PCR Quantitation of Fetal Cells in Maternal Blood in Normal and Aneuploid Pregnancies*

Diana W. Bianchi,[1] John M. Williams,[3] Lisa M. Sullivan,[2] Frederick W. Hanson,[4] Katherine W. Klinger,[5] and Anthony P. Shuber[5]

[1]Departments of Pediatrics, Obstetrics and Gynecology, New England Medical Center and Tufts University School of Medicine, and [2]Section of General Internal Medicine Research Unit, Boston Medical Center, Boston; [3]Genzyme Corporation, Cambridge, MA; [4]The Prenatal Diagnostic and Imaging Center, Sacramento; and [5]Genzyme Genetics, Framingham, MA

Summary

Fetal cells in maternal blood are a noninvasive source of fetal genetic material for prenatal diagnosis. We determined the number of fetal-cell DNA equivalents present in maternal whole-blood samples to deduce whether this number is affected by fetal karyotype. Peripheral blood samples were obtained from 199 women carrying chromosomally normal fetuses and from 31 women with male aneuploid fetuses. Male fetal-cell DNA-equivalent quantitation was determined by PCR amplification of a Y chromosome–specific sequence and was compared with PCR product amplified from known concentrations of male DNA run simultaneously. The mean number of male fetal-cell DNA equivalents detected in 16-ml blood samples from 90 women bearing a 46,XY fetus was 19 (range 0–91). The mean number of male fetal-cell DNA equivalents detected in 109 women bearing a 46,XX fetus was 2 (range 0–24). The mean number of male fetal-cell DNA equivalents detected when the fetus was male compared with when the fetus was female was highly significant ($P = .0001$). More fetal cells were detected in maternal blood when the fetus was aneuploid. The mean number of male fetal-cell DNA equivalents detected when the fetal karyotype was 47,XY,+21 was 110 (range 0.1–650), which was significantly higher than the number of male fetal-cell DNA equivalents detected in 46,XY fetuses ($P = .0001$). Feto-maternal transfusion of nucleated cells appears to be influenced by fetal karyotype. The sixfold elevation of fetal cells observed in maternal blood when the fetus had trisomy 21 indicates that noninvasive cytogenetic diagnosis of trisomy 21 should be feasible.

Introduction

Feto-maternal transfusion of erythrocytes, with its consequence of maternal alloimmunization and fetal anemia, has been appreciated since the 1940s (Allen 1982). The opportunity to use these transfused fetal cells for noninvasive prenatal cytogenetic diagnosis has only recently been realized (Price et al. 1991; Bianchi et al. 1992; Simpson and Elias 1993; Gänshirt-Ahlert et al. 1993; Bianchi 1995). Currently, invasive prenatal diagnostic procedures such as chorionic-villus sampling and amniocentesis are usually offered to women >35 years of age, in the absence of specific fetal abnormalities, positive family history, or other indications of elevated risk. Screening on the basis of maternal age detects <20% of fetuses with trisomy 21 (Verloes et al. 1995), whereas measurement of maternal serum alpha-fetoprotein, human chorionic gonadotropin, and unconjugated estriol detects 65% of fetuses with trisomy 21, at a calculated false-positive rate of 5% (Wald et al. 1995).

As an alternative, we and others are exploring the feasibility and clinical applicability of screening for common fetal aneuploidies by performing FISH analysis of interphase fetal cells circulating in maternal blood. The sensitivity and specificity of this approach is presently being addressed in a multicenter clinical trial that is supported by the National Institutes of Health (de la Cruz et al. 1995).

