Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 705–708, January 1996
Medical Sciences

Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum

(pregnancy/chimerism/CD34/CD38)

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Communicated by Leonard A. Herzenberg, Stanford University School of Medicine, Stanford, CA, October 11, 1995
(received for review June 5, 1995)

ABSTRACT **Rare nucleated fetal cells circulate within maternal blood. Noninvasive prenatal diagnosis by isolation and genetic analysis of these cells is currently being undertaken. We sought to determine if genetic evidence existed for persistent circulation of fetal cells from prior pregnancies. Venous blood samples were obtained from 32 pregnant women and 8 nonpregnant women who had given birth to males 6 months to 27 years earlier. Mononuclear cells were sorted by flow cytometry using antibodies to CD antigens 3, 4, 5, 19, 23, 34, and 38. DNA within sorted cells, amplified by PCR for Y chromosome sequences, was considered predictive of a male fetus or evidence of persistent male fetal cells. In the 32 pregnancies, male DNA was detected in 13 of 19 women carrying a male fetus. In 4 of 13 pregnancies with female fetuses, male DNA was also detected. All of the 4 women had prior pregnancies; 2 of the 4 had prior males and the other 2 had terminations of pregnancy. In 6 of the 8 nonpregnant women, male DNA was detected in $CD34^+CD38^+$ cells, even in a woman who had her last son 27 years prior to blood sampling. Our data demonstrate the continued maternal circulation of fetal $CD34^+$ or $CD34^+CD38^+$ cells from a prior pregnancy. The prolonged persistence of fetal progenitor cells may represent a human analogue of the microchimerism described in the mouse and may have significance in development of tolerance of the fetus. Pregnancy may thus establish a long-term, low-grade chimeric state in the human female.**

Multiple investigators have now confirmed the existence of fetal cells in maternal blood, a finding first clearly demonstrated by Herzenberg *et al.* (1) in 1979, by fluorescence-activated cell sorting of fetal cells bearing uniquely paternally inherited cell surface antigens. More recently, molecular genetic techniques such as PCR and fluorescence *in situ* hybridization have enhanced the detection of fetal-specific gene sequences in candidate fetal cells isolated from maternal blood, as summarized in recent review articles (2, 3). A multicenter clinical evaluation, comparing the diagnostic accuracy of genetic analysis of fetal cells circulating in maternal blood with cells obtained by amniocentesis or chorionic villus sampling, is currently being funded by the National Institutes of Health. The question of whether fetal cells in maternal blood originate from a current or prior pregnancy is important, not only as it pertains to diagnostic considerations but also as a fundamental issue in reproductive immunobiology.

Schröder *et al.* (4) originally described the persistence of fetal leukocytes in the maternal circulation after delivery. In this important observation, interphase Y body fluorescence was used to determine the frequency of male fetal cells in maternal blood and the kinetics of their subsequent disappearance. In a group of 20 primigravidas sampled on more than one occasion postpartum, quinacrine fluorescent signals ("Y body") were seen at a frequency of 0.01–0.1% of lymphocytes. These frequencies were noted to remain "practically unchanged" up until 1 year after delivery, when the study ended. In a related study, Ciaranfi *et al.* (5) extended the observations of Schröder *et al.* (4) to include samples from women 5–7 years postpartum (5). In a series of 62 women who delivered a male infant, more than half had detectable male lymphocytes 2 years after birth. In some cases, metaphases with a Y chromosome were visualized 5 years postpartum. These studies, although intriguing, were performed in the 1970s using techniques less sensitive and less accurate than those available today.

During this decade, investigators using PCR amplification of Y chromosome-specific sequences to identify fetal cells in maternal blood noted "false positive" results—i.e., detection of male DNA when the fetus was female (6, 7). Although laboratory contamination as a source of true false positives can be a concern (8), it appears that, in some cases, the male DNA was a real finding, possibly originating from a prior pregnancy (9). Other investigators studied postpartum maternal samples without using cell separation techniques to enrich for specific subpopulations of fetal cells and concluded that fetal cells did not persist postpartum (10).

