Detection of Maternal Cells in Human Umbilical Cord Blood Using
Fluorescence In Situ Hybridization

By Jeff M. Hall, Patricia Lingenfelter, Sharon L. Adams, Daniel Lasser, John A. Hansen, and Michael A. Bean

Cord blood is a potential source of hematopoietic stem cells for transplantation and is being used on a growing number of patients. However, there are concerns that cord blood might be contaminated with maternal cells that could lead to graft-versus-host disease. To ascertain the extent to which maternal cell contamination of cord blood occurs, we examined 49 cord blood samples from male babies for maternal cells by fluorescence in situ hybridization using probes to the X and Y chromosomes. A minimum of 1,000 nuclei were scored from each sample, and maternal cells were found in 7 of the 49 cord bloods, at levels ranging from 0.04% to 1.0%. In addition, in 39 and 27 of the cord blood samples, respectively, we examined the CD8+ and CD34+ cell populations for maternal cells. Maternal cells were found in 5 of the 39 CD8+ fractions and in 1 of the 27 CD34+ fractions, at levels similar to that found in the unseparated mononuclear fraction or the CD8 or CD34+ fractions in 10 of the 49 cord blood samples (20%). These results show that maternal cells are present in a substantial number of cord bloods, and that some of these maternal cells are T cells.

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Cord Blood WAS FIRST PROPOSED as a source of hematopoietic stem cells for use in transplantation by Broxmeyer et al. in 1989, and the first patient was treated using cord blood from an HLA-identical sibling in the same year. Cord blood transplants have now been used in over 50 cases to treat diseases such as Fanconi’s anemia, leukemia, lymphoma, and aplastic anemia, and banking of cord blood for transplantation is currently being proposed or is actually underway at several sites. However concerns have been expressed about contamination of cord or placental blood with maternal cells, particularly T-cells, which might lead to problems with graft-versus-host disease (GVHD). The presence of maternal cells in cord blood could be the result of minor leakage during pregnancy, leakage caused by trauma during parturition, or specific trafficking by motile cells during pregnancy. Examples of maternal to fetal hemorrhage exist in the literature, although large scale bleeds are relatively uncommon. Nonetheless, even small numbers of maternal cells could contain reactive T cells that might be capable of later clonal expansion against host tissues.

In the present study, we have used fluorescence in situ hybridization (FISH) to screen male cord blood samples for the presence of female cells of maternal origin. Although tedious, the use of FISH to detect rare cells offers direct visual confirmation of the maternal female cell in a large population of male cord blood cells. We examined cells from 49 unseparated male cord bloods, as well as the CD34 and CD8 fractions from a large subset of the same cord bloods to determine if maternally derived cells were present in any of these populations. The CD34 fraction was examined because it contains the primitive hematopoietic stem cells required for engraftment, whereas the CD8 fraction was examined because it could contain cytotoxic T cells.

MATERIALS AND METHODS

Umbilical cords were obtained at the time of delivery from women who had experienced no complications during pregnancy or at the time of delivery. Cord blood samples were collected as follows: immediately after delivery, the umbilical cord was doubly clamped, the exterior of the cord was cleaned of any maternal blood by rinsing with 70% ethanol, and 30 mL of cord blood was withdrawn from between the two clamps using a needle and syringe. The cord blood was added to a 50-mL conical tube containing 3 mL of acid citrate dextrose, and passed through a 70-μm cell filter to remove any clots before use. Mononuclear cells were isolated from the cord blood on a Ficoll gradient (density, 1.077 g/mL). Antibody incubations were performed on ice using either 20 μg/mL biotinylated anti-CD34 manufactured by CeliPro Inc (Bothell, WA), or 0.5 μg/mL anti-CD8 (Gen Trak Inc, Plymouth Meeting, PA), followed by incubation with 4 μg/mL biotinylated rat-antimouse IgG (Zymed, South San Francisco, CA). Avidin-biotin immunofluorescence analysis of the CD8- or CD34+ cell fractions was accomplished as per instructions, using the CeliPro CEPRA Laboratory Cell Separation System.

Nuclei were prepared for in situ hybridization by resuspending the cells in 100 μL of 0.075 mol/L KCI and incubating them at 37°C for 15 minutes. Ten microliters of 3:1 methanol:acetic acid was added to the cells, the cells were centrifuged, and the pellet was resuspended and washed twice in 100 μL of the methanol:acetic acid solution. Samples were stored at 4°C until slides were prepared. Nuclei were dropped onto methanol cleaned slides and allowed to dry under moist heat.