Review of the published literature has suggested that variable numbers of fetal cells are detectable in maternal blood, range 3–74, in 15–40-ml maternal blood samples (Elias et al. 1992; Wessman et al. 1992; Hawes et al. 1994; Reading et al. 1995; Takabayashi et al. 1995). Our own studies, performed on 20-ml maternal venous blood samples, obtained from women with cytogenetically normal fetuses and enriched for the presence of fetal cells by means of density-gradient centrifugation and flow sorting, revealed a mean fetal-cell number of 21, as ascertained by quantitative PCR techniques (Bianchi et al. 1994). In contrast, other groups, using dissimilar cell-separation methods, demonstrated significantly higher numbers of fetal cells by FISH analysis using chromosome-specific probes, but their patient populations consisted of women with aneuploid fetuses (Elias

et al. 1992; Gänshirt-Ahlert et al. 1993). Because of the relatively small numbers of patients studied in each report, it was impossible to determine whether the discrepancies were due to laboratory technique, patient population, or other, unknown factors. The purpose of the present study, therefore, was to determine as accurately as possible the number of fetal cells circulating in maternal whole-blood samples, thereby deducing whether this number is influenced primarily by fetal karyotype, which affects the biology of the fetoplacental unit, or by laboratory method of cell separation. To perform the work, we developed a technique of quantitation of male fetal-cell DNA equivalents, using PCR amplification of maternal venous blood samples. Our results, based on a large study sample, suggest that fetal cells are present at low levels when the fetus has a normal karyotype and that increased feto-maternal transfusion of nucleated cells occurs when the fetus has a chromosome abnormality.

Material and Methods

Maternal Blood Samples

A total of 230 venous blood samples were studied. Study patients were recruited from clinical sites all over the United States. All women underwent prenatal cytogenetic diagnosis either by amniocentesis (226 cases) or chorionic villus sampling (CVS) (4 cases). In 199 samples, the fetus had a normal karyotype. Of these, 190 were obtained preamniocentesis, and 9 were obtained postamniocentesis. In samples obtained from 31 pregnant women, the fetal karyotype was abnormal and male. Four of these samples were obtained preprocedure, and 27 were obtained postprocedure, at the time when the women were receiving genetic counseling regarding the abnormal diagnosis. Of the 27 samples obtained postprocedure, 4 were drawn post-CVS (mean 12.8 d, range 7–20 d), and 23 were drawn postamniocentesis (mean 14.6 d, range 5–55 d).

After informed consent was obtained under a protocol approved by New England Medical Center and collaborating institutions, ~16 ml of blood were drawn into two vacutainer tubes containing acid citrate dextrose solution A (ACDA) as an anticoagulant. In all cases, gestational age of the fetus was confirmed by sonographic examination. In the 199 fetuses with a normal karyotype, blood samples were processed and results analyzed without prior knowledge of fetal gender. Fetal karyotype was confirmed in the referring physician's office, on a minimum of two separate occasions. When a discrepancy in the fetal gender was noted between the two reports, a third verification was requested, with the infant's gender subsequently being confirmed at birth. In all cases the discrepancies were due to secretarial transcription error, not cytogenetic laboratory error.

Validation of Whole-Blood–PCR Method

Three separate experiments were performed, with two to five replicates (35 total samples) of small numbers (0, 25, 50, or 100) of either Raji cells (a male B-lymphoblastoid cell line), male fetal nucleated blood cells, or male fetal liver cells (Advanced Biosciences Resources), mixed into 8 ml of adult female peripheral blood. No difference in quantitation of male fetal-cell DNA equivalents was observed by the PCR method, on the basis of the type of model fetal cell employed (data not shown). Additional samples containing 0, 5, 10, 25, 50, or 100 Raji cells were then subjected to DNA extraction and amplification conditions, followed by quantitation of Y DNA and statistical analysis, and were compared with fluorescent-microscopy enumeration of Hoechst 33342–stained nuclei in the constructed cell mixture.

PCR

The contents of each 8-ml vacutainer tube were mixed with 30 ml of $1 \times$ erythrocyte lysis buffer (ELB) ($10 \times$ ELB = 82.9 g of NH_4Cl, 10.0 g $KHCO_3$, and 0.2 g of EDTA in 1 liter of dH_2O, pH = 7.4) and were centrifuged, and the supernatant was discarded twice. The remaining pellet was resuspended in nuclear lysis buffer (5 ml of 1 M Tris pH 7.5, 40 ml of 5 M NaCl, 2 ml of 0.5 M EDTA, and 453 ml of dH_2O) with 660 μl of 10% SDS solution and 1.65 ml of 20 mg proteinase K solution/ml and was kept at 37°C overnight and then was subjected to high salt extraction and precipitation using isopropanol and to further purification using a 50% solution of Chelex resin (Bio-Rad). Final resuspension occurred in 2 ml of Tris-EDTA buffer. Extreme caution was observed, to prevent introduction of previously amplified product into the maternal DNA.

