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 procedures 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 leukaemic cells in bone marrow or peripheral blood samples of potential leukaemia 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-amino-uracil-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 staining 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 leukaemic 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 leukaemic cells in bone marrow or peripheral blood samples of leukaemia 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. By comparison, fetal 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 detected this cell number by cell counting on a Coulter counter, then incubated the sample with AUR/DANS, and analysed the whole sample on a Partec flow cytometer as described above.9 A normal total blood cytocentrifuge 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 possible by sequential analysis of DNA distribution and TK activity. For DNA distribution we analysed about 30 000 cells; for TK activity (AUR/DANS positive) we analysed about 40 million cells. We found that in normal samples 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 13th week of pregnancy, and we concluded that the calculated fetal:maternal cell ratio is about 1:400 000. These data were in conflict with our earlier observation that the detection limit of our cytofluorometric TK assay varies between 1 cell in 90 000 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 cytofluorometric TK assay 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. (2) 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. (3) 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.10 Still, all these data only provide evidence that the cells found are fetal 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 fetal cells exhibit a specific fluorescence, we think that it should be possible to enrich fetal cells through flow activated cell sorting.
Experiments in this direction are currently under investigation in our laboratory.

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Segregation analysis 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 3′ untranslated region of the gene encoding myotonin kinase (DMPK) on chromosome 19q.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.4 An attractive hypothesis which has been proposed to explain this observation is the occurrence of meiotic drive,5,6 that is, a sex specific distortion of meiotic recombination which may occur 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 allow the replacement of female meiosis in new parents by male meiosis but not in male meiosis, in contrast to the two previous studies. These conflicting results show that the origin of meiotic drive is controversial.

In this study, we have analyzed 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 (1) the total proportion of affected versus normal offspring and (2) the sex ratio among affected and normal descendants.

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 (X\(^2\)= 0.74) and 206 affected versus 202 normal born to affected fathers (X\(^2\)= 0.79). In addition, the analysis of the sex ratio showed that among the normal offspring of affected DM parents the proportion of males did not differ from expected (57 males:50 females of maternal origin, X\(^2\)= 0.42, p=0.52; 97 males:103 females, X\(^2\)= 0.186, p=0.67).

Therefore no excess of DM patients was observed in Brazilian DM families as reported in Italian and Spanish DM pedigrees.10 One possible explanation is that in the present sample a proportion of subjects classified as normal, in particular affected fathers, (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 (X\(^2\)= 1.41, p=0.24) for the offspring of affected mothers but was at the limit of significance (X\(^2\)= 3.80, p=0.05) for the offspring of affected fathers, probably owing to the sample size. Indeed, when the descend-

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<th>Table 1</th>
<th>Proportion of affected and unaffected offspring born to DM parents</th>
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<td><strong>Present study</strong></td>
<td><strong>Gennarelli et al.</strong></td>
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<td><strong>Sons</strong></td>
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<td>Parental</td>
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<td><strong>Daughters</strong></td>
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<td><strong>Male DM versus normal offspring: maternal p= 0.24; paternal p= 0.05; total: male + paternal p=0.02.</strong></td>
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<td>Data from the present study are shown in bold and from Gennarelli et al. in parentheses.</td>
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Interestingly, in a previous study of Brazilian DM families we had observed a significantly greater proportion of affected DM males than females (237 males:174 females, X\(^2\)= 6.59, p=0.002), without 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 reported by others.2,7 A finding 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, X\(^2\)= 6.59, p=0.002).

Given that the male mutation rate is significantly higher than that of the female mutation rate, 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.8 These findings support the hypothesis that the mutant 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 that characterize 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 of the high reproductive fitness in DM males (525 offspring/210 fathers, mean 2.5 child per father) than in DM females (372 offspring/178 mothers, mean 2.08 child per mother) was also reported in the study published by Gennarelli et al.8 One possible explanation for this reproductive sex difference is that DM males are on average less severely affected than DM females. Indeed, in both studies the DM expansion was significantly larger in the group of DM affected males (range from 66 to 1700 repeats in the present study, 90 to 2000 in the study of Gennarelli et al.) than in the group of affected females (ranging from 30 to 900 repeats 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 multiparental data 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 males have a slightly higher relative fertility than DM females which is not lost through negative selection. Other population studies will be very important to confirm this hypothesis.