RAPID COMMUNICATION

Search for Maternal Cells in Human Umbilical Cord Blood by Polymerase Chain Reaction Amplification of Two Minisatellite Sequences

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Since our first report in 1989, 26 transplants by means of umbilical cord blood have been reported. Furthermore, systematic studies of the feasibility of using banked placental blood for bone-marrow reconstitution of unrelated recipients on a large scale are in progress worldwide. However, already by 1989, it was pointed out that the use of cord blood might be hampered by contamination of neonatal blood with maternal cells contributing unacceptably to graft-versus-host disease (GVHD). In the present study, we used the polymerase chain reaction (PCR) amplification of 2 minisatellite sequences (33.6 and MS 51) to address this question. The sensitivity of PCR amplification of minisatellite sequences is known to be 1% to 0.1%. This sensitivity has been confirmed in the present study, in which a dilution analysis was performed for each experiment in which cell separation was performed. The inclusion of the dilution experiment in these analyses allowed us to estimate the relative amount of contaminating maternal cells, if any. Among 47 cases (31 whole blood analyses, 10 gradient separations, and 6 subpopulation separations), the coamplification of the 2 minisatellites sequences allowed the discrimination of maternal and neonate alleles in 42 cases (89%). In 1 case, we were able to detect a child-specific allele in a mother’s whole blood sample, thus validating our approach to search for maternal cells in cord blood. In a single other case, we were able to detect a maternal-specific allele in the cord blood sample. This maternal-specific allele was detected in the whole blood, polymorphonuclear cell fraction, and lymphocyte fractions. Comparison of the signal intensity obtained with these 3 cord blood samples to the result of the dilution experiment performed in the same analysis led to an estimate of 1 to 5% maternal cells in the polymorphonuclear cell fraction and 0.1% to 1% maternal cells in the whole blood and lymphocyte cell fractions. In conclusion, our study indicates that maternal cells are very rarely present in the cord blood collected at birth because we detected them in only 1 of 47 cases. More importantly, when detected, they were present at very low level in the lymphocyte cell fraction (0.1% to 1%). However, although small, this amount of cells may result in GVHD in a susceptible recipient. Because the method we used allows the detection of maternal cells within cord blood from 10^14 nucleated cells, it would thus be of interest in a cord blood banking perspective. © 1994 by The American Society of Hematology.

TRANSPLANTATION of HLA-matched bone marrow is currently the treatment of choice for selected patients with aplastic anemia, leukemia, and inherited disorders of the bone marrow. The availability of HLA-identical sibling donors limits this option to roughly a quarter of otherwise suitable patients. Therefore, registries of HLA-typed individuals willing to donate bone marrow have been set up in Europe, the United States, and elsewhere. However, even in the large bone marrow donor registries that have been developed worldwide, the efficiency search for an HLA-matched unrelated donor is only 20%.1 In 1989, Broxmeyer et al2 made the suggestion that the neonatal blood retained in the placenta might contain sufficient hematopoietic stem cells to serve as a transplant inoculum. A few months later, we reported the first case of a patient grafted by means of umbilical cord blood from an HLA-identical sibling.3 Since then, more than 20 such transplants have been performed worldwide (E. Gluckman, personal communication, November 1993). Furthermore, because the numbers of stem/progenitor cells in placental blood are in the range associated with successfully transplanted adult bone marrow,4,6 placental blood has the potential to overcome some of the limitations of the current system of registries for unrelated donor procurement. Systematic studies of the feasibility of using banked placental blood for bone marrow reconstitution of unrelated recipients seem, therefore, timely and warranted. At least three such studies are in progress worldwide.7,9

However, already by 1989, two editorials pointed out that, even if one cord blood can be sufficient to allow reconstitution after allogeneic transplantation, its use might be hampered by contamination of neonatal blood with maternal cells.10,11 This possibility was raised because some blood leakage from fetus/neonate to the mother occurs in up to 50% of births, usually during parturition, and, occasionally, clinically significant leakage occurs from mother to fetus. The incidence of relatively minor leakages from maternal to the fetal circulation during parturition is not known, but was assumed to be high.10 The magnitude of maternal blood contamination could be increased by the collection procedure, which tries to collect optimal amounts of blood. More importantly, mature maternal T lymphocytes incompletely matched with the histocompatibility antigens of the recipient could contribute unacceptably to graft-versus-host disease (GVHD) after transplant.10 Graft-versus-host disease has been limited or absent thus far in the small number of
children transplanted with cord blood, but all, except one, had received a matched sibling transplant and it is well known that the incidence and severity of GVHD is reduced in transplanted children. Therefore, in the evaluation of placent/cord blood as a source tissue for hematopoietic reconstitution, the putative contamination of the graft by maternal cells, as well as its frequency and magnitude, remain open questions.7

