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# Rapid Clearance of Fetal DNA from Maternal Plasma

Y. M. Dennis Lo,[1] Jun Zhang,[1] Tse N. Leung,[2] Tze K. Lau,[2] Allan M. Z. Chang,[2] and N. Magnus Hjelm[1]

Departments of [1]Chemical Pathology and [2]Obstetrics and Gynecology, Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong

## Summary

**Fetal DNA has been detected in maternal plasma during pregnancy. We investigated the clearance of circulating fetal DNA after delivery, using quantitative PCR analysis of the sex-determining region Y gene as a marker for male fetuses. We analyzed plasma samples from 12 women 1–42 d after delivery of male babies and found that circulating fetal DNA was undetectable by day 1 after delivery. To obtain a higher time-resolution picture of fetal DNA clearance, we performed serial sampling of eight women, which indicated that most women (seven) had undetectable levels of circulating fetal DNA by 2 h postpartum. The mean half-life for circulating fetal DNA was 16.3 min (range 4–30 min). Plasma nucleases were found to account for only part of the clearance of plasma fetal DNA. The rapid turnover of circulating DNA suggests that plasma DNA analysis may be less susceptible to false-positive results, which result from carryover from previous pregnancies, than is the detection of fetal cells in maternal blood; also, rapid turnover may be useful for the monitoring of feto-maternal events with rapid dynamics. These results also may have implications for the study of other types of nonhost DNA in plasma, such as circulating tumor-derived and graft-derived DNA in oncology and transplant patients, respectively.**

## Introduction

The two-way transfer of nucleated cells between the mother and fetus is now a well-established phenomenon (Walknowska et al. 1969; Lo et al. 1989, 1996). In clinical use, the transfer of fetal cells into maternal blood provides a source of fetal genetic material for noninvasive prenatal diagnosis (Bianchi et al. 1990; Simpson and Elias 1995; Cheung et al. 1996). After delivery, most fetal cells are cleared by 2–3 mo postpartum (Lo et al. 1993; Hamada et al. 1994; Thomas et al. 1995). By the use of cell sorting and sensitive PCR assays, fetal hematopoietic progenitor cells have been shown to persist in some women, even decades after delivery (Bianchi et al. 1996). The latter phenomenon has been proposed to be associated with certain autoimmune disorders (Artlett et al. 1998; Nelson et al. 1998).

We have shown recently that, in addition to the presence of fetal cells in maternal blood, cell-free fetal DNA is also present in maternal circulation (Lo et al. 1997). By means of a quantitative PCR assay, fetal DNA has been demonstrated to be present in high concentrations in maternal plasma (Lo et al. 1998*a*). This observation suggests that plasma fetal DNA analysis may have clinical applications in the noninvasive prenatal diagnosis of certain disorders, including sex-linked diseases and fetal hemolytic disease resulting from Rh blood group incompatibility (Lo et al. 1997; Bianchi 1998). Little is known about the parameters governing the level of circulating fetal DNA, except that it tends to increase as gestation progresses, especially toward the end of pregnancy (Lo et al. 1998*a*).

In the present study, we investigated the clearance of fetal DNA from maternal plasma after delivery. If a steady-state situation is assumed, the kinetics of fetal DNA clearance will allow estimation of the rate of fetal DNA release into maternal circulation. The rate of clearance will also provide information as to the applicability of fetal DNA measurement in the study of the dynamic processes involved in the handling of circulating DNA during pregnancy. We also aim to obtain data about the role of plasma nucleases in the clearance of fetal DNA from maternal circulation.

Our study relied on the recent development of a real-time quantitative PCR assay for fetal-derived DNA sequences in maternal plasma (Lo et al. 1998*a*). For fetal DNA detection, we used the sex-determining region Y (SRY) gene on the Y chromosome, as a marker for male fetuses. In the first stage of our project, we studied 12 women with male babies, at various times after delivery.

