A new cytofluorometric approach to detect fetal cells in the maternal circulation

Fetal trophoblasts, lymphocytes, and erythrocytes are present in the peripheral blood of women during pregnancy. Since they are a potential source of material for prenatal diagnosis of fetal inherited disorders, several different approaches have been described to detect or enrich these fetal cells. However, there is a problem because of the scarcity of fetal cells in the maternal circulation.1

A comparable problem is the detection of leukemic cells in bone marrow or peripheral blood samples of potential leukemia patients. We have recently established a new cytofluorometric assay for the simultaneous measurement of DNA content and intracellular thymidine kinase (TK) activity.2 In this assay the cells are incubated with the fluorescent thymidine analogue N-dansyl-aminouracil-deoxyribose (AUR/DANS), which we have shown earlier to be a substrate for TK. The amount of intracellular accumulation of this analogue reflects the TK activity of the cells analysed. After staining of the DNA with ethidium bromide, we detect both fluoroscences on a flow cytometer (for the protocol of synthesis and purification of the fluorescent thymidine analogue and for details of the cytofluorometric method see references 4 and 5). Using this assay we have previously shown that normal adult peripheral blood cells do not exhibit TK activity, whereas transformed cells, such as leukemic cells for example, express high levels of TK activity. This enabled us to detect cytofluorometrically the neoplastic cells in a high excess of normal cells because of their high TK activity.6 Recently, we successfully used this approach specifically to detect leukemic cells in bone marrow or peripheral blood samples of leukemic patients.7

Using this cytofluorometric assay, we analysed cell samples from patients at different gestational ages undergoing chorionic villus sampling or isolation of fetal umbilical cord blood for standard obstetric indications. These cells were directly analysed without previous cultivation. Maternal cells from peripheral blood samples were obtained by density gradient centrifugation on Ficoll-Paque. The blood was mixed with phosphate buffered saline in a tube at the bottom of which Ficoll-Paque was gently layered. After centrifugation, the cells at the interface between the plasma and the red cells were collected and analysed.8 Permission to use parts of the material obtained for the present study was obtained from the institutional review board. We found that primary fetal trophoblasts and fetal cord blood cells express high levels of intracellular TK activity, whereas adult peripheral blood samples do not contain any detectable TK activity (fig 1). The percentage of TK positive cells in relation to the total number of cells used for the FACS analysis (calculated by cell counting) was about 85% in the trophoblast samples and between 55% and 70% in the fetal cord blood samples. These data have been confirmed by measuring TK activity with the traditional radioactive enzyme assay (for details of this assay see reference 6, data not shown). This observation prompted us to speculate whether it would be possible to detect fetal cells in the circulation of pregnant women using this assay.

When we analysed peripheral blood samples from six different pregnant women we always observed a second cell population with higher intracellular TK activity (fig 1). Since this second cell population was never detected in blood of non-pregnant women or male patients (we have analysed over 30 different blood samples), we concluded that this cell population with higher TK activity is very likely of fetal origin. Here it is important to note that in peripheral blood samples we cytofluorometrically analysed about 40 million cells. We first counted the cell number by cell counting on a Coulter counter, then incubated the sample with AuDR/DANS, and analysed the whole sample on a Partec cytofluorometer as described above.9 A normal adult peripheral blood cytofluorometric measurement contains data from between 10 000 to 100 000 events for data storage reasons. The analyses described here of such large cell numbers at once were performed by sequential analysis of DNA distribution and TK activity. For DNA distribution we analysed about 30 000 cells; for TK activity we analysed about 40 million cells. The latter was possible since only very few cells in the sample were TK positive and accordingly only those appeared on the flow cytometer. All the women analysed were between the 11th and 15th week of pregnancy, and the calculated fetal/maternal cell ratio is about 1:400 000.10 These data were in conflict with our earlier observation that the detection limit of our cytofluorometric TK assay varies between 1 cell in 50 000 and 1 in 100 000 (unpublished results). To investigate whether the sensitivity of detection of fetal cells in maternal blood is higher than 1 in 1 000 000 or whether the ratio of fetal cells in maternal cells is higher than previously described, we made different dilutions of fetal cord blood cells or trophoblasts in peripheral blood of non-pregnant adults. By this approach we found that some samples indeed enabled us to detect one fetal cell with high TK activity in 400 000 maternal cells without detectable TK activity (data not shown).

