Acetylcholinesterase and Fetal Malformations: Modified Qualitative Technique for Diagnosis of Neural Tube Defects

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A single-blind study involving amniotic-fluid samples from 214 pregnancies of known outcome confirms that an electrophoretically distinct isoenzyme of acetylcholinesterase is associated with aetial fetal neural tube defects. Furthermore, only one of 13 amniotic-fluid samples with false-positive results for alpha-fetoprotein showed the characteristic isoenzyme, indicating that qualitative acetylcholinesterase assessment can decrease the proportion of false positives from the alpha-fetoprotein assay. We have also identified this characteristic isoenzyme in amniotic fluids from pregnancies in which other serious fetal defects occurred. A detailed electrophoresis protocol for identifying this characteristic isoenzyme is described.

Additional Keyphrases: isoenzymes · fetal status · electrophoresis, polyacrylamide disc gel

Qualitative assessment of acetylcholinesterase (AChE) in amniotic fluid has been suggested as helpful in second-trimester prenatal diagnosis (1-3). We present a modified technique for doing so and demonstrate its performance. This technique is effective both for reinforcing true-positive results for alpha-fetoprotein (AFP) associated with fetal open neural tube defects and in correctly reclassifying amniotic-fluid samples with falsely increased AFP concentrations.

Materials and Methods

Samples. Amniotic fluid was sampled during the second trimester from 214 pregnancies and stored at -25°C until the outcome was known. Specimens were then assayed by a modification of the qualitative AChE test reported by Smith et al. (2). These specimens were chosen to include examples of various fetal lesions associated with increases in AFP, as well as blood-contaminated samples and clear samples from otherwise normal pregnancies. All amniotic-fluid samples were tested without prior knowledge of AFP concentrations, pregnancy outcome, or extent of blood contamination from the mother or the fetus.

Samples were assayed for AFP by electroimmunodiffusion (rocket) as described by Laurell (4).

Qualitative assay for acetylcholinesterase. For polyacrylamide disc gel electrophoresis we used a Biochrom 2103 power supply (LKB Instruments Inc., Rockville, MD 20852) with a Model 51514 gel-column-electrophoresis unit (Gelman Instrument Co., Ann Arbor, MI 48106).

Tube preparation. Allow Pyrex tubes, 6.0 (i.d.) x 65 mm, lightly flame-polished and etch-marked 10 mm from one end, to soak in a strong detergent (Contrad 70-C6327; Scientific Products, McGraw Park, IL 60085) for 30 min, remove, and thoroughly rinse them with distilled water. Then dip the tubes several times into 10-fold diluted Photo Flo 200 (Eastman Kodak Co., Rochester, NY 14650). After drying, insert them vertically into moistened 15-mm vaccine-bottle stoppers such that the etch mark is 10 mm from the top of the tube.

Gel preparation. Prepare two stock solutions, as follows: Solution A contains 30 g of acrylamide monomer and 1 g of N,N'-methylene-bisacrylamide (both from Bio-Rad Labs., Richmond, CA 94804), in 123 mL of distilled water; Solution B contains 1.5 g of tris(hydroxymethyl)aminomethane (Tris) and 7.25 g of glycine in 245 mL of distilled water.

For enough gel to fill 12 tubes, mix 6.5 mL of A with 2.5 mL of B and add this mixture to a freshly prepared solution of 14 mg of ammonium persulfate (Bio-Rad) in 11 mL of distilled water; then add 7 μL of N,N,N',N'-tetramethylethylenediamine (Bio-Rad). Use this solution to fill each of the prepared tubes up to the etch mark. Using a syringe (no. 710N; Hamilton Co., Reno, NV 89150), overlay the acrylamide mix in each tube with water, being especially careful to avoid any mixing. After 20 min, shake out the overlayered water and position the tubes in the upper chamber grommets of a gel column-electrophoresis unit.

Sample preparation. Every amniotic-fluid sample is analyzed in duplicate. Mix a 50-μL aliquot of each sample with 25 μL of an equimolar solution of glycine and water and 25 μL of a marker protein solution consisting of 1 g of bovine serum albumin and 0.1 g of Reactive Blue 2 (R4502; Sigma Chemical Co., St. Louis, MO 63178) per liter. The marker protein allows one to observe the progress of the electrophoresis in each tube.

In each electrophoretic assay, include as controls amniotic fluid samples known to be negative and positive for neural tube defects.

Electrophoresis. The electrophoresis method is a modification of that of Clarke (5). The buffer consists of 29 g of glycine and 6 g of Tris in 975 mL of distilled water and 5 mL of 1 mol/L HCl; dilute 10-fold and adjust the pH to 7.5. (Make pH adjustments with dropwise additions of 1 mol/L NaOH or 1 mol/L HCl.) Pretreat the gels by electrophoresing at 1 mA/tube for approximately 1 h without sample. Then carefully layer previously prepared 100-μL sample mixtures onto the gels, beneath the electrophoresis buffer, with a Hamilton no. 710N syringe. Perform the electrophoresis at constant current for 15 min at 0.5 mA/tube, then at 2 mA/tube until the stained albumin band has reached the lower end of the gel.