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Address for correspondence and reprints: Dr. Y. M. Dennis Lo, Department of Chemical Pathology, Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong SAR. E-mail: loym@cuhk.edu.hk

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Having established that fetal DNA is cleared very rapidly after parturition, we then carried out serial sampling of 8 women, to obtain a high time-resolution picture of fetal DNA clearance. These data are valuable for furthering our understanding of the feto-maternal transfer of nucleic acids.

## Subjects and Methods

### *Subjects*

Pregnant women attending the Department of Obstetrics and Gynecology at the Prince of Wales Hospital, Shatin, Hong Kong, were recruited, and informed consent was obtained. Approval for the study was obtained from the Research Ethics Committee of the Chinese University of Hong Kong. For the first part of the project, healthy pregnant women were recruited just after the onset of labor. Samples of maternal peripheral blood (5–10 ml) were collected into tubes containing EDTA. Only subjects who subsequently delivered male babies vaginally were followed-up, and blood was obtained by venesection, at a single time point, after delivery. Ten subjects who subsequently delivered female fetuses were recruited as negative controls. Blood samples were obtained from subjects at one of the following time points: 6 wk, 1 wk, or 1 d after delivery. For the second part of the project, subjects were recruited just before undergoing elective cesarean section. These women were free of any medical disease or any antenatal complications, and their elective cesarean sections were indicated because of previous cesareans or (in one case) because of abnormal fetal lie. The single case of abnormal fetal lie (subject S3) had not been subjected to external cephalic version. An ultrasound scan was done, to ascertain fetal sex (subsequently confirmed at delivery), and only subjects determined to be carrying male babies were studied. Maternal peripheral blood samples (5 ml) were collected, before cesarean section, into an EDTA-containing tube. After delivery of the baby, 2 ml maternal peripheral blood was collected into an EDTA tube at 5, 15, 30, 45, 60, and 120 min postpartum. A total of 8 subjects with male babies were studied. For the 10 subjects recruited for the study of the role of plasma nucleases, 5 ml maternal blood was collected into a plain tube before cesarean section and at 2 h after delivery.

### *Sample Preparation*

Blood samples from subjects, obtained at a single postdelivery time point, were processed as described elsewhere (Lo et al. 1998*a*). For subjects recruited for the serial sampling project and for the study of the role of plasma nucleases, a researcher was present at the time of collection of the pre- and postdelivery samples, which were transported instantly to the laboratory, for immediate processing. Blood samples were centrifuged at 3,000 *g,* and plasma was removed and transferred into plain polypropylene tubes. The plasma samples were recentrifuged at 3,000 *g,* and the supernatants were collected into fresh polypropylene tubes. The samples were stored at −20°C until further processing.

### *DNA Extraction from Plasma Samples*

DNA from plasma samples was extracted by use of a QIAamp Blood Kit (Qiagen); the blood and body fluid protocol, as recommended by the manufacturer, was followed (Chen et al. 1996). For subjects whose blood was sampled at a single postdelivery time point, an 800-$\mu$l plasma sample was used for DNA extraction; 200 $\mu$l plasma was used, for DNA extraction, for subjects whose blood was sampled at multiple postdelivery time points and for subjects recruited for the study of the role of plasma nucleases.

### *Real-Time Quantitative PCR*

Real-time quantitative PCR analysis was done as described elsewhere, by use of a 7700 Sequence Detector (PE Applied Biosystems), which is essentially a combined thermal cycler and fluorescence detector with the ability to monitor the progress of individual PCR reactions optically (Heid et al. 1996; Lo et al. 1998*a*). The amplification and product reporting system used is based on the 5′ nuclease assay (TaqMan assay; Perkin-Elmer; Holland et al. 1991). In this system, apart from the two amplification primers as used in conventional PCR, a dual-labeled fluorogenic hybridization probe is also included (Lee et al. 1993; Livak et al. 1995). One fluorescent dye serves as a reporter (6-carboxyfluorescein), and its emission spectrum is quenched by a second fluorescent dye (6-carboxy-tetramethylrhodamine). During the extension phase of PCR, the 5′-to-3′ exonuclease activity of the *Taq* DNA polymerase cleaves the reporter from the probe, thus releasing it from the quencher, resulting in an increase in fluorescent emission at 518 nm. This sequence detector is able to measure the fluorescent spectra of the 96 amplification wells continuously during DNA amplification, and data are captured onto a Macintosh computer (Apple Computer).