Because the Y chromosome is absent in the mother, it was chosen as a marker to quantify the number of male fetal-cell DNA equivalents originating in male fetuses. PCR was performed by use of the primers 49a-06-Y (5'-CTT/TCT/TTT/CAG/GCA/TTT/CCT/GCT/TAT-3')and 49a-07-Y (5'-GTT/CTA/CAG/AAA/AGT/TAT/TGC/CAA/GTA-3'), which map to the long arm of the Y chromosome (Lucotte et al. 1991). The total reaction volume consisted of 10 μl of "hot start" premix (8 μl of dH_2O, 1 μl of $1 \times$ Taq buffer, and 1 μl of Taq polymerase), 10 μl of DNA, 21 μl of dH_2O, 4 μl $10 \times$ Taq buffer, 2.5 μl of $20 \times$ dNTPs, 2 μl of 10 μM Y primers, and 0.5 μl of ^{32}P dCTP. Amplification consisted of 10–30 min at 95°C, followed by 34 cycles at 95°C for 30 s, 55°C for 30 s, and 74°C for 30 s, followed by a 15°C holding temperature. For each reaction, a standard curve was run simultaneously with known amounts of added male genomic DNA (50–400 pg). Amplified product was separated by electrophoresis in a 2% Seakem ME agarose gel, at 175 mA constant current for $1\frac{1}{2}$ h. The gel was then dried and was exposed to the phosphorimager screen for 25 min, followed by

exposure to x-ray film for 2–6 h at −80°C. Results were quantitated by use of a phosphorimager (Molecular Dynamics). Average counts per minute (cpm) were determined for each reaction product by drawing a rectangle around the largest band and subtracting the background cpm in the reaction blanks. The number of cpm in the test samples was either plotted directly against the curve of the amplified product in the male standard DNA or calculated by use of a factor derived from the number of cpm measured divided by the number of picograms of male standard DNA and was converted to a cell number by use of the formula 5 pg of DNA/cell. Results were reported per 8-ml tube of blood and were extrapolated upward or downward to the equivalent of 16 ml if more or less blood originally had been taken from the patient.

Statistical Analysis

Statistical analyses were performed by use of Statistical Analysis Software (SAS). In the first phase of analysis, descriptive statistics were generated for all study variables. Graphical analyses were utilized to understand the distributional properties of study variables. In the second phase, a series of statistical tests were performed. Two-independent-samples t-tests were used to examine differences, in the mean number of male cells detected, between normal karyotypes. Analysis of variance (ANOVA) was used to test for differences, in the mean numbers of male cells detected, among abnormal karyotypes, and least-squares means were compared to assess significance between pairs of abnormal karyotypes. Although the two-independent-samples t-test has been shown to be robust in the presence of skewed distributions (Sullivan and D'Agostino 1992), since the distri-
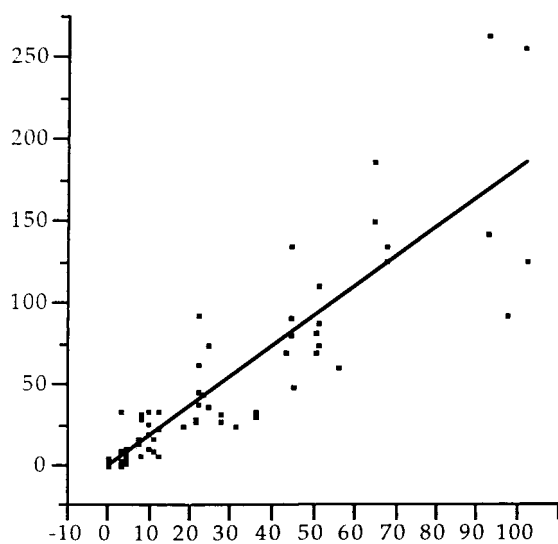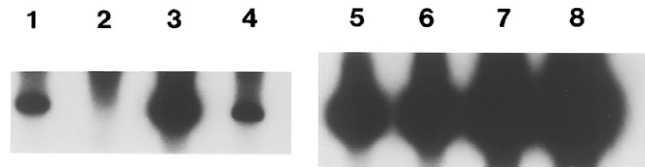
Figure 2   Autoradiograph of amplified PCR product and male standard DNA used for quantitation. These reactions were run simultaneously. Lanes 1 and 4 represent aliquots from the same maternal sample, with 13 and 16 male fetal-cell DNA equivalents detected in the respective lanes. Lane 2 represents a negative control with no added male cells (0 male cells detected). Lane 3 represents a positive control with 142 male cells detected. Lanes 5–8 represent products generated from known amounts (50–400 pg) of male DNA.