The study reported here occurred in two parts. Initially, we used a monoclonal antibody to cluster differentiation antigen (CD) 34 to isolate fetal hematopoietic stem cells from the blood of pregnant women. The surprising results, described below, suggested that the gender of the circulating $CD34^+$ cells did not always correlate with the fetal gender of the current pregnancy. This led to the hypothesis that fetal $CD34^+$ cells could persist from a prior pregnancy. We tested this hypothesis by fluorescence-activated cell sorting of four specific subpopulations of mononuclear cells from women who were not presently pregnant but who had previously given birth to a male infant. Although we were initially interested in the immunophenotypic identification of the persisting fetal cells because of the potential for diagnostic errors in prenatal genetic diagnosis, our results suggest a broader significance. Pregnancy may establish a long-term, low-grade chimeric state in the human female.

METHODS

Patients. In the initial study, venous blood samples (20 ml) were obtained in EDTA or citrated dextrose solution A (ACDA) from 32 pregnant women (9.5–19 weeks of gestation) prior to genetic amniocentesis for advanced maternal age. In the second phase of the study, venous blood samples (20 ml)

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were obtained from 8 women between 30 and 65 years of age who were not pregnant, defined clinically by regular menses or having already entered menopause. They had given birth to between one and six male offspring 6 months to 27 years before blood sampling. Informed consent was obtained, and the protocol was approved by the institutional review boards at Beth Israel, Brigham and Women's, Children's, and New England Medical Center hospitals. All of the women in the study were healthy. Of the women studied who were not pregnant at the time of the blood sampling, none had a history of having received blood transfusions.

Fluorescence-Activated Cell Sorting. Mononuclear cells were isolated from the peripheral blood by dilution in Hanks' balanced salt solution and Ficoll/Hypaque (Pharmacia) gradient centrifugation at a density of 1.077 g/ml. Approximately 2×10^7 mononuclear cells per pregnant patient were incubated with monoclonal antibody to CD34 (clones My10 or 8G12; Becton Dickinson). The mononuclear cells from the nonpregnant women were divided into aliquots and incubated for 30 min on ice with fluorescently conjugated monoclonal antibodies that recognize mature B-cell antigens (CD19 or CD23), T-cell antigens (CD3, CD4, or CD5), hematopoietic stem cell antigen (CD34), or antigens expressed on committed lymphoid and myeloid progenitor cells (CD34 and CD38) (Becton Dickinson).

Fetal cell analysis and sorting were performed on a Becton Dickinson FACStar^{PLUS} flow cytometer equipped with LYSIS II analytic software. The gain was standardized manually using fluorescent beads and chicken erythrocytes. A small aliquot (50 μl) of the patient's mononuclear cells was incubated with fluorescent-labeled antibodies to keyhole limpet hemocyanin (Becton Dickinson), an antigen not expressed on human cells, to establish background fluorescence due to nonspecific antibody binding. During sorting, fluorescent (antibody-positive) and nonfluorescent (antibody-negative) cells were determined by physical separation on a logarithmic scale. Candidate fetal cells and sheath fluid were sorted by flow cytometry into 1.5-ml centrifuge tubes and frozen at −20°C. A solution of 10% household bleach (Clorox) in distilled water was flushed through the flow cytometer tubing for 10 min between samples to remove any adherent cells from a prior sort. In experiments involving the nonpregnant patients, it was critical to eliminate the possibility that the male DNA detected originated in any adherent cells from previous experiments. Thus, the initial sort consisted of a control sample from the mononuclear cells of a female who had never been pregnant. Only if the sample control sorted cells were devoid of male DNA were the subsequent sorted cells further analyzed for the presence of male DNA.