Primer and probe sequences for the SRY and the $\beta$-globin genes were as described elsewhere (Lo et al. 1998*a*). TaqMan amplification reactions were set up in a reaction volume of 50 $\mu$l, with components (except TaqMan probes and amplification primers) supplied in a TaqMan PCR Core Reagent Kit (Perkin-Elmer). TaqMan probes were custom-synthesized by PE Applied Biosystems. PCR primers were synthesized by Life Technologies. Each reaction contained 5 $\mu$l 10 × buffer A; 300 nM each amplification primer; 100 nM of the corresponding TaqMan probe; 4 mM $MgCl_2$; 200 $\mu$M each

dATP, dCTP, and dGTP; 400 μM dUTP; 1.25 U AmpliTaq Gold; and 0.5 U AmpErase uracil *N*-glycosylase. Five microliters of the extracted plasma DNA were used for amplification. DNA amplifications were performed in 96-well reaction plates that were frosted by the manufacturer to prevent light reflection and that were closed with caps designed to prevent light scattering (Perkin-Elmer). Each sample was analyzed in duplicate. A calibration curve was run in parallel and in duplicate with each analysis. The conversion factor of 6.6 pg DNA/cell (Saiki et al. 1988) was used to express the results as copy numbers.

An identical thermal profile was used for both the SRY and β-globin TaqMan systems. Thermal cycling was initiated with a 2-min incubation at 50°C for the uracil *N*-glycosylase to act, followed by a first denaturation step of 10 min at 95°C. Next, 40 cycles of 95°C for 15 s and 60°C for 1 min were performed.

Amplification data collected by the 7700 Sequence Detector and stored in the Macintosh computer were then analyzed by means of the Sequence Detection System software (PE Applied Biosystems). The mean quantity of each duplicate was used for further concentration calculation. The concentration, expressed in copies per milliliter, was calculated by use of the equation

$$C = Q\left(\frac{V_{DNA}}{V_{PCR}}\right)\left(\frac{1}{V_{ext}}\right),$$

where $C$ = target concentration in plasma (copies per milliliter); $Q$ = target quantity (copies), determined by sequence detector in a PCR; $V_{DNA}$ = total volume of DNA, obtained after extraction, typically 50 μl per Qiagen extraction; $V_{PCR}$ = volume of DNA solution used for PCR, typically 5 μl; and $V_{ext}$ = volume of plasma extracted, either 0.2 ml or 0.8 ml.

### *Anticontamination Measures*

Strict precautions against PCR contamination were taken (Kwok and Higuchi 1989). Aerosol-resistant pipette tips were used for all liquid handling. Separate areas were used for setup of amplification reactions, addition of DNA template, and execution of amplification reactions. The 7700 Sequence Detector offered an extra level of protection, because its optical detection system obviated the need to reopen the reaction tubes after completion of the amplification reactions, thus minimizing the possibility of carryover contamination. In addition, the TaqMan assay included a further anticontamination measure in the form of preamplification treatment with uracil *N*-glycosylase, which destroyed uracil-containing PCR products (Longo et al. 1990). Multiple negative-water blanks were included in each analysis.

## Results

### *Subjects Studied before Delivery and at One Time Point after Delivery*

Twelve subjects with male babies were studied. Plasma samples collected before delivery revealed the presence of fetal-derived SRY sequences, in all instances (table 1). Fetal DNA was not detected in any of the postpartum samples with collection times of 1–42 d after delivery. β-globin TaqMan PCR was done for all pre- and postdelivery samples, which demonstrated the presence of amplifiable DNA in all instances. None of the predelivery plasma samples taken from the 10 women who gave birth to female fetuses had detectable SRY signals.