bution of the number of male fetal-cell DNA equivalents was not normal, we also investigated nonparametric tests. In all cases the results of the parametric and nonparametric tests were consistent. Thus, results of the parametric tests are presented here. Finally, gestational age and whether the sample was obtained pre- or postinvasive procedure were considered as covariates. The relationship between gestational age and the number of male cells detected was evaluated by use of correlation analysis, and the relationship between whether the sample was obtained pre- or postprocedure and the number of male cells detected was evaluated by use of two-independent-samples t-tests and ANOVA.

Results

Quantitation in Constructed Cell Mixtures

The sensitivity and accuracy of PCR quantitation of male fetal-cell DNA equivalents in whole blood was determined on constructed cell mixtures. These artificial cell mixtures were designed to mimic a small feto-maternal hemorrhage and consisted of the same volume of blood processed for bona fide maternal samples. A correlation coefficient of .89 was observed for the number of male fetal-cell DNA equivalents detected by PCR and the estimated number of cells spiked into constructed cell mixtures (fig. 1).
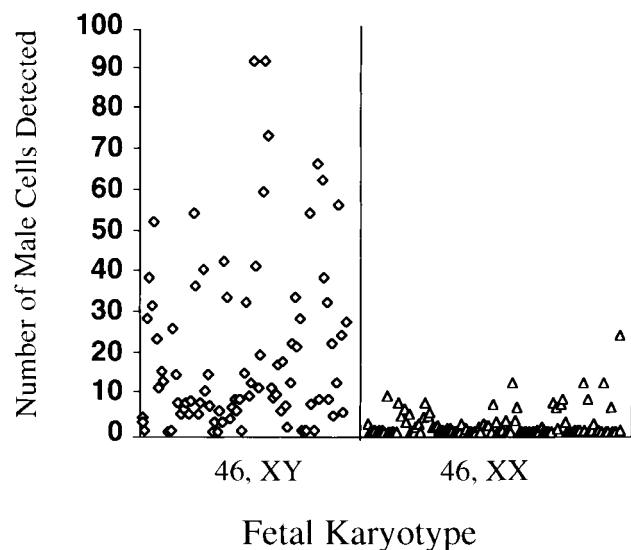
Quantitation in Normal Pregnancies

Of the 199 samples obtained from women with cytogenetically normal prenatal karyotypes, 90 carried 46,XY fetuses and 109 carried 46,XX fetuses. Figure 2 demonstrates the amplified products used for quantitation. The mean number of male fetal-cell DNA equivalents detected in the samples obtained from the 90 women bearing 46,XY fetuses was 19 (range 0–91). Eighty-four percent of women carrying a 46,XY fetus had detectable male DNA at the level of ⩾2 cell equivalents (table 1 and fig. 3). An additional 15.3% of women had male DNA detected in them, but it was at the level of <2 cell equivalents. The mean number of male cells

Figure 1   Dot plot demonstrating relationship between number of nucleated male cells in artificial mixture (*x*-axis), as determined by fluorescent-microscopic enumeration, and average number of male-cell DNA equivalents, as detected by PCR (*y*-axis).