The nuclei on the slides were denaturated in a solution of 70% formamide, 2X saline sodium citrate (0.15 mol/L NaCl, 0.015 mol/L sodium citrate, pH 7.0) at 74°C for 5 minutes. The slides were then dehydrated in successive washes of 70%, 80%, 90%, and 100% ethanol and allowed to air dry. Spectrum CEP Direct Chromosome Enumeration Probes for the X and Y chromosomes were obtained from Immagenetics (Framingham, MA) and used as directed by the manufacturer’s protocol. Nuclei were counterstained with a 4,6-diamino-2-phenyl-indole (DAPI)/antifade solution.

Male cord blood samples were examined for the presence of male cells by FISH, using the red signal of the Spectrum Orange CEP X probe to identify the X chromosome and the green signal of the Spectrum Green CEP Y probe for the Y chromosome. Nuclei were scored as male when they exhibited both an X and Y signal, whereas nuclei were scored as female when they exhibited no Y signal and had two X signals. In artificial mixtures of male peripheral blood lymphocytes spiked into female peripheral blood lymphocytes, it was determined that there was a direct linear relationship between the presence and the scoring of male cells down to one male cell in...
cord bloods by an artifact involving both events occurring simultaneously in the 49 cord blood samples (14%), and the ratio of the CD8 fraction to the CD34 fraction in the 5,000 nuclei scored. Maternal cells were observed in the unfractionated cord blood sample CB24 is shown in Fig 1. An example of two maternal cells observed in the 5,000 nuclei that were scored.

RESULTS

We examined approximately 1,000 nuclei from Ficoll-separated mononuclear cells from each of 49 collected cord bloods by FISH for the presence of maternal XX cells. As shown in Table 1, 18 maternal XX cells were observed in 48,987 nuclei screened. Maternal cells were observed in 7 of the 49 cord blood samples (14%), and the ratio of XX cells to XY cells in the 7 positives ranged from 1/2,499 to 1/1102. An example of two maternal XX cells observed in the unfractionated cord blood sample CB24 is shown in Fig 1.

We also examined the CD8 and CD34 populations of the cord blood for the presence of maternal cells. Enrichment for these populations was accomplished using solid-phase avidin immunoaffinity selection of cells labeled with biotinylated anti-CD8 or anti-CD34 monoclonal antibodies. Purity, as determined by fluorescence-activated cell sorting analysis of a randomly chosen subset from these selected samples, showed populations with a purity of 90% or better in the CD8 fraction and a purity of 50% or better in the CD34 fraction. A minimum of 1,000 nuclei were scored in each fractionated sample, although in some of the CD34-selected fractions, in which less than 1,000 total nuclei were obtained, all the nuclei were scored. As shown in Table 1, 26 XX maternal cells were observed in 39,704 nuclei in the CD8 fraction, whereas only 1 XX maternal cell was observed in the 25,663 nuclei in the CD34 fraction. We calculated the statistical significance of the total number of maternal cells observed per total cells examined in comparison between the unfractionated, CD8, and CD34 fractions. The $P$ values for the different cell fractions, as determined by Chi-square analysis are: unfractionated versus CD8, $P = .056$; unfractionated versus CD34, $P = .008$; CD8 versus CD34, $P = .001$. Maternal cells were observed in 5 of the 39 CD8 fractionated samples (14%), and in 1 of the 27 CD34 fractionated samples (4%). A summary of the 10 samples in which we observed maternal cells in either the unfractionated cells or CD8 or CD34 fractions is shown in Table 2.

### Table 1. Summary of Maternal Cell Detection in Unfractionated and CD8 and CD34 Selected Fractions of Cord Blood Using FISH

<table>
<thead>
<tr>
<th>Cell Fraction</th>
<th>No. of Maternal Cells/No. of Total Cells</th>
<th>No. of Samples With Maternal Cells/No. of Samples Recorded</th>
<th>% of Samples With Maternal Cells</th>
<th>% of Maternal Cells*</th>
<th>Median Value of Maternal Cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated</td>
<td>18/48,987</td>
<td>7/48</td>
<td>15</td>
<td>0.04-1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>CD8</td>
<td>26/39,704</td>
<td>5/39</td>
<td>13</td>
<td>0.1-1.2</td>
<td>0.5</td>
</tr>
<tr>
<td>CD34</td>
<td>1/25,663</td>
<td>1/27</td>
<td>4</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

* Expressed as a percent of maternal cells in the total cells, in the samples containing observed maternal cells.