### *Sequential Follow-Up after Delivery*

Eight women were recruited just before undergoing elective cesarean section. Plasma samples were obtained before cesarean section and at 5, 15, 30, 45, 60, and 120 min after delivery. β-globin TaqMan PCR was done for all pre- and postdelivery samples, which demonstrated the presence of amplifiable DNA in all instances. The fetal DNA concentration in maternal plasma was determined by use of real-time quantitative PCR (fig. 1). In three instances (subjects S1, S3, and S6), there was a rise in plasma fetal DNA concentration shortly after delivery, at 5 min, compared with the predelivery value. Seven of the eight women had no detectable plasma fetal DNA by 120 min postpartum. The remaining subject (S6) had 90% of maternal plasma fetal DNA cleared by 120 min after delivery. The mean time taken to reduce the peak plasma fetal DNA concentration by 50% was 16.3 min (range 4–30 min).

### *Role of Plasma Nucleases*

To study the role of plasma nucleases in the clearance of fetal DNA from maternal plasma, we collected pe-

**Table 1**

**Detection of Fetal DNA in Maternal Plasma**

| Case | Predelivery (copies/ml) | Postdelivery (copies/ml) | Time postdelivery (days) |
|---|---|---|---|
| P1 | 29.0 | 0 | 42 |
| P2 | 112.5 | 0 | 42 |
| P3 | 80.0 | 0 | 7 |
| P4 | 337.5 | 0 | 7 |
| P5 | 27.5 | 0 | 7 |
| P6 | 41.3 | 0 | 7 |
| P7 | 33.8 | 0 | 7 |
| P8 | 50.3 | 0 | 1 |
| P9 | 63.5 | 0 | 1 |
| P10 | 176.1 | 0 | 1 |
| P11 | 99.4 | 0 | 1 |
| P12 | 3,024.0 | 0 | 1 |

