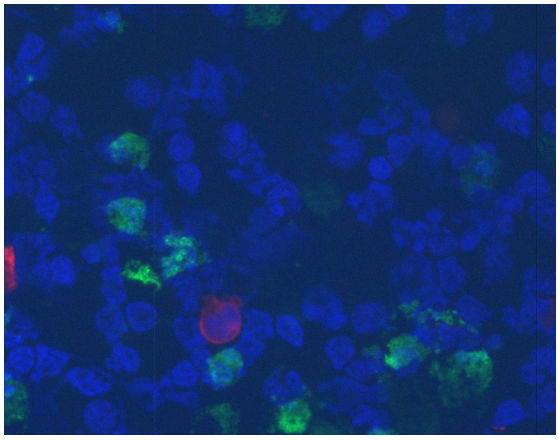

Figure 2b
Patient sample stained with a combination of mAb SN230G6 anti-HLA-A2 and DAPI: 1 fetal MNC positive for HLA-A2 (red membrane staining) against a background of HLA-A2 negative maternal MNCs.

100% positive. The correlation between the theoretical and detected concentrations of fetal MNCs was good (r^2=0.99), especially in the lower ranges. These results are given in table 2.

Fetal MNC quantification in patient samples
Postpartum, mAb SN230G6 detected extremely small numbers of fetal MNCs, which decreased further in time (table 1). The clearance rate of fetal MNCs from maternal blood is given in figure 1c. An HLA-A2 positive fetal MNC (red membrane staining) against a background of negative maternal MNCs is shown in figure 2a and 2b. At two years postpartum no fetal MNCs could be identified.

Antibody formation in the mother
In the maternal serum sample taken on day 4 postpartum, HLA antibodies against HLA-A2 were detected. Samples taken earlier were negative. Fifteen days postpartum antibodies against HLA-B40 were found in addition.

Discussion

Little is known about the lifespan of fetal blood cells transfused into the maternal circulation. We studied the clearance rates of AFP and fetal RBCs and fetal MNCs in maternal blood samples at several points of time postpartum in a patient diagnosed with large FMH aiming to contribute to the discussion on the persistence of fetal cells. Our results indicate that AFP is cleared very rapidly after large FMH and that ABO and Rh compatible fetal RBCs have a lifespan of approximately 80 days in maternal blood. As compared to fetal RBCs, fetal MNCs are either transferred to maternal blood in extremely small amounts or cleared very rapidly from the maternal circulation.

Our technique to detect fetal MNCs using a staining with a mAb against the paternally derived HLA alloantigen provides a possibility to study microchimerism on a cellular basis in all pregnancies irrespective of the fetal sex. The mAb SN230G6 is specific for the diaminoacid GE 62-63 sequence, a determinant shared between HLA-A2 and HLA-B17. The absence of this determinant on any of the HLA alleles of the mother allowed the unequivocal detection of fetal MNCs on the basis of cell surface expression of HLA-A2. The principle of discrimination between fetal and maternal MNCs based on a paternally inherited HLA alloantigen by fluorescence activated cell sorting has been used previously.[24]

Passage of fetal cells into the maternal circulation does occur at the fetomaternal interface. In the human hemochorial placenta the villi are surrounded by maternal

intervillous blood. Under normal physiological conditions the fetal and maternal circulations are separated by the so-called placental barrier, consisting of the following layers: (1) a continuous layer of syncytiotrophoblast cells, (2) an initially (in the first trimester) complete, but later on (second and third trimester) discontinuous layer of cytotrophoblast cells, (3) a trophoblastic basal lamina, (4) connective tissue derived from the extra-embryonic mesoderm, and (5) the fetal endothelium.[25] Throughout pregnancy the placental barrier becomes progressively thinner while simultaneously fetal blood flow and blood pressure increase as the villous tree enlarges.[26] Due to spontaneous or induced disruption of the placental barrier fetal plasma and blood cells including their precursors will leak into the maternal circulation.

In our patient we found an extremely high AFP concentration in the first postpartum sample underlining the large FMH volume. In the following postpartum samples a relatively rapid decline of the AFP concentration was observed. AFP is a major serum glycoprotein synthesized during fetal life mainly by the yolk sac and trophoblast early in the first trimester followed shortly thereafter by the fetal liver. In the human fetus the concentration of AFP peaks at 13 weeks of gestation (3-4 mg/ml), falls to about 50 μg/ml at term and disappears after birth.[27] The AFP concentration in adults is approximately 5 μg/l. The primary roles of AFP are indirectly regulation of cell growth by controlling apoptosis, involvement in inflammatory reactions and immunoregulation.[28] At term the placenta does not synthesize AFP and with an intact placental barrier the presence of AFP in the placenta is a reflection of transplacental transport via a receptor-mediated mechanism.[29,30] Elevated levels of AFP in maternal blood during pregnancy are associated with fetal malformations, such as neural tube defects and with placental tissue damage and fetomaternal hemorrhage. The large influx of the AFP into the maternal circulation as observed in our patient is explained by the breakdown of the placental barrier and corresponds with the amount of transfused fetal blood. AFP disappeared from the maternal circulation within 1 month.

