Prenatal Diagnosis of Cystic Fibrosis: False Negative Result with the 4-Methylumbelliferyl-p-guanidinobenzoate Assay for Proteases in Amniotic Fluid

J. R. Green1, M. J. Lentze1, E. Rossi1, D. Sidiropoulos2, and G. Schubiger3

1 Universitäts-Kinderklinik, Inselspital, CH-3010 Bern, Switzerland,
2 Universitäts-Frauenklinik, CH-3012 Bern, Switzerland,
3 Kinderklinik, CH-6004 Luzern, Switzerland

Abstract. The promising method for the prenatal detection of cystic fibrosis (CF), developed by Nadler's group [6, 7], has recently attracted considerable interest but there is a lack of confirmatory reports from other laboratories. We have established the assay for methylumbelliferyl-guanidinobenzoate-reactive proteases (MUGB assay) and used it, together with isoelectric focusing and gel filtration, to screen retrospectively 84 second and third trimester amniotic fluids, one of which was known to be from a CF pregnancy.

The control values for MUGB-reactive protease were 4.25 ± 1.36 and 6.12 ± 1.04 nmoles/mg protein (mean ± 1 SD) for second and third trimester fluids, respectively, compared to 5.64 nmoles/mg protein for the third trimester CF fluid. Gel filtration resolved two peaks of MUGB-reactive protease of approximately 50 000 and 10 000 molecular weight from all samples. Insufficient CF fluid remained for isoelectric focusing and the results obtained with normal amniotic fluids were inconclusive due to a highly variable background staining. Thus, with two methods a false-negative result has been obtained, suggesting that further refinement of these techniques may be required before they can be routinely applied for the prenatal diagnosis of CF.

Key words: Cystic fibrosis – Prenatal diagnosis – MUGB-reactive protease

Introduction

In a series of publications over the past two years Nadler and co-workers have described a promising new method for the prenatal detection of cystic fibrosis (CF) and have successfully diagnosed prospectively the outcome of 13 at-risk pregnancies from couples who had previously had a CF child [6, 7, 13, 14, 16]. The main method is based on the measurement in amniotic fluid of undefined protease enzymes by titration of their active sites with 4-methylumbelliferyl-p-guanidinobenzoate (MUGB) in the presence and absence of benzamidine to correct for non-specific hydrolysis of the substrate. Amniotic fluids from fetuses affected with CF appeared to have lower levels of MUGB-reactive proteases than normal controls and this was associated with the absence of a protease band from isoelectric focusing gels and an abnormal elution pattern in gel filtration.

Although the MUGB-assay has aroused considerable interest and several groups have published preliminary results obtained with the method [1, 3, 11], there have been no detailed accounts of attempts to reproduce it in other independent laboratories. We have established the assay by following the method published by Nadler and his co-workers, and now report some of the technical problems encountered in trying to repeat the work as well as the retrospective detection of a false-negative fluid from a confirmed CF pregnancy.

Methods

All the methods used were essentially those described or cited by Nadler's group, with only minor modifications as dictated by the availability of different equipment. Amniotic fluids were collected as part of a routine amniocentesis programme, the cells were removed by centrifugation and the supernatants were analysed as coded unknown samples in the laboratory.

MUGB Assay

The MUGB-assay was performed as described by Walsh et al. [16], except that all volumes were increased 2.5-fold for more convenient handling and to reduce pipetting errors. Fifty microlitres of amniotic fluid was mixed with either 50 µl of distilled water or 50 µl of 200 mM benzamidine hydrochloride (Merck) dissolved in distilled water, and then incubated for 2 h in the dark at room temperature (20°C) with 50 µl of MUGB-substrate solution comprising 200 µM 4-methylumbelliferyl-p-guanidinobenzoate (Sigma) in barbiturate buffer (0.1 M sodium 5,5-diethylbarbiturate, pH adjusted to 8.3 with 5 N HCl, containing 4% by volume of dimethylsulphoxide—DMSO—in which the MUGB was first dissolved). The reaction was terminated by the addition of 5 ml of the same buffer without MUGB. The fluorescence was measured immediately in a 1 cm² cell on a Perkin-Elmer model 1000 spectrofluorimeter with an excitation wavelength of 365 nm and an emission wavelength of 450 nm. Blanks contained 50 µl of distilled water, plus either 50 µl of 200 mM benzamidine hydrochloride or 50 µl of distilled water, and 50 µl of MUGB-substrate solution. All samples and blanks were set up in triplicate. To avoid inaccuracies that could arise from quenching of fluorescence transfer, separate standard curves for 4-methylumbelliferylone were constructed with and without benzamidine hydrochloride, but both containing MUGB-substrate solution so that conditions closely resembled those in the assay (i.e. a low amount of free
methylumbelliferone in the presence of a relatively high amount of MUGB). These sets of tubes contained 50 μl of distilled water, 50 μl of 200 μM MUGB-substrate solution, either 50 μl of 200 mM benzamidine hydrochloride or 50 μl distilled water, and 5 ml of barbiturate buffer containing 4% DMSO and a range of amounts of free 4-methylumbelliferone from 0 to approximately 4.0 nmoles per tube. Protein was measured by the method of Lowry et al. [5] using crystalline bovine serum albumin as standard.