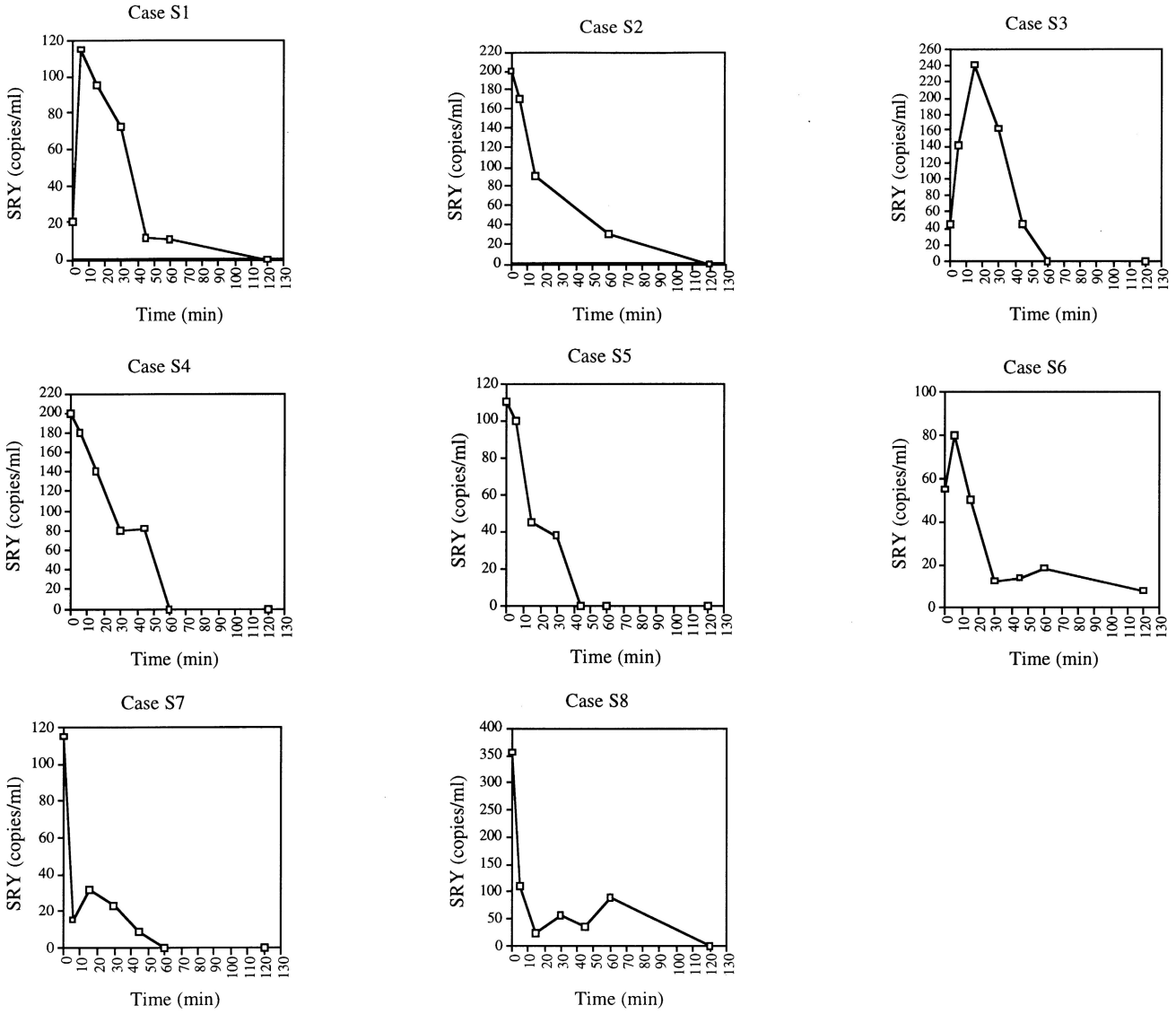


**Figure 1** Sequential follow-up of circulating fetal-derived SRY sequence in plasma of women, after cesarean section. Time (min) after delivery is plotted on the *X*-axis, and the plasma SRY level (copies/ml) is plotted on the *Y*-axis. Time of "0" denotes the predelivery sample.

ripheral blood samples, before cesarean section, from 10 women carrying male fetuses. The blood samples were collected into plain tubes, transported instantly to the laboratory, and processed immediately. After incubation at 37°C for 2 h, samples from three subjects (N1, N5, and N9) had plasma fetal DNA concentrations >90% of preincubation values (fig. 2). The remaining seven subjects' samples had concentrations with a range of 31%–74% of the values before incubation (fig. 2). At 2 h after delivery, a second peripheral blood sample was obtained from each of these women. Nine subjects did not have detectable circulating fetal DNA in their plasma. The remaining subject (N6) had a circulating fetal DNA level at 12% of that of the predelivery sample.

## Discussion

In this study, we present the first data on the clearance of fetal DNA from maternal plasma. Previous work on the clearance of fetal cells from maternal blood indicates that the clearance in most subjects takes place over a period of weeks (Lo et al. 1993; Hamada et al. 1994; Thomas et al. 1995) and that, in certain persons, fetal hematopoietic progenitors are detectable, even decades after delivery (Bianchi et al. 1996). These data prompted us to start our investigation into fetal DNA clearance at 6 wk (42 d) after delivery. When no fetal DNA was detectable in maternal plasma at 6 wk, we gradually reduced the time interval studied, to gauge the time

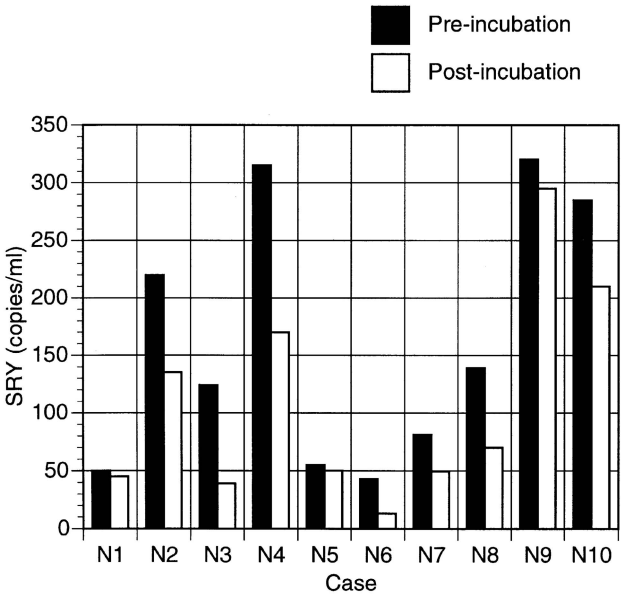


**Figure 2** Effect of plasma nucleases on the clearance of circulating fetal DNA. Blood samples were obtained from 10 women (N1–N10), before delivery. Plasma SRY levels (copies/ml) were assayed, both before and after incubation, at 37°C for 2 h.