Table 1

Distribution of Number of Cells Detected, According to Karyotype

	PROPORTION IN WHICH NO. OF DETECTED MALE-CELL DNA EQUIVALENTS =[a] (%)				
KARYOTYPE	.0–2.0	2.1–5.0	5.1–10.0	10.1–25.0	≥25.1
46,XX	74.3	11.0	11.0	3.7	0
46,XY	15.6	11.1	22.2	22.2	28.9
47,XY,+21	11.1	0	11.1	11.1	66.7

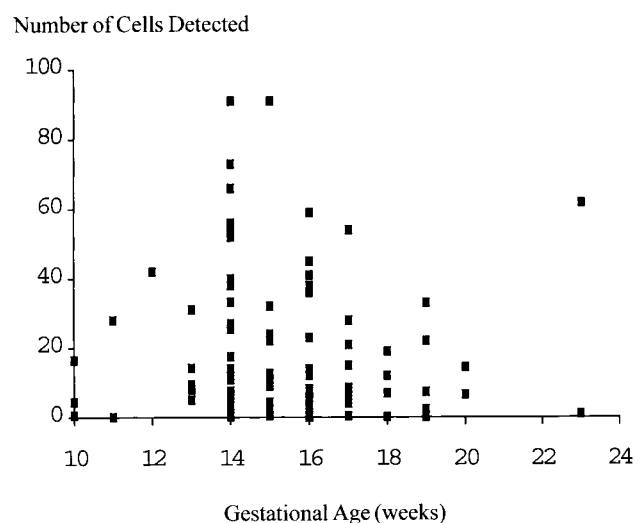[a] Note that, for each karyotype, the row sums to 100%.

detected in the samples drawn from the 109 women carrying 46,XX fetuses was 2 (range 0–24). The number of male fetal-cell DNA equivalents detected when the fetus was male versus when the fetus was female was highly significant ($P = .0001$). The mean gestational ages at which blood samples were obtained were 15.7 and 15.5 wk for 46,XX and 46,XY fetuses, respectively, and were not statistically different ($P = .46$) (fig. 4). In male fetuses, no significant correlation was observed between the number of male cells detected and gestational age at the time of sampling (11–32 wk) ($r = -.07832$; $P = .46$). Because amniocentesis could potentially cause feto-maternal hemorrhage, samples were also analyzed according to when they were obtained. No differences were observed in the mean number of male cells detected pre- versus postamniocentesis, in 46,XX fetuses and 46,XY fetuses ($P = .92$ and .25, respectively) (fig. 5).

Quantitation in Aneuploid Pregnancies

Significantly more fetal-cell DNA equivalents were detected in blood samples from mothers carrying aneuploid fetuses, compared with mothers carrying normal fetuses (88 vs. 10; $P = .0001$) (fig. 6). The mean numbers and ranges of male fetal-cell DNA equivalents detected, according to abnormal karyotype, are shown in table 2. The mean gestational age of the abnormal fetuses was 18.5 wk, which did significantly differ from that of the normal fetuses ($P = .006$), although the 3-wk difference is not of clinical importance. After adjustment for gestational age, however, the values of the male fetal-cell DNA equivalents remained highly significantly elevated in the abnormal fetuses ($P = .0001$). The mean number of cells detected when the karyotype was 47,XY,+21 was 110 (range 0.1–650), which was significantly higher than the mean number of fetal cells detected in normal male (46,XY) fetuses ($P = .0001$). The distribution of numbers of cells detected in 46,XX and 46,XY fetuses versus 47,XY,+21 fetuses is given in table 1. Sixty-six percent of trisomy 21 fetuses had >25 fetal cells de-
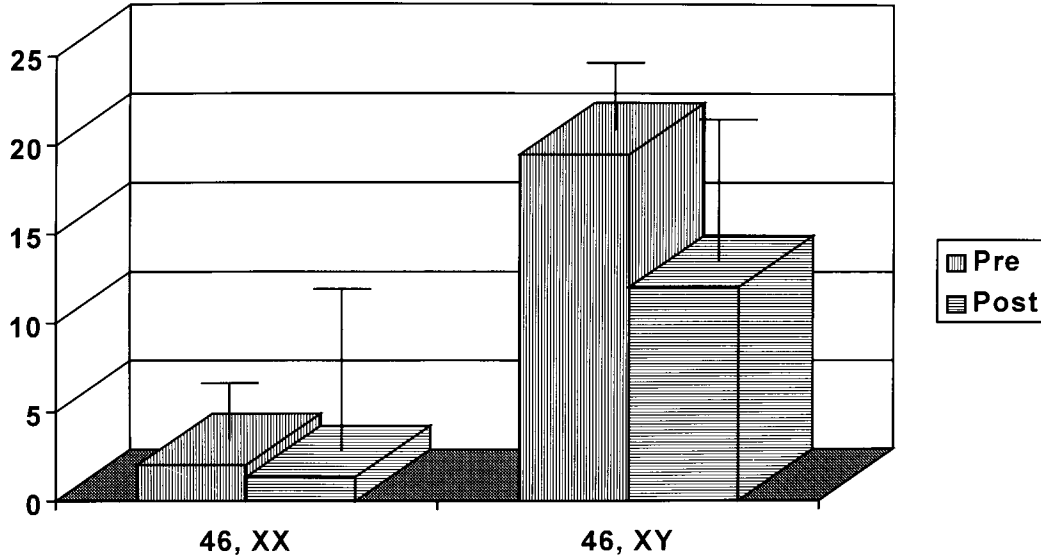