Gel Filtration

A 1-ml portion of each amniotic fluid was chromatographed at 4°C on a column (95 cm x 1.6 cm) of Bioigel A-0.5 m, 100–200 mesh, eluted with 10 mM disodium hydrogen phosphate, pH adjusted to 7.7 with 1 M phosphoric acid, at a flow rate of 12 ml/hr. This procedure differs from that of Walsh et al. [16] in that they used a shorter column (35 x 1.5 cm) loaded with 5 ml of four-fold concentrated amniotic fluid. Fractions were collected every 20 min and assayed for MUGB-reactive proteases by incubating 200 μl of eluate with 200 μl of MUGB-substrate solution for 2 h in the dark at room temperature. The reaction was terminated by the addition of 3 ml of barbiturate buffer, containing 4% DMSO, and the fluorescence read as described above. The column was calibrated with the Bio-Rad gel filtration kit of standard molecular weight markers.

Isoelectric Focusing

Isoelectric focusing was carried out in 5% polyacrylamide gels, 7 cm long, by a method similar to that described by Rao and Nadler [9] except that instead of LKB ampholine, 0.8% Pharmalyte (Pharmacia AB, Sweden) pH range 4.0–6.5, was used and the gels were cast and run according to this manufacturer's instructions. In place of a piece of cheese-cloth [9], the gel was supported at the anode end by a 1 cm plug of 10% polyacrylamide made up in 10 mM glutamic acid without ampholine. The anode solution was 10 mM glutamic acid and the cathode solution was 10 mM histidine in contrast to the 0.2% H2SO4 and 0.4% diethanolamine electrode solutions used by Rao and Nadler [9]. In order to detect any enzymic activity on the gels after focusing, amniotic fluid samples were concentrated 20 times in an Amicon CS-15 disposable ultrafiltration cell, and 50 μl of concentrate mixed with ampholine and sucrose was transferred into fresh staining solution and incubated for a further 2 h. This procedure differs from that of Walsh et al. [16] in that instead of LKB ampholine, 0.8% Pharmalyte, each focusing run was calibrated with a standard mix of pure proteins of known pI values (Pharmacia pI calibration kit, low pI range 2.5–6.5). These gels were fixed with a solution of 17.25 g of sulphosalicylic acid and 57.5 g of trichloroacetic acid dissolved in 500 ml of 30% methanol, and then stained for protein with Coomassie Blue.

Case Report

The false-negative CF fluid studied in this investigation was obtained from a 19-year-old primigravida, hospitalized at weeks 27 and 33 because of bleeding and threatened abortion. Amniocentesis was performed at week 33 to clarify a discrepancy between foetal size and calculated gestational age detected by ultrasound. Analysis of the amniotic fluid indicated retared foetal development: low lecithin/sphingomyelin ratio, low amylase, normal levels of bile acids and alpha-fetoprotein. CF was suspected shortly after birth (prematurely at week 36, 2.4 kg) from a positive meconium-albumin test and a viscous meconium. The disease was confirmed by two pathological sweat tests at 3 and 6 months of age, and this was consistent with clinical symptoms of impaired digestion, distended abdomen and recurrent bronchitis.

Results and Discussion

MUGB Assay

During preliminary attempts to establish the assay it was noticed that the MUGB-substrate solution was unstable in the presence of high concentrations of benzamidine hydrochloride (Fig. 1). This effect was only observed in tubes that had been incubated with benzamidine hydrochloride for 2 h before the addition of 5 ml of barbiturate buffer. If the benzamidine hydrochloride was immediately diluted out with buffer, as for the standard curves, no instability of MUGB was observed for up to 4 h. No explanation for this effect has been found. Initially it was suspected that the acidity of the benzamidine hydrochloride solution could be the cause, but adjustment of the pH to 8.3 before addition to the MUGB-substrate solution did not prevent decomposition of the latter. Schwartz and Brandt [11] have reported similar difficulties with benzamidine hydrochloride in the MUGB-assay and recommended the use of p-nitrophenylguanidinobenzoate as an alternative inhibitor.

The standard curves for 4-methylumbelliferone were linear throughout the range 0–4.0 nmoles and stable for at least 4 h under the conditions described, but the relative fluorescence was about 5% lower in the presence of benzamidine hydrochloride (Fig. 2). Therefore two standard curves are required to calculate accurately the difference between the amounts of MUGB released in the presence and absence of benzamidine hydrochloride.

The results for the assay of MUGB-reactive proteases in amniotic fluid are summarized in Table 1. The mean control values, expressed either as per ml fluid or per mg protein, are approximately double those obtained by Nadler's group [6, 14, 16]. However, our mean control value and range for MUGB activity per mg protein in second trimester fluids (4.25 ± 1.36, 2.33–7.97) are very close to those published recently [11] by Schwartz and Brandt (4.6 ± 1.0, 2.6–7.5). Further analysis of the data shows that our results for the hydrolysis of MUGB in the absence of benzamidine are similar to those