1,000 female cells. However, because scoring 1,000 cells manually takes about 45 minutes at the microscope, the physical demands of the task precluded the examination of dilutions much lower than 1 per 1,000. Therefore, a minimum of 1,000 nuclei were scored from each sample as being either XX or XY. Five adult male blood samples were also examined for the presence of nuclei with an XX signal to address the possible concern that XX nuclei observed in the cord blood samples could be the artifactual result of an XY nucleus with a loss of the Y signal and a gain of a second X signal. Both of these events are independent, and each has been estimated to occur at $\sim 1$ event in 500. Therefore, the statistical frequency of an artifact involving both events occurring simultaneously in the same nucleus should be in the range of 1 in 250,000. In the five adult male bloods that were looked at using FISH, no XX nuclei were observed in the 5,000 nuclei that were scored.

DISCUSSION

We have used FISH to determine if maternal cells are found in cord blood and have found them present in 7 of 49 unfractionated cord blood samples, at levels between 1/2,499 and 1/100. We observed similar levels of maternal blood contamination in 5 of 39 CD8 fractionated samples, ranging from 1/1,052 to 1/83, indicating that T cells are among the maternal cells that are found in the cord blood. We also observed contamination with maternal cells in 1 of the 26 CD34 fractionated samples, at a level of 1/898. In all, 10 of the 49 cord blood samples (20%), exhibited maternal cells in at least one of the three populations scored. The sensitivity of the scoring method used in this paper is limited by the total number of nuclei that could be scored. Therefore, there may well have been some cord bloods with maternal cell contamination at or below 1/1,000, which we would not have detected.

Of the 10 cord bloods exhibiting maternal cells, 8 had the unfractionated cells and the CD8 and/or CD34 fractions examined as well, yet only 3 of these 8 had maternal cells in more than one fraction (Table 2). This is likely to be the result of the low numbers of maternal cells observed in any one fraction and the statistical chance of observing the same low levels in 1,000 cells of another fraction. The lower frequency of maternal cells observed in the CD34-enriched samples compared with either the unfractionated or CD8 fractionated samples may be caused by the fact that any maternal CD34 cells that have entered the neonatal circulation will have been diluted into the much higher levels of neonatal CD34 cells. Alternatively, if specific trafficking of cell types is responsible for the presence of maternal cells in the cord blood, our results may indicate that CD34 cells are less likely to cross the uterine/placental interface. Our results indicate that the degree of contamination of cord blood by maternal cells is reduced by selecting CD34+ cells. The level of maternal cells decreased from 18 maternal cells observed in 48,987 total cells in the unfractionated cord bloods to 1 maternal cell in 25,663 cells in the CD34 fraction. Because the average purity of the CD34-enriched fraction was 50%, the probability that the single maternal cell we observed wasn’t a CD34 cell is also 50%. Thus, if the pres-
ence of maternal cells in cord blood results in negative side effects after transplantation, a CD34 enrichment of the cord blood would be useful to reduce the level of maternal cells.

The presence of maternal cells provides a note of caution in assuming that all cord blood samples contain a homogeneous population of donor cells. However, it is unclear whether such low levels of contaminating cells have a negative effect in the use of harvested cord blood in transplantation. Two important issues are as follows: (1) are these levels theoretically significant; and (2) if these levels are theoretically significant, has GVHD been observed in practice in cord blood transplants? To answer the first point, it has been estimated that a cord blood transplant involving a 20-kg child as the recipient would require a minimum of $1 \times 10^7$ CD34 cells. If the CD34 cells are 1% of the nucleated cells of the cord blood, a total of $1 \times 10^9$ cells would be required. If maternal cells were present in the cord blood at levels of 0.1%, there would be $1 \times 10^6$ maternal cells in such a graft. It has been calculated that at least $1 \times 10^5$ T cells/kg are required for GVHD, and thus, the samples in which we have observed maternal cells are at the borderline of clinical significance for GVHD. There have been few reports of GVHD in ~50 cord blood transplantations that have been done thus far, although the majority of these have used related host-donor pairs. The possibility also exists that any maternal T cells present in the cord blood are suppressed in their function and are not able to mount a graft-versus-host response. Such suppression may be a normal part of pregnancy, and may be an added benefit to using cord blood in transplantation.