Figure 3 Dot plots demonstrating number of male fetal-cell DNA equivalents detected in 16-ml maternal samples from women carrying fetuses with normal karyotypes. The range of the number of male fetal-cell DNA equivalents detected is shown for 46,XY (*left*) and 46,XX fetuses.

Figure 4 Number of male-cell DNA equivalents detected, as a function of gestational age. Data shown are for 46,XY fetuses only.
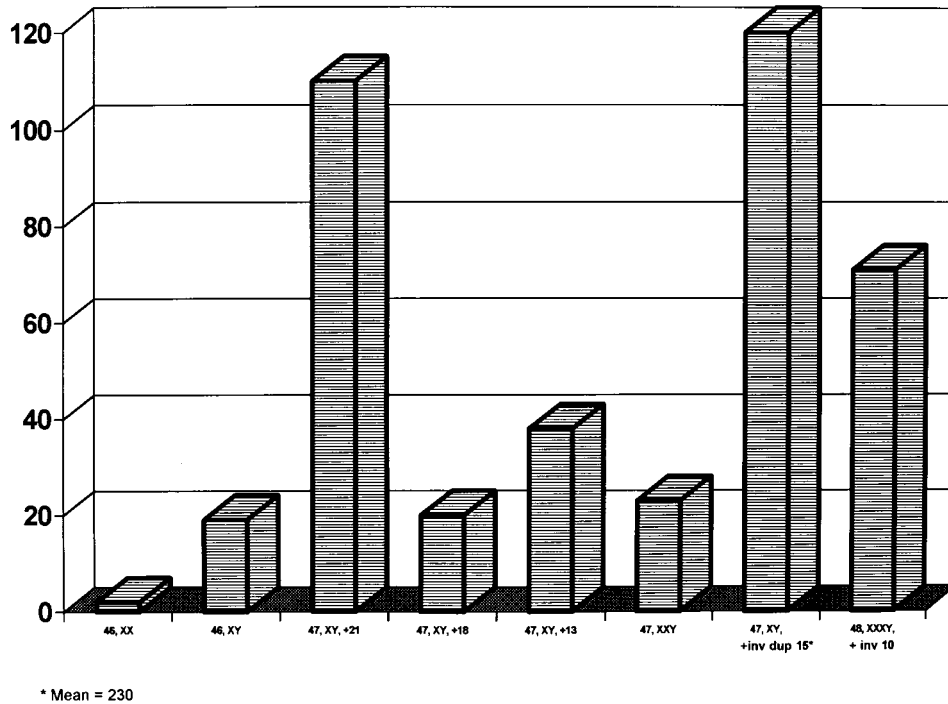
Figure 5   Bar graph demonstrating mean number of male fetal-cell DNA equivalents detected in 16-ml maternal samples obtained from women carrying normal fetuses pre- and postamniocentesis. The numbers of samples represented in this graph are as follows: 46,XX-pre, 105; 46,XX-post, 4; 46,XY-pre, 85; and 46,XY-post, 5.