PCR. Extreme caution was observed in the preparation of sorted samples for subsequent PCR amplification, due to the known risks of PCR product carryover and false positive amplification (8). Each experiment had internal positive and negative controls designed to detect PCR contamination. Our criteria for an experimental sample to be considered positive for the presence of male DNA were that (*i*) there was no male DNA in the reagent (no added DNA) control lane and (*ii*) 0.01 ng of control male genomic DNA (approximately one male cell) generated an amplification product of the appropriate size for the primer pair used in the experiment (198, 291, or 397 bp). The technicians were blinded to patient identity. PCR experiments were performed over a period of several years.

For the pregnant patients, DNA was crudely extracted by boiling sorted cells for 5 min. PCR was performed with primers complementary to probe Y49a, a Y chromosome-specific sequence (11). The details of the PCR amplification, separation by electrophoresis, Southern transfer, and hybridization have been described (6). Autoradiographs were interpreted as positive or negative for the presence of the amplified 291- or 397-bp fragment before the results of the fetal gender were known from prenatal cytogenetic analysis. The presence of the amplified fragment was considered predictive of a male fetus.

In the interval between the first and second phases of the study, we became aware of the application of nested PCR to the detection of rare cells (7). This technique has the advantage of suppressing nonspecific background amplification, permitting detection of rare cell populations without requiring Southern transfer and hybridization to detect a PCR product. In the second phase of the study, the sorted cells were boiled. Individual PCRs were set up according to the method of Lo *et al.* (7), using the previously reported external primers Y 1.5 and 1.6. After initial PCR for 40 cycles, a 2-μl aliquot of the first reaction was added to a second reaction using the flanking primers Y 1.7 and 1.8 (7). The second reaction proceeded for 25 cycles. Amplification products were analyzed on a 2% agarose gel for the presence of a 198-bp product.

RESULTS

The results of the initial study are listed in Table 1. Of the 32 pregnancies, 19 had male and 13 had female fetuses. The male-specific amplification product was detected in 17 sorted samples amplified before fetal gender was determined by prenatal karyotype. Of the 17 fetuses predicted to be male, 13 were male and 4 were female as determined from results of cytogenetic studies on amniocytes. In the four false positive cases, there was no evidence of PCR contamination in the reagent control. Review of the prior pregnancy histories of these four women indicated that two of the women each had one male infant prior to sampling for the current study. The other two women had two and four elective terminations, respectively, prior to the current pregnancy with a female fetus.

The results of the study involving the nonpregnant women are shown in Table 2. There was no evidence of false positive amplification in reagent controls or female cells sorted prior to experimental samples. No male DNA was detected in cells expressing the B-cell antigen CD19 or CD23. One woman had detectable male DNA in her $CD3^+$ as well as her $CD34^+CD38^+$ cells. Male DNA was detected in the sorted $CD34^+CD38^+$ cells from six of the eight women (Fig. 1). Male DNA was not detected in the women who had most recently delivered males (6 and 10 months prior to sampling). The persistence of male $CD34^+CD38^+$ cells was demonstrated in the woman who had delivered her last son 27 years prior to sampling.

DISCUSSION

We have presented data on four women who were pregnant with female fetuses and six women who were not pregnant that demonstrate the continued circulation of male $CD34^+$ or $CD34^+CD38^+$ cells from a previous pregnancy. Although controversy exists in the literature regarding the exact length of time that male DNA can be detected in postpartum blood samples obtained from women who delivered male infants (range of 3–8 months) (9, 10), these previous studies were performed on unpurified maternal DNA. In the present study, we utilized the fluorescence-activated cell sorter to isolate specific subpopulations of cells. The results cannot be compared to previous studies because we selectively amplified only

Table 1. Detection of male DNA in $CD34^+$ cells sorted from pregnant women

Parameter	Current pregnancy	
	Male fetus	Female fetus
Male DNA detected	13	4
No male DNA detected	6	9
Total	19	13

$P = 0.0702$, by Fisher's exact test.