Micro-chimerism may exist transiently at the time of birth or even before delivery and may play a role in immunotolerance both during and after pregnancy. The demonstration of neonatal exposure to maternal lymphoid cells

Table 2. Summary of Cord Blood Samples Containing Maternal Blood Cells

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Unfractionated</th>
<th>CD8</th>
<th>CD34</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB10</td>
<td>0/1,055</td>
<td>1/1,052</td>
<td>0/889</td>
</tr>
<tr>
<td>CB14</td>
<td>1/1,102</td>
<td>12/1,000</td>
<td>ND</td>
</tr>
<tr>
<td>CB28</td>
<td>1/2,498</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CB29</td>
<td>1/1,005</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CB42</td>
<td>0/1,018</td>
<td>2/1,109</td>
<td>1/898</td>
</tr>
<tr>
<td>CB43</td>
<td>1/1,111</td>
<td>0/1,062</td>
<td>0/1,002</td>
</tr>
<tr>
<td>CB46</td>
<td>1/1,004</td>
<td>5/1,008</td>
<td>0/459</td>
</tr>
<tr>
<td>CB47</td>
<td>2/1,051</td>
<td>0/1,001</td>
<td>0/1,016</td>
</tr>
<tr>
<td>CB48</td>
<td>1/602</td>
<td>0/1,035</td>
<td>0/459</td>
</tr>
<tr>
<td>CB49</td>
<td>0/1,009</td>
<td>6/1,001</td>
<td>0/922</td>
</tr>
</tbody>
</table>

Values represent the number of XX maternal cells found over the total number of nuclei scored.

Abbreviation: ND, not done.
reported here suggests a mechanism for the establishment of acquired tolerance to noninherited maternal antigens (NIMAs), which were first observed in highly sensitized end-stage renal patients. Class et al. postulated that the NIMA effect may be caused by an in utero exposure to noninherited maternal HLA antigens leading to an altered immune responsiveness to these antigens later in life. Such an attenuation of the immune response could be caused by exposure to even small numbers of allogenic lymphoid cells at or before birth. The demonstration of neonatal exposure to maternal cells may also offer one explanation why not all babies of human immunodeficiency virus (HIV)-infected mothers become infected with the HIV virus. It is possible that this transmission is dependent on the passage of some minimal number of HIV-infected maternal cells into the neonatal circulation.

These data show the presence of maternal cells more often than the results obtained in two other recent studies show. The first of these, by Socie et al., used polymerase chain reaction (PCR) to screen cord blood for maternal cells using minisatellite DNA markers. In that study, only 1 cord blood in 47 was found to exhibit a PCR signal from the mother, and in six samples that were fractionated for B and T cells, no maternal PCR signal was observed. It has been our experience that when two populations of DNA are amplified using primers that are common to both populations, the ratios can actually be depressed further by continued rounds of PCR. In a report by Wang et al., who used polymorphic microsatellite markers to monitor for GVHD, the sensitivity of their PCR assay dropped off when the minor population reached 10% or less of the total, although more recent work has shown a sensitivity to 1% using a fluorescent gel scanning procedure for detection. In a second, smaller study of six cord bloods by Kogler et al., in which three of the cord bloods were examined by PCR and three by FISH, no maternal cells were found. This is most likely the result of the small sample size in that study.

The maternal cells observed in this study may merely be the result of spillage at time of birth, however they may also be the result of maternal to fetal leakage or trafficking during pregnancy. A number of studies have shown the reverse phenomenon, the passage of fetal cells into the maternal circulation during pregnancy, lending support to the concept of a limited exchange of cells across the placenta/uterine interface. It would be of considerable interest to sample fetal blood for the presence of maternal cells before delivery, and also to undertake follow-up sampling after birth in those cases where cord blood samples are shown to contain maternal cells to determine if there is persistence of these maternal cells and, if so, for what length of time.

REFERENCES

Detection of maternal cells in human umbilical cord blood using fluorescence in situ hybridization

JM Hall, P Lingenfelter, SL Adams, D Lasser, JA Hansen and MA Bean