tected. Similarly, the difference between the number of male fetal-cell DNA equivalents detected in 47,XY,+inv(dup)15 and 46,XY fetuses was also highly significant ($P = .0001$). In pairwise comparisons, no significant differences were observed between 46,XY and either 47,XY,+13, 47,XXY, or 47,XY,+18. However, the lack of significance may be due to small numbers—and, therefore, low power in the statistical tests.

To ascertain whether inclusion of the samples from the four women who underwent CVS biased the data,

* Mean = 230

Figure 6   Bar graph demonstrating mean number of male fetal-cell DNA equivalents detected in maternal samples, stratified by fetal karyotype. Note that the highest number of male fetal-cell DNA equivalents is detected when the fetus has 47,XY,+21 or 47,XY,+inv(dup)15. The asterisk (*) indicates that the values for 47,XY,+inv(dup)15 are off the scale, with a mean value of 230.

Table 2

Number of Male-Cell DNA Equivalents Detected in Aneuploid Cases

Fetal Karyotype (No. of Samples)	Mean ± SD (Range) of Male-Cell DNA Equivalents Detected
47,XY,+21 (18)	110 ± 162 (.1–650)
47,XY,+18 (6)	20 ± 25 (0–58)
47,XY,+13 (2)	38 ± 54 (.1–76)
47,XXY (2)	23 ± 2 (21–24)
47,XY,+inv(dup)15 (2)	230 ± 269 (40–420)
48,XXXY,+inv10 (1)	7

we analyzed these samples separately. Of the four samples, three were from women whose fetuses had 47,XY,+21 and one was from a woman whose male fetus had trisomy 18. The number of male fetal-cell DNA equivalents for the first three samples was 9, 22, and 91, respectively. The number of fetal-cell DNA equivalents for the single 47,XY,+18 sample was 58. As stated above, the mean number of fetal-cell DNA equivalents in all maternal samples carrying fetuses with trisomy 21 was 110. Removal of the three post-CVS patients raised the mean value for trisomy 21 to 123. Removal of the trisomy 18 patient reduced the mean for trisomy 18 from 20 to 12. Therefore, inclusion of the samples obtained from women who were postprocedure CVS did not significantly affect the results and conclusions.

Discussion

We have described a quantitative fetal-cell DNA-equivalent assay designed to detect rare numbers of fetal cells circulating in maternal blood. Because this assay was performed on maternal whole-blood samples, as opposed to samples enriched for the presence of a specific fetal-cell type by a variety of cell-separation techniques, the fundamental question of how many fetal cells were present in a starting sample could be addressed. This assay detected DNA derived from all fetal nucleated cells in the sample, including trophoblasts, erythroblasts, lymphocytes, and granulocytes.

Our results indicate that male fetal DNA is detectable in 99.3% of pregnant women carrying male fetuses, when a 16-ml blood sample is assayed. No unique marker could be used to distinguish female fetal cells from maternal cells in a similar PCR-based assay; however, we assume that the same proportion of female fetal cells would be detected in maternal blood if such a marker were available.

In the fetuses with normal chromosomes, a highly significant difference was observed in the number of male fetal-cell DNA equivalents detected when the fetus was male versus when it was female. This database rep-

resents the largest population of maternal samples studied to date by use of a single uniform assay. The data demonstrate that feto-maternal transfusion of nucleated cells is a common phenomenon but that the mean number of fetal cells detected in a 16-ml sample is perhaps lower than previously had been expected. On average, it appears that ~1 nucleated fetal-cell DNA equivalent/ml of maternal whole blood is present when the fetus has a normal karyotype.