frame of fetal DNA clearance. This approach allowed us to determine that fetal DNA is cleared very rapidly from maternal plasma, with the first phase of our project indicating that fetal DNA became undetectable by 1 d after delivery.

In the second part of the project, we used serial blood sampling of 8 pregnant women carrying male fetuses who were delivered by cesarean section. We chose women undergoing cesarean section, because the time of delivery could be easily and accurately determined in these subjects, and because the time of blood sampling could be planned in advance. These data confirm the rapid clearance of fetal DNA from maternal plasma. Indeed, seven of the eight women had undetectable plasma fetal DNA by 2 h postpartum. The single subject (S6) with detectable plasma fetal DNA had 90% of circulating fetal DNA cleared by 2 h postpartum. In three instances (subjects S1, S3, and S6), there was a noticeable rise in plasma fetal DNA concentration shortly after delivery, at 5 min, compared with the predelivery value. This phenomenon may be a result of delivery-associated processes operating in these subjects, which could have resulted in increased direct liberation of fetal DNA or in increased indirect fetal DNA release, secondary to the destruction of fetal nucleated cells, after their entry into maternal blood (e.g., because of feto-maternal hemorrhage). Review of the obstetric notes indicates that, of the eight women, three (subjects S1, S3, and S8) underwent manual removal of the placenta, whereas the remaining five had the placenta removed by the more gentle procedure of controlled cord traction. It is possible that the potentially more traumatic procedure of manual removal precipitated a release of fetal DNA into the maternal circulation in the two examples in which the largest rise in fetal DNA after the procedure was demonstrated (subjects S1 and S3). Study of the curves revealed that fetal DNA clearance appeared to occur in two phases, with different kinetics: an initial, more rapid phase, followed by a slower phase. This observation suggests that more than one mechanism may be involved in the clearance of circulating fetal DNA.

Our data demonstrate the rapid clearance of circulating fetal DNA after delivery. Assuming there are no abrupt changes in circulating fetal DNA clearance associated with delivery, one may be able to extrapolate our findings to the predelivery state, so as to gain mechanistic insights about the physiology of DNA transfer from the fetus to the mother. On the basis of this assumption, in spite of the rapid clearance of fetal DNA in maternal plasma, previous work has shown that fetal DNA is present in high concentrations and is readily detectable in maternal circulation during pregnancy (Lo et al. 1998*a*). These observations suggest that, to maintain a steady state, fetal DNA must be liberated in large quantities into maternal circulation. Our previous work has shown that the mean maternal plasma fetal DNA concentration in the third trimester of pregnancy is 292 copies/ml (Lo et al. 1998*a*). If a plasma volume of 2,500 ml is assumed, the total amount of fetal DNA that is present in maternal circulation is $292 \times 2{,}500$ copies $= 7.3 \times 10^5$ copies. With a mean plasma half-life of 16.3 min, 50% of these $7.3 \times 10^5$ copies (i.e., $3.65 \times 10^5$ copies) will be cleared in 16.3 min. The mean fetal DNA clearance rate is estimated to be $2.24 \times 10^4$ copies/min. In the steady state, the liberation rate of fetal DNA should be equal to this clearance rate. Therefore, our calculations suggest that fetal DNA is liberated at a mean rate of $2.24 \times 10^4$ copies/min into the maternal circulation. It should be noted that these calculations represent an average only and that there are significant variations in the fetal DNA clearance rate (and the liberation rate) among individual cases, as evidenced by the range of half-lives observed. Nonetheless, these baseline data would be very useful to guide future studies on the pathologic or physiologic parameters affecting the liberation or clearance of fetal DNA from maternal blood. An interesting example is pregnancies complicated by Rh blood group incompatibility, in which increased liberation of fetal DNA may occur because of hemolysis of fetal nucleated red cells.