Table 2. Results of studies on women who were not pregnant at time of blood sampling

	Clinical history					Detection of 198-bp fragment in sorted cells			
Patient	No. of pregnancies	Male infants	Female infants	Tab/Sab	Interval between sampling and most recent male	B cells ($CD19^+23^+$)	T cells ($CD3^+4^+5^+$)	Lymphoid progenitor ($CD34^+38^+$)	Hematopoietic stem cell ($CD34^+$)
1	4	3	1	0	1 year	−	−	+	−
2	3	1	2	0	7 years	ND	ND	+	−
3	2	2	0	0	2 years	ND	ND	+	+
4	3	2	1	0	3 years	−	−	+	ND
5	10	6	3	1	27 years	−	ND	+	−
6	3	2	0	1	6 years	−	+	+	ND
7	4	2	1	1	10 months	−	−	−	−
8	1	1	0	0	6 months	−	−	−	−

ND, not done; Tab, therapeutic abortion; Sab, spontaneous abortion.

a very small fraction of the original maternal blood sample. The results are significant because they identify a potential diagnostic complication for genetic analysis using fetal cells circulating in maternal blood, the persistence of cells from a prior pregnancy. If fetal cells are to be used for genetic diagnosis, several strategies should be employed to be certain that the cells being analyzed derive from the current pregnancy. Depletion methods that selectively remove T cells, $CD34^+$, or $CD34^+CD38^+$ cells could be incorporated into fetal cell isolation protocols. Alternatively, positive selection methods could be directed toward a highly differentiated fetal cell type that is unlikely to proliferate, such as the fetal nucleated erythrocyte (12). Both methods may become necessary to ensure diagnostic accuracy.

In this study, male cells were detected qualitatively by the PCR amplification of Y chromosome-specific sequences. No attempts were made to quantify the number of male cells detected in the samples obtained from the pregnant or nonpregnant women. The fact that the persisting cells were detected only by Southern hybridization or nested PCR techniques implies that they are extremely rare and probably circulate in maternal blood at much lower frequencies than Schröder *et al.* (4) originally suggested. We have independently described techniques of fetal cells quantitation by PCR that might be applied to future experiments to address the question of frequency (13).

The fact that male $CD34^+CD38^+$ cells were detectable as long as 27 years after the birth of a male infant was unexpected. An analogous situation has been described in the mouse, using a uniquely paternal cytogenetic marker chromosome, T6, that is transmitted to offspring. Gaillard *et al.* (14) demonstrated that during murine pregnancy, there was a considerable concentration of fetal cells bearing the T6 chromosome in the maternal spleen (3–6% of total cells). Additionally, these cells were shown to be capable of division, survived postpartum, and increased in number in a second pregnancy. The term "microchimerism" has been proposed to describe the apparently stable long-term survival and proliferation of allogeneic fetal cells in the maternal mouse without induction of graft versus host disease (15–18). Liégois *et al.* (15) demonstrated the presence of the paternal T6 marker chromosome in mouse maternal spleen and bone marrow, even when the subsequent pregnancies were induced by other murine species that did not carry the T6 chromosome. Thus, the murine maternal lymphoid organs were shown to be an important area where fetal antigens were recognized. In 1983, Liégois (17) speculated that microchimerism might play a role in the induction or maintenance of tolerance to the fetus during murine pregnancy. In the same volume, Masseyeff *et al.* (18) extended their observations to humans and speculated that fetal cells in maternal blood preferentially homed to lymphoid organs where they possessed suppressor characteristics.