The observation that low numbers of male fetal cells could be detected in some blood samples from women carrying normal female fetuses was unexpected. This phenomenon was observed in 28/109 (25.7%) of women carrying female fetuses, although only 16/109 (14.7%) of women had >5 male cells detected and only 4/109 women (3.7%) had ⩾10 fetal cells measured. Possible sources of male cells in the blood samples from pregnant women include a vanishing-twin gestation, previous maternal blood transfusions from a male donor, or the history of a prior pregnancy with a male fetus. The long-term persistence of fetal CD34+CD38+ cells postpartum has recently been demonstrated elsewhere (Bianchi et al. 1996). In the current study, no information was available regarding the maternal transfusion history. Review of the prior pregnancy history was retrospectively ascertained. In women carrying 46,XX fetuses and with no prior history of a male child, the mean number of male cells detected was 1. In women carrying 46,XX fetuses, there was also no association between the number of prior male children and the number of male fetal-cell DNA equivalents detected. Interestingly, however, there was an association between the age of the most recently delivered son and the number of male fetal-cell DNA equivalents. The women who were currently pregnant with female fetuses whose sons were ⩾8 years of age had the most male cells detected.

The most important conclusion from this study is that there is a significantly higher number of fetal cells present in maternal blood when the fetus has trisomy 21. Trisomy 21, which occurs in 1/1,000 births, is the most common autosomal aneuploidy among live borns that results in serious mental retardation and congenital anomalies. The sixfold elevation in fetal cells observed in the maternal circulation when the fetus had trisomy 21 should bode well for noninvasive cytogenetic screening for trisomy 21. This phenomenon is in agreement with pathologic studies that demonstrate placental abnormalities such as immaturity and hydrops in cases of trisomies 21, 13, and 18 (Labbé et al. 1989).

The other cases of aneuploidy represented in our database suggest that increased feto-maternal transfusion also occurs in other fetal cytogenetic abnormalities, such as 47,XXY (Klinefelter syndrome) and 47,XY,+inv-(dup)15. In our experience, cases of trisomy 18 were the most variable in number of fetal cells detected. The number of cases remains small, so no definitive conclu-

sions can be made at present. Although in this study we did not receive blood samples from any women with a male triploid fetus (69,XXY or 69,XYY), our previous experience in performing FISH using chromosome-specific probes on samples from women carrying female triploid fetuses (69,XXX) suggests that, in triploidy, feto-maternal transfusion is also increased (Zheng et al. 1995). This is not surprising, considering the well-documented placental and hematologic abnormalities that occur in triploid fetuses (Strobel and Brandt 1989; Kuhlmann et al. 1990).

Because most of the samples from women carrying abnormal fetuses were obtained at a mean of 2 wk postamniocentesis, the question of amniocentesis effect was also addressed; and no differences were seen. We recognize the potential bias due to the majority of aneuploid fetuses being ascertained postprocedure. Unfortunately, the relatively low prevalence of aneuploidy makes it difficult to identify affected fetuses prior to amniocentesis or CVS. However, if the increased number of fetal cells detected in cases of trisomy 21 were due to the amniocentesis procedure, then we would have seen equivalent postprocedural increases in the cases of trisomies 18 and 13. Futhermore, we studied three women carrying male fetuses sequentially, and in all three cases the mean number of fetal cells decreased after the amniocentesis (data not shown).

These data help to resolve some of the inconsistencies observed in prior studies of fetal cells in maternal blood. Given the low numbers of fetal cells present in most maternal samples when the fetus is cytogenetically normal, any loss deriving from fetal cell–enrichment techniques may result in the inability to detect fetal cells. Similarly, the data explain why the best detection of fetal cells in maternal blood has occurred in laboratories working with samples from women carrying aneuploid pregnancies (Elias et al. 1992; Gänshirt-Ahlert et al. 1993). The precise knowledge of the number of fetal cells expected in a maternal sample will help to clearly focus strategies for their isolation and analysis. Knowing that relatively few cells will be present in most maternal samples studied dictates that techniques that emphasize fetal-cell yield are more important than fetal-cell purity. Importantly, our data add to the growing body of information regarding fundamental differences in the biology of the fetoplacental unit in aneuploid gestations.

Acknowledgments

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