LETTERS TO
THE EDITOR

Genotype-phenotype correlation in five cystic fibrosis patients homozygous for the 621+1G→T mutation

Cystic fibrosis (CF) is the most common, lethal, autosomal recessive childhood disorder in the white population, occurring in about 1 in 2500 live births. The incidence (1975-1995) is 1 in 936 live births, with a carrier rate of 1 in 15 inhabitants in Saguenay Lac-Saint-Jean (SLSJ), a geographically isolated region in north-eastern Quebec (unpublished results).

One hundred and sixty-six patients distributed in 143 families are known at the "Clinique de la Fibrose Kystique" in Chicoutimi (Saguenay), which is the referral centre for the whole region (285 000 inhabitants) and has followed all the CF patients, but one, since 1973. Molecular characterisation has been performed on all identified living CF patients (unpublished results). Three mutations account for 87.6% of the CF chromosomes: these are the AF508 (57.6%), 621+1G→T (21.7%), and A455E (8.3%) mutations. It appears that the presence of the 621+1G→T mutation in the SLSJ population is the result of a founder effect.

We have so far identified five patients homozygous for the 621+1G→T mutation who have been followed for at least nine years. Data collected on each patient were extracted from the files kept at the CF clinic in Chicoutimi where the patients come on a regular basis (approximately every two months) as outpatients for advice, follow up, and treatment. This medical visit consists of a physical examination, a growth and nutritional status evaluation, pulmonary functional tests by spirometry, and a sputum culture. This phase of data gathering has been described in detail elsewhere.

The main characteristics of the five CF patients are presented in table 1. There were two males and three females, all alive and French Canadian from the Saguenay Lac-Saint-Jean region. Their mean age in 1996 was 14.2 years, the oldest being 23 years old. Four patients were diagnosed in the first three months of life, including two diagnosed at birth because of a meconium ileus. All patients experienced distal intestinal obstructive syndrome (DIOS). All five patients were pancreatic insufficient. The mean sweat chloride concentration was 112.4 mEq/l. The mean value for all the 46 CF patients homozygous for the AF508 mutation was 102.7 mEq/l (SD 8.5). The difference between both groups was statistically significant (two tailed t-test=2.5, p=0.016).

The clinical status of all five patients has remained excellent since diagnosis, as shown by the Shwachman scores equal to or higher than 85. The three oldest patients, including two colonized by Pseudomonas aeruginosa, had normal pulmonary function tests. Patient 3 had a milder pulmonary function. However, the results of his functional tests shown in table 1 were obtained two months after diagnosis of diabetes mellitus in September 1996. The last tests performed, in June 1996, before diagnosis of diabetes, were almost normal (FVC=98% and FEV1=92% of the predicted values). It is believed that the onset of diabetes mellitus in patient 5 is not related to cystic fibrosis because he had a sister who also developed insulin dependent diabetes, therefore suggesting that they had familial juvenile diabetes.

Based on the assumption that two "severe" alleles causing pancreatic insufficiency were necessary for pancreatic insufficiency to be manifest, the 621+1G→T mutation was considered to be a "severe" allele causing pancreatic insufficiency. All five homozygous patients are pancreatic insufficient, confirming that the 621+1G→T mutation is a "severe" allele. Except for one patient who was recently diagnosed with diabetes mellitus and experienced shortly after the onset a drop in his pulmonary function test results, the other patients are doing well and have a very stable condition, especially for their pulmonary condition. The three oldest patients have normal pulmonary function.

Two other CF patients homozygous for the 621+1G→T mutation have been described. Cheddale et al (1996) reported a 24 year old man of Welsh/English ancestry who was diagnosed at the age of 7 years. He was pancreatic insufficient and developed "considerable chest disease". Compliance with treatment had been poor and the "Clinical Cystic Fibrosis Functional" tests showed the FVC and the FEV1 respectively at 41% and 24% of the predicted values. Wilt et al (1996) reported a 21 year old woman of Polish origin who was diagnosed at the age of 10 years, although she had suffered from bronchitis and sinusitis since she was 1 year old. She was pancreatic insufficient and developed gastro-oesophageal reflux with oesophagitis. The reported two tailed P values for the FEV1 tests showed the FVC at 71% and the FEV1 at 35% of the predicted values.

The five CF patients from Saguenay Lac-Saint-Jean have a much better pulmonary condition than those two previously described. We do not know whether it is because of the much younger age at diagnosis, more aggressive therapy, better compliance with treatment, or other genes modifying the CFTR function.

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Table 1 Phenotypic characteristics of five patients homozygous for the 621+1G→T mutation

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<th>Patient No</th>
<th>Family No</th>
<th>Year of birth</th>
<th>Sex</th>
<th>Status</th>
<th>Age (s)</th>
<th>Current weight (kg)</th>
<th>Current height (cm)</th>
<th>Age at diagnosis (mth)</th>
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*Familial juvenile diabetes?
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.

A comparable problem is the detection of leukemic cells in bone marrow or peripheral blood samples of potential leukemic patients. We have recently established a new cytofluorometric assay for the simultaneous measurement of DNA content and intracellular thymidine kinase (TK) activity. In this assay the cells are incubated with the fluorescent thymidine analogue N-dansyl-amino-uracil-deoxyribose (AUDR; 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. Recently, we successfully used this approach specifically to detect leukemic cells in bone marrow or peripheral blood samples of leukemia patients.

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. Primary 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 glycerol-covered. After centrifugation, the cells at the interface between the plasma and the red cells were collected and analysed. 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 limited this 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.

A normal fetal flow 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 realized by sequential analysis of DNA distribution and TK activity. For DNA distribution we analysed about 30 000 cells; for TK activity (AudR/DANS positive) we analysed about 40 million cells. However, 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 detected 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 in 10 000 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 measurement 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. Still, all these data only provide evidence that the cells found are of fetal origin 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.