Fetal cells have been detected in the human maternal circulation as early as 4 weeks and 5 days postconception (19). The origins of these cells are presently unknown. It is tempting to hypothesize that active cellular traffic across the placenta early in gestation is important and perhaps necessary in inducing tolerance to the human fetus. The establishment of fetal $CD34^+$ or $CD34^+CD38^+$ cells in maternal lymphoid organs or bone marrow may help to maintain tolerance of the fetal graft in a manner analogous to allogeneic organ transplantation. Starzl and co-workers (20, 21) have demonstrated chimerism resulting from widespread seeding of donor dendritic and hemopoietic cells that emanate from whole organs being transplanted, such as kidney, liver, or intestine. They have postulated that bidirectional cell migration and repopu-

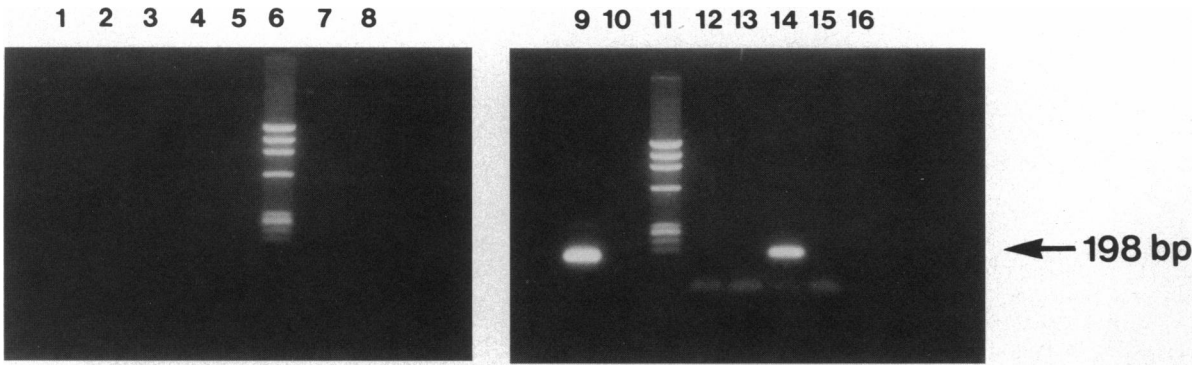

FIG. 1. Demonstration of a 198-bp amplified product, consistent with the presence of male DNA, in $CD34^+CD38^+$ cells sorted by flow cytometry from a woman who was not pregnant but had previously given birth to a male infant. Lanes: 1, female control; 2, $CD4^+$ sorted cells; 3, $CD4^-$ sorted cells; 4, $CD5^+$ sorted cells; 5, $CD5^-$ sorted cells; 6, molecular weight marker ϕX174; 7, empty lane; 8, reagent control; 9, 10 pg of male DNA; 10, empty lane; 11, molecular weight marker ϕX174; 12, $CD23^+$ sorted cells; 13, $CD23^-$ sorted cells; 14, $CD34^+CD38^+$ sorted cells; 15, $CD34^-CD38^-$ sorted cells; 16, empty lane.

lation is the first step in the acquisition of donor-specific tolerance and, ultimately, successful graft acceptance. The human pregnancy may also benefit from similar one-way or even two-way traffic.

The development of microchimerism in human pregnancies needs to be further investigated in larger numbers of multiparous women. Fetal cells could be engrafting in maternal lymphoid organs or bone marrow. Alternatively, the progenitor cells could remain in the circulation and continue to divide for years. These hypotheses could be tested by obtaining blood and bone marrow samples from women who have had chromosomally abnormal fetuses. Important questions need to be asked, such as what is the clinical significance, if any, of becoming chimeric? Is there any evidence of low-grade, chronic, graft versus host disease in parous women due to microchimerism? Is there any relationship between pregnancy and the subsequent development of autoimmune disorders? The eight nonpregnant women described here were all healthy. The ability to detect rare populations of fetal cells via sensitive techniques such as fluorescence-activated cell sorting and PCR will permit further exploration of these important issues.

This work was supported by a Basil O'Connor Starter Scholar Grant from the March of Dimes Birth Defects Foundation to D.W.B. and National Institutes of Health Grant P01-HD18658.

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