In the present study, we used the polymerase chain reaction (PCR) amplification of minisatellite sequences to address this question. In our hands, using this sensitive technique, maternal cell contamination of neonate blood is a very rare event and of a low magnitude when it occurred.

MATERIALS AND METHODS

Collection of Maternal and Umbilical Cord Blood Samples

The technique for cord blood collection has already been described in details.11,14 In brief, immediately upon delivery, the umbilical cord was double clamped and transected 5 to 7 cm from the umbilicus. The umbilical vein was catheterized aseptically and blood was collected by gravity in a plastic bag (MacoPharma, Roubaix, France) containing citrate phosphate dextran. Samples were collected after full-term vaginal delivery and after obtaining the mother’s informed consent. In all cases, a 2.5 to 5 mL sample of mother whole blood was collected at the same time.

Method

Preparation of DNA samples. Genomic DNA was extracted from mother whole blood according to classical methods. For cord blood samples, genomic DNA was extracted using the same technique, either from whole blood samples (31 cases) or after lymphocyte and polymorphonuclear cell separation (10 cases), as previously described.15 In a third set of experiments, DNA was extracted from purified lymphocyte subpopulations, either after immunomagnetic separation or after cell sorting (see infra).

Purification of cord blood lymphocyte subpopulations. Twelve different cord blood samples were processed. Of these, sufficient DNA for PCR analysis could be recovered from purified lymphocyte subpopulation in 6 cases (Table 1). The techniques used for lymphocyte subpopulations purification were as follows. A mean of 5 mL was retained for whole cord blood DNA extraction. Mononuclear cells from 45 mL of whole cord blood were separated by Ficoll gradient centrifugation (d = 1.077; Pharmacia, Uppsala, Sweden) and were processed for purification as described above, either immediately (3 cases) or after overnight plastic adherence at 10⁴ cells/mL in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 20% fetal calf serum (GIBCO) to remove monocytes (3 cases). Sorting of lymphocytes obtained either directly after Ficoll gradient centrifugation or after recovery of the nonadherent cells was performed using Dyna Beads (Dynal International, Oslo, Norway) coated with an antibody specific for B (CD19; Dynal No. 111 031) or T lymphocytes (CD 2; Dynal No. 111 012) in 4 different cases. Separation procedures were performed according to manufacturer protocol. In the 2 other cases, T cells were purified by FACS (Ortho 50; Ortho, Westwood, MA) after being stained with anti-CD3 antibody (IOT3; Immunotech, Marseille, France). In each experiment, purity of sorted cells was greater than 90%.

PCR amplification and molecular probes. PCR amplification of minisatellite sequences 33.6 and MS 51 was performed as described by Jeffreys et al.16,17 Samples were handled with gloves in a laminar vertical air flow hood using positive displacement pipettes exclusively assigned to PCR experiments. After amplification, samples were analyzed by Southern blotting as previously described.15 The 33.6 and the MS 51 probes were labeled by random priming (Amersham Megaprime DNA labeling system), as previously described.18 As described by Roux et al,19 a dilution experiment of mother DNA in cord blood DNA (10%, 5%, 1%, 0.1%, and 0.01%) was included in each experiment in which lymphocyte/polymorphonuclear and lymphocyte subpopulation separation was performed to allow a quantitative estimation that takes into account the allele size-dependent efficacy of PCR amplification.

RESULTS

Analysis of Whole Cord Blood Samples

Thirty-one different mother/cord blood pairs were studied. Of these, 28 (90%) were informative at the 33.6 0 and MS 51 loci (ie, at least one different allele at one locus was observed between the mother and the child). The 33.6 and MS 51 probes were informative in 79% and 61% pairs, respectively. We were unable to detect a maternal-specific allele in any of the 31 cord blood samples. On the contrary, we could detect the child specific allele in the mother whole blood sample in 1 case (Unique Cord Blood Number [UCBN] 48346) (Fig 1).