The data presented here suggest that the second cell population with higher TK activity reflects cells of fetal origin. (1) We show that trophoblasts and fetal cord blood cells express high intracellular levels of TK activity, whereas normal adult peripheral blood is totally TK negative. It has earlier been shown that circulating cells of fetal origin include trophoblasts, fetal lymphocytes, nucleated red blood cells, and haematopoietic stem cells.11 The small cell population with high TK activity specifically occurs in peripheral blood samples of pregnant women and not in the circulation of non-pregnant subjects. (2) The observed ratio of TK positive cells in the blood of pregnant women looks very similar to the earlier reported fetal/maternal cell ratio, estimated by other methods.12 Still, all these data only provide evidence that the cells are fetal cells and experiments to prove conclusively that these TK positive cells are indeed fetal need to be done.

However, we describe a promising new approach specifically to detect fetal cells in the maternal circulation. Since in this assay the cells exhibit a specific fluorescence, we think that it should be possible to enrich fetal cells through flow activated cell sorting.

Figure 1  Simultaneous cytofluorometric detection of DNA content and thymidine kinase activity in living cells. About 10 000 primary trophoblasts and 30 000 fetal cord blood cells were cytofluorometrically analysed as described in the text. In the case of adult peripheral blood of a non-pregnant and a 12 week pregnant woman, about 30 000 cells were analysed for DNA distribution and about 40 million lymphocytes were analysed for TK activity. (A) Distribution of DNA content in relation to the TK activity of (A) Trophoblasts (B) Fetal cord blood (not pregnant) (C) Fetal cord blood (pregnant). (B) Two dimensional presentation of DNA content and TK activity (ordinate, note that increasing enzyme activity goes down the axis).

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Segregation distortion of the CTG repeats at the myotonic dystrophy (DM) locus: new data from Brazilian DM families

Myotonic dystrophy (DM) is an autosomal dominant condition caused by a (CTG)n expansion in the DMPK gene on chromosome 19q13.1. The clinical course is highly variable and may include frontal baldness, cataract, progressive muscular weakness and atrophy, male infertility, diabetes, and mental retardation. An intriguing question is how a disease which usually decreases reproductive fitness within a few generations has been maintained in the population with a relatively high prevalence, considering the rarity of new mutations. An attractive hypothesis which has been proposed to explain this observation is the occurrence of meiotic drive,1 that is, a sex specific distortion of genetic transmission. A possible explanation is that when there is a negative selection against one or more class of gametes or when they are unable to conjugate according to the usual meiotic pattern) favouring the transmission of larger repeat alleles. This mechanism would lead to a preferential transmission of the enlarged repeat alleles lost through negative selection, thus maintaining the prevalence of these conditions in the population.

Metiotic drive favouring the transmission of larger alleles was observed in the DMPK locus when normal mouse meiosis was first suggested in 1994 for normal subjects2 and supported by Gennarelli et al3 in affected DM families. More recently, Chakraborty et al4 reported a preferential transmission of the larger alleles in normal female meiosis but not in male meiosis, in contrast to the first two studies. These conflicting results show that the origin of meiotic drive is controversial.

In our previous study, we have analysed the total number of affected versus normal subjects in 69 Brazilian families with DM and compared in the offspring of affected fathers versus affected mothers the total proportion of affected versus normal offspring and (2) the sex ratio among affected and normal descendents. As seen in table 1, there was no segregation distortion, that is, no significant difference from the expected mendelian ratios. The total proportion of affected versus normal offspring of maternal compared to paternal origin was 102 affected versus 107 unaffected born to DM mothers (χ²=0.74, p=0.79) and 206 affected versus 200 normal born to affected fathers (χ²=0.07, p=0.79). In addition, the analysis of the sex ratio showed that among the normal offspring of affected DM parents the proportion of males versus females did not differ from expected (50 males:50 females of maternal origin, χ²=0.42, p=0.52; 97 males:103 females, χ²=0.186, p=0.67).