The rapid turnover of plasma fetal DNA implies that its level provides an almost real-time picture of fetal DNA production and clearance and, thus, may be useful for monitoring feto-maternal events having rapid dy-

namics. Fetal cells in maternal blood, the concentrations of which change relatively slowly over the course of weeks (Hamada et al. 1994), may not be as good a marker for these events. One possible example is the monitoring of the occurrence and resolution of feto-maternal hemorrhage, in conjunction with established methods of detecting fetal hemoglobin–containing cells (e.g., the Kleihauer test). The SRY PCR system, described in the present study, is applicable only to pregnancies involving male fetuses. The application of plasma PCR to pregnancies involving female fetuses will require the development of quantitative PCR systems that are applicable to autosomal polymorphic loci. Potential loci suitable for this type of analysis have been described elsewhere (Lo et al. 1996).

For prenatal diagnostic purposes, the rapid clearance of fetal DNA in maternal plasma makes the approach described in the present study less susceptible to false-positive results that are caused by the persistence of fetal DNA from one pregnancy into the next. The persistence of fetal cells has been raised as a potential source of false positivity when fetal cells in maternal blood are used for noninvasive prenatal diagnosis (Hsieh et al. 1993; Bianchi et al. 1996).

Other work has demonstrated the existence of nucleases in the plasma of humans (Herriott et al. 1961). Experiments involving the injection of purified DNA into animals has indicated a role for plasma nucleases in the degradation of the injected DNA (Paoletti et al. 1963; Gosse et al. 1965; Emlen and Mannik 1984). The significance of these data with regard to the clearance of circulating fetal DNA is unclear, because the starting material in these animal experiments are DNA preparations that have been purified highly and that are derived from other species. To elucidate the role of plasma nucleases in the clearance of circulating fetal DNA, we incubated plasma from 10 pregnant women at 37°C, after blood collection. We used nonanticoagulated blood for this experiment, to avoid the potential inhibitory effect of anticoagulants on plasma nucleases (Herriott et al. 1961). To avoid the release of nuclease inhibitors after blood clotting (Frost and Lachmann 1968), venesection was done in the presence of a researcher, who immediately transported the blood samples to the laboratory, where it was processed promptly. In vitro incubation of fresh plasma samples for 2 h at 37°C resulted in the incomplete removal of plasma fetal DNA in all instances. In contrast, the analysis of a second maternal blood sample, collected 2 h after delivery, revealed the complete clearance of circulating fetal DNA in 9 of the 10 subjects. These data show that plasma nucleases play only a partial role in the removal of circulating fetal DNA in most subjects and suggest that other organ systems also contribute toward the clearance of circulating fetal DNA. Previous work done on the basis of the injection of extracted, foreign DNA into experimental animals variously has implicated the liver, spleen (Chused et al. 1972; Emlen and Mannik 1978), and kidney (Tsumita and Iwanaga 1963) as being involved in the removal of circulating DNA. Further work to elucidate the important organ systems in fetal DNA clearance may require the study of subjects with known disorders in the respective organ systems, such as persons with chronic liver disease or renal failure.

The presence of fetal DNA in maternal plasma is only one of a number of clinical scenarios in which nonhost DNA can be found in the plasma of human subjects. The other two situations are in oncology and transplant patients, in whom tumor-derived and donor-derived DNA has been demonstrated to circulate in the peripheral blood (Chen et al. 1996; Nawroz et al. 1996; Lo et al. 1998*b*). It is likely that, analogous to the rapid clearance of fetal DNA from maternal blood, tumor-derived and organ donor–derived DNA may also be rapidly removed. The rapid kinetics of circulating DNA potentially will make plasma-based molecular diagnostics very useful for monitoring dynamic changes in patients with cancer and in transplant recipients under specific clinical circumstances. One possible application is in the monitoring of potential complications of cancer chemotherapy, for example, the tumor lysis syndrome.

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