Analysis of Cord Blood After Lymphocyte and Polymorphonuclear Cell Separation by Ficoll Gradient Centrifugation

In 10 different mother/cord blood pairs, maternal cells were searched after cell separation by Ficoll gradient centrifugation. For each cord blood, three different samples were analyzed: whole blood, lymphocyte cell fraction, and polymorphonuclear cell fraction. Furthermore, for each experiment, a dilution of the mother DNA in the child DNA was performed, leading to a maternal-specific allele detection sensitivity of 1% overall (range, 1% to 0.1%). Of these 10 pairs, 8 were informative at the 33.6 0 and MS 51 loci. The 33.6 and MS 51 probes were informative in 8 and 5 pairs, respectively.

In a single case (UCBN 53078) we were able to detect a maternal-specific allele in the cord blood samples. This maternal-specific allele was present in the three cell fractions (whole blood, polymorphonuclear cells, and lymphocytes). Comparison of the signal intensity obtained with these 3 cord blood samples to the result of the dilution experiment performed in the same analysis led to an estimate of 1% to...
of 6 pairs (100%) were informative at the 33.6 or/and MS 51 loci. The 33.6 and MS 51 probes were informative in 6 and 4 pairs, respectively. We were unable to detect a maternal-specific allele in any of the 6 cord blood samples (data not shown).

**DISCUSSION**

The lack of vascular continuity between maternal and fetal circulation and the presence of an intact trophoblastic barrier throughout the whole gestation impose a considerable restriction on the nature and extent of cell traffic across the placenta. In normal pregnancy, it is imperative that there is no significant influx of immunocompetent maternal lymphoid cells into the fetus to prevent GVHD. The fact that neonatal GVHD is an extremely rare clinical condition is evidence for the effectiveness of the barrier in normal pregnancy. Even in the few reported cases of GVHD, there are invariably complicating factors, such as fetal immuno-deficiency. In the experimental murine system, data concerning immune traffic from the mother to the fetus are conflicting, but traffic seems to occur at a very low frequency, if any. Thus, at least in humans and rodents, maternal nucleated cells do not seem to readily cross the placental barrier. However, as pointed out by Billington, the techniques that have been used might have been of insufficient sensitivity to detect a very small number of cells, and/or the fetal immune system, through cytotoxic antibodies and/or cytotoxic or suppressive cells, is able to rapidly eliminate any maternal lymphocytes that succeed in breaching the barrier.

In the present study, we used PCR amplification of 2 minisatellite sequences to assess the presence, if any, of maternal cells within the cord blood of neonates. We as well as others have estimated the sensitivity of PCR amplification of minisatellite sequences (ie, the ability to detect cells representing a small percentage of the population) to be 1% to 0.1%. This sensitivity has been confirmed in the present study, where a dilution analysis was performed for each experiment in which cell separation was performed. Furthermore, the inclusion of dilution experiment in these analyses allowed us to estimate the relative amount of contaminating maternal cells, if present.

The overall informativity of the 2 minisatellite probes we used in this study was 82% and 62% for the 33.6 and MS 51 loci.
probe, respectively. These results fit with those published by Jeffrey et al. In the 47 cases (31 whole blood analyses, 10 Ficoll gradient separations, and 6 subpopulation separations) in which sufficient DNA could be obtained, the coamplification of these 2 minisatellite sequences allowed the discrimination of maternal and neonate alleles in 42 cases (89%). The analysis of 5 other mother/cord blood pairs was not informative because of common alleles at both the 33.6 and MS 51 loci. The use of a third polymorphic probe, such as YNZ22, might have allowed us to have an informative profile for these 5 other mother/cord blood pairs. In fact, in a study of chimerism after allogeneic bone marrow transplantation, we have shown that the use of this third probe in addition to the 33.6 and MS 51 minisatellite probes allowed us to differentiate all 19 analyzed donor/recipient pairs. In addition to the search for maternal cells in human umbilical cord blood, one of the aim of this study was to know if a single PCR procedure (coamplification of the 2 minisatellite sequences) was a reliable tool (informative in the majority of cases) in a cord blood banking perspective. This turned out to be the case because this technique gives informative profiles in nearly 90% of the cases. Furthermore, because a crucial point in cord blood banking is the number of cells available for biologic study (if cord blood is used for transplantation), the use of this sensitive and informative technique that does not need a large number of cells (100 ng of DNA, ie, 1.5 × 10^6 cells) might be of interest in such a perspective.