Therefore no excess of DM patients was observed in the present study in both families as reported in Italian and Spanish DM pedigrees. One possible explanation is that in the present sample a proportion of subjects classified as normal, in particular in the older generations (and who are not available for DNA analysis), may carry the mutant allele. However, among DM patients there were more affected male than female offspring, both of maternal (57 males:45 females) and of paternal origin (117 males:89 females). This difference did not reach the level of significance (χ²=1.41, p=0.24) for the offspring of affected mothers but was at the limit of significance (χ²=3.80, p=0.05) for the offspring of affected fathers, probably owing to the sample size. Indeed, when the descendents of DM mothers were analysed together with those of DM fathers, the excess of affected males was statistically significant (174 males:134 females, χ²=5.19, p=0.02).

These findings support the observation of Gennarelli et al who reported that the mutant allele is transmitted to sons but not to daughters. Indeed, as seen in table 1, when we analysed our data together with those published by these authors, there was a more significant excess of affected DM males; the total number of affected offspring (of both maternal and paternal origin) was 149 males:137 females (χ²=3.77, p=0.05). Also, in accordance with these authors, we observed that although the mutant allele is preferentially transmitted to male offspring by both sexes, the distortion reached a level of significance only in male to male transmission.

Interestingly, in a previous study of Brazilian DM families we had observed a significantly greater proportion of affected DM males than females, and this was also reported by others,5 a finding for which we had no biological explanation (except a bias of ascertainment). This observation was confirmed in the present study since among 878 subjects, there was a statistically significant excess of affected males as compared to affected females (237 males:174 females, χ²=6.5, p=0.002), but no sex ratio distortion among unaffected relatives (234 males:233 females).

The present observation confirming that the mutant parental allele is significantly more often transmitted to sons than to daughters provides an explanation for the excess of affected males in the group of Brazilian families. It is noteworthy that the preferential transmission of the mutant allele to sons than to daughters, independently of the sex of the transmitting parent, was also found in Brazilian Machado-Joseph disease families.6 These findings suggest that the mutated allele may have a selective advantage at the pre- or postzygotic level. An interesting and unexpected finding is that although male infertility (but not female infertility) is one of the features observed in myotonic dystrophy, we have found in the present study that DM affected males have on average more children (406 offspring/114 fathers, mean=3.6 children per affected father) than DM mothers (209 offspring/78 mothers, mean=2.7 children per affected mother), although the proportion of affected patients who reproduced was similar for both sexes (78/174=0.45 females and 114/237=0.48 males). A possible explanation for this observation is that in the group of DM affected mothers (range from 66 to 1700 parents in the present study, 90 to 2000 in the study of Gennarelli et al) but than in the group of affected fathers (ranging from 66 to 900 parents in the present study, 54 to 1100 in the study of Gennarelli et al). Interestingly, the estimated relative fitness of our DM males was also slightly greater than that observed in our maternal population, as reported in the last census for the population of São Paulo (3.46 per male, age range 23-72). Therefore, it is tempting to speculate that if confirmed that DM men have a slightly higher relative fitness than the normal population associated with the observed preferential transmission of enlarged alleles to males, this would provide an explanation for maintaining the frequency of mutant alleles which are lost through negative selection. Other population studies will be very important to confirm this hypothesis.

| Table 1 | Proportion of affected and unaffected offspring born to DM parents |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | DM mothers, n=78 (178) |               |                  |                  |
|                  | Normal          | Total          | Normal          | Total          |
| **Sons**         |                  |                 |                 |                 |
| 117 (166)        | 97 (105)        | 214 (271)      | 57 (112)        | 57 (71)        | 114 (183)       |
| 199 (149)        | 103 (251)       | 302 (552)      | 102 (207)       | 107 (165)      | 209 (372)       |
| **Totals**       | 316 (314)       | 616 (901)      | 159 (219)       | 164 (236)      | 323 (608)       |

DM male versus normal offspring: maternal p=0.24; paternal p=0.05; total: maternal+p=0.02. Data from the present study are shown in bold and from Gennarelli et al3 in parentheses.
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