The transfused volume of ABO and Rh compatible fetal RBCs was estimated at 142 ml or 284 ml fetal whole blood. Given a fetoplacental volume at term of 125 ml/kg, this fetus has lost 75% of its circulating volume, which corresponds well with the extremely low hemoglobin level at birth.[22] Precise calculation of the FMH volume is important in case of Rh incompatibility between mother and child. The transfused RBCs had a maximum lifespan of approximately 77 days and a $t_{½}$ of 15 days. The clearance rate of fetal RBCs from maternal blood after FMH depends on a number of facts: the ABO and Rh compatibility, administration of anti-D immunoglobulin and the time of entry in the maternal circulation. Results on the lifespan of ABO Rh compatible fetal RBCs described in literature showed different clearance rates. Some

studies report a shorter lifespan compared to adult RBCs corresponding with our results.[31,32] Two other studies on this subject report a fetal RBC lifespan equal to adult RBCs.[3,33] Differences in the observed lifespan of fetal RBCs in maternal blood are most likely due to a varying age distribution of cells entering the maternal circulation.

Many factors may influence the transfer of fetal cells into maternal blood during and after pregnancy. Fetomaternal cell trafficking is increased in conditions such as preeclampsia, intra-uterine growth restriction, fetal abnormalities and termination of pregnancy.[34-37] The amount of fetal to maternal cell transfer may be influenced by histocompatibility. Certain maternal HLA class II alleles, such as HLA-DQ A1*0501 were found to be more frequently associated with fetal cell microchimerism.[38,39] Also, very little is known about the role of maternal antibody formation against fetal specific HLA alloantigens in fetal cell microchimerism.

Different types of fetal nucleated cells can be identified and isolated: trophoblasts, nucleated erythroid cells, and lymphocytes including lymphoid progenitor cells. The biological implications of fetomaternal cell trafficking are currently being explored. Various research groups working on fetal cell isolation for the purpose of non-invasive prenatal genetic diagnosis found evidence for the survival of fetal progenitor cells in the circulation of women many years after delivery.[40-43] This finding led to the hypothesis that fetal cells persisting in maternal blood and tissues are involved in the pathogenesis of auto-immune disease in women after their child-bearing years. So far various techniques and strategies have been used for the detection of rare cells. In studies focussing on the detection of intact fetal cells different cell separation protocols have been applied using fluorescence activated cell sorting (FACS) and magnetic activated cell sorting (MACS) to enrich for specific subpopulations of fetal cells followed by fluorescence *in situ* hybridization (FISH) with chromosome specific probes and PCR to amplify fetal specific gene sequences based on the genotype of the parents.[44,45] One of the limitations of these techniques is the not yet optimal sensitivity and specificity. Therefore, fetal cell microchimerism is still difficult to detect using techniques currently available.

The frequency of fetal cells in the maternal circulation increases with the gestational age.[4] The number of fetal nucleated cells in maternal blood is estimated to be 1-6 cells/ml in uncomplicated second-trimester pregnancies.[36,37] At near-term all women have circulating fetal cells in their blood.[46] After delivery the number of fetal nucleated cells rapidly decreases. Fetal cell microchimerism can be detected in as many as 90% of healthy woman after delivery and thus is a widespread phenomenon, but is difficult to detect.[41] The majority of fetal MNCs are mature and they most probably become apoptotic as a consequence of the maternal T cell response, providing an explanation for the very low frequency at which these cells are found. It is currently believed that fetal cells, which persist in maternal blood and

other organs, must have stem-cell-like properties.[20] The hypothesis is that these cells persist in a maternal stem cell niche, where they are able to engraft. Later in life they may repopulate and home to a damaged organ in case of injury and thus contribute to the maternal repair response.

In conclusion, following large FMH the AFP, fetal RBCs and fetal MNCs show different clearance rates from maternal blood. We demonstrate a new technique to detect fetal MNCs by the use of a mAb directed against the paternally derived alloantigen HLA-A2. This approach is independent of the fetal sex. Our findings underline the low frequency of fetal MNCs circulating in maternal blood postpartum.

Acknowledgments

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