In 1 case (UCBN 48346), we were able to detect a child-specific allele in the mother's whole blood sample. The existence of fetal cells within the mother's blood flow during pregnancy is now a rather well-recognized phenomenon. Fetal cells in the mother's blood can be detected from 15 weeks of pregnancy and reach a level of about 0.1% during the third trimester of pregnancy. We were indeed able to detect fetal cells in maternal peripheral blood, thus validating our approach to search for maternal cells in cord blood.

In 1 case (UCBN 53078) we were able to detect a maternal-specific allele in the cord blood sample. Careful review of clinical data concerning the pregnancy, delivery, and perinatal life of this child indicated that he was an unremarkable case. A careful review of the data collection form was also unremarkable. Although we cannot completely rule out a minor problem in the collection procedure that would have resulted in some of the mother's blood dripping into the collection kit, two points should be underlined: (1) the system we use is a closed system (aimed to minimize any maternal cell contamination), and (2) to further minimize this contamination risk, we only use gravity collection without any attempt to flush placental vessels. The maternal-specific allele was present in the three cell fractions (whole blood, polymorphonuclear cells, and lymphocytes). Comparison of the signal intensity obtained with these 3 cord blood samples to the result of the dilution experiment performed in the same analysis led to an estimate of 1% to 5% maternal cells in the polymorphonuclear cell fraction and 0.1% to 1% maternal cells in the whole blood and lymphocyte cell fractions. Thus, the question raised in the recent review of Rubinstein, “Are maternal lymphocytes present and, if so, how often and at what levels?” is answered, within the sensitivity limit of the method used. In our experience, maternal cells are very rarely present within the cord blood collected at birth because we detected them in only 1 of 47 cases (2%). More importantly, when detected, they were present at very low levels in the lymphocyte cell fraction (0.1% to 1%), thus questioning the effect on GVHD such a small amount of cells might have if such material is used in a clinical setting of transplantation. However, although small, this amount of cells may result in GVHD in a susceptible recipient because as few as 10^6 lymphocytes have been reported to induce transfusion-associated GVHD. Taking an average of 10 × 10^6 nonnucleated cells per cord blood sample with 7 × 10^6 lymphocytes, this lead to an estimate of 7 × 10^9 to 7 × 10^6 lymphocytes of maternal origin (5 × 10^6 to 5 × 10^3 T lymphocytes). Because GVHD is mainly mediated via T lymphocytes, it would be of interest to know the amount of contaminating maternal T cells in this lymphocyte subpopulation. Unfortunately, we did not detect any maternal cells in the 6 cases that were studied after lymphocyte subpopulation separation. Data concerning the presence of maternal cells in the cord blood are scarce. In the above-mentioned review, Rubinstein stated that, in his experience on 17 cord bloods studied by PCR amplification of noninherited HLA antigens, he did not detect any maternal DNA in cord blood samples. Broxmeyer et al reported that, after cord blood transplantation, maternal T cells could not be detected in any of 3 graft patients. Therefore, this study of nearly 50 different cord blood is, to the best of our knowledge, the largest study performed in a physiologic setting and underlines the need to search for maternal cell contamination within the cord blood if used as a source of transplantable cells. Because the method we used allows the detection of maternal cells within the cord blood from about 10^9 nucleated cells (100 ng of DNA), it would be of interest in a cord blood banking perspective.

ACKNOWLEDGMENT

We are grateful to Dr. A. Jeffrey for the gift of 33.6 and MS 51 probes and to Dr. Chaouat for throughout discussion of the immunology of pregnancy and help for bibliography. We thank E. Van Nifertik for collecting the samples, and V. Sibella (PCR analyses) and M.C. Gendron (FACS analysis) for excellent technical assistance.

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