Enzymic development. After electrophoresis, remove the gels from the glass tubes and place them in 13 x 75 mm test tubes. Duplicate gels are separated into two identical groups. The same procedure is used to develop both groups except that one is incubated in the presence of cholinesterase inhibitor [1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide, A 9013; Sigma Chemical Co., St. Louis, MO 63178], 50 μmol/L, placed in the incubation medium before incubation, to identify areas of AChE activity.

The development medium is basically that of Koelle (6).
Prepare the following standard solutions: for solution C, dissolve 12.2 g of maleic acid in 300 mL of distilled water, adjust the pH to 6.5, and dilute to 500 mL with distilled water; for solution D dissolve 3 g of CuSO₄·5H₂O in 100 mL of distilled water; for solution E dissolve 4.5 g of glycine in 100 mL of distilled water. To prepare the pre-incubation mixture, mix 100 mL of Solution C with 72 g of sodium sulfate and 200 mL of distilled water (pH adjusted to 6.5). Fill each test tube with the pre-incubation mixture, and let stand. After at least 30 min, pour off the solution, replacing it with the incubation mixture, freshly made from 20 mL of C, 2 mL of D, 2 mL of E, 0.2 mL of 1 mol/L MgCl₂, 36 mL of distilled water, 14.4 g of sodium sulfate, and 70 mg of acetylthiocholine iodide (Sigma). Adjust the pH to 6.5. Incubate at room temperature until white, distinct bands appear in the positive control (6-20 h). Stop the reaction by replacing the incubation mixture with distilled water.

A photograph of the gels (e.g., Figure 1) provides a distinct image of the precipitates and is a permanent record. It is not necessary to stain the bands.

**Results**

A specific AChE isoenzyme is found in association with fetal open neural tube defects and other serious fetal malformations. We have confirmed that this isoenzyme has identical electrophoretic mobility to an AChE isoenzyme in cerebrospinal fluid, labeled “B” and “Z” by Smith et al. (2). Identification of the diagnostic AChE isoenzyme rests on two criteria: (a) electrophoretic identity with the diagnostic AChE isoenzyme in a positive control and (b) complete inhibition by the cholinesterase inhibitor (Figure 1).

Table 1 summarizes our results with 214 second-trimester samples of amniotic fluid in our single-blind study. Pregnancies in the upper group of Table 1 were not associated with intrauterine death, spontaneous abortion, fetal open neural tube defects, or fetal malformations. With one exception, the 153 samples in this group were correctly classified by AChE analysis. The single false-positive AChE result was for a sample grossly contaminated with blood from both the fetus and the mother. Of the 51 samples in the lower group, which were associated with open neural tube defects (anecephaly, open spina bifida, or encephalocele), 50 were correctly classified. The sole open neural tube defect-affected pregnancy that was misclassified by the qualitative AChE assay was also misclassified by the AFP test. Results from samples involving certain other major fetal malformations indicate that the qualitative AChE assay, like the AFP assay, is not absolutely specific for fetal open neural tube defects; five cases of omphalocele/gastrochisis all yielded positive AChE results, as did a single case of Turner’s syndrome with hygroma. The diagnostic AChE isoenzyme was also present in the hygroma fluid. The diagnostic AChE band found in conjunction with a macerated fetus suggests still another possible clinical correlation.

**Discussion**

Measurement of AFP in amniotic fluid evidently allows identification of 98% of fetal open neural tube defects between 13 and 24 weeks of pregnancy (7). However, 0.48% of unaffected singleton pregnancies not associated with miscarriage also give positive results, some of which are due to fetal blood contamination during amniocentesis (7). An amniotic fluid test less sensitive to fetal blood contamination would be useful as an adjunct to the AFP test and could decrease the proportion of misclassified pregnancies.

Smith et al. (2), using a quantitative assay, reported significantly increased concentrations of AChE activity in second-trimester samples of amniotic fluid associated with fetal open neural tube defects. There is considerable overlap, however, between AChE activities or normal and neural tube defect-affected pregnancies (2, 8-10). Smith (1) has also suggested a qualitative gel electrophoresis test for diagnosing fetal open neural tube defects because an electrophoretically distinct AChE isoenzyme acts as a marker for those condi-
tions. Our modifications of the technique described by Smith et al. (2) include increasing the gel concentration from 60 to 80 g/L, decreasing the pH of the electrophoresis buffer from 8.0 to 7.5, and decreasing the cholinesterase inhibitor A9013 (Sigma) to $5 \times 10^{-5}$ mol/L to minimize nonspecific esterase inhibition. These changes produce sharply defined bands of AChE activity, thus facilitating evaluation of the gels.

Our observations support the use of this qualitative AChE assay as a worthwhile addition to laboratory diagnosis of open neural tube defects. Furthermore, certain other major fetal malformations are identified by this technique. Our sample of omphalocele/gastroschisis cases is too small to give a reliable estimate of detection frequency. This technique appears to be especially helpful in sorting out samples contaminated with fetal blood. As yet, there is insufficient experience with the assay to assign false-positive and false-negative rates. The single false-positive AChE result we found occurred in a grossly contaminated sample, raising the possibility that extensive blood contamination may, on occasion, elicit false-positive AChE results. Our observations suggest that the qualitative AChE assay may provide an effective means of decreasing false-positive classifications of neural tube defect.

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References


