Detection of Errors in Methylmalonyl-CoA Metabolism by Using Amniotic Fluid

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We report a method for rapid prenatal detection of methylmalonic acidemia, consisting of measuring methylmalonyl-CoA mutase (EC 5.4.99.2) activity in non-cultured amniotic cells and measuring the concentration of methylmalonate in the amniotic fluid. Immediate stabilization of the mutase activity in the non-cultured amniotic cell by its coenzyme, adenosylcobalamin, and use of methylmalonyl-CoA with high specific activity gives mutase activity comparable to that of cultured amniotic cells or normal fibroblasts. Consequently, findings of low mutase activity and a high concentration of methylmalonate in the amniotic fluid allows accurate diagnosis of the vitamin B12-responsive form of methylmalonic acidemia. These results can be obtained in two days. For the vitamin B12-nonresponsive form, the correct diagnosis depends upon finding amniotic fluid methylmalonate, because cells from these patients will display normal methylmalonyl-CoA mutase activity after adenosylcobalamin is added. Problems in interpreting data on bloody samples and the limitations of the method are discussed.

Additional Keyphrases: inherited disorders • enzyme activity • vitamin B12 • methylmalonic acidemia • prenatal diagnosis • fetal status

Since the original description (1) of the hereditary defect, methylmalonic acidemia, at least six distinct variants have been characterized (2-6). In all of these, abnormal activity of the responsible enzyme can be detected in skin fibroblasts. As a result, cultured amniotic fluid cells can be used effectively for diagnoses in utero. Several additional factors unique to this inborn error of metabolism make it possible to evaluate the fetus by means other than cultured cells. For example, in an affected pregnancy, both amniotic fluid and maternal urine (7-10) contain excess methylmalonate, because methylmalonate is not readily metabolized and, as a consequence, accumulates in both fetal and maternal fluids (7). Decreased activity of methylmalonyl-CoA mutase (EC 5.4.99.2, hereafter simply called "mutase") is the cause of this inborn error of metabolism. Mutase is a remarkably stable enzyme; its activity being preserved in long-frozen postmortem tissue (4) and in hepatic tissue left at room temperature for as long as two days (personal observation). Thus it should be possible to diagnose methylmalonic acidemia prenatally by measuring mutase activity in non-cultured amniotic cells and methylmalonate in amniotic fluid. The following report describes a method for rapid prenatal diagnosis of methylmalonic acidemia in a fetus by measuring mutase activity in non-cultured cells as well as amniotic fluid methylmalonate.

Materials and Methods

Reagents

Racemic [methyl-14C]malonyl-CoA was synthesized by the enzymatic method of Whitaker and Giorgio (11). Adenosylcobalamin was a gift from Dr. Robert Abeles (Brandeis University).

The following reagents were obtained from the company indicated in parentheses:

Ham's F-12 media, fetal calf serum and trypsin (Grant Island Biological Co., Santa Clara, Calif. 95050).

Tissue culture flask 25 cm² (Falcon Plastics, Oxnard, Calif. 93030).

Sodium Penicillin-G (Squibb and Sons, Inc., Princeton, N. J. 08540).

Streptomycin sulfate (Eli Lilly and Co., Indianapolis, Ind. 46206).

Ethylmalonic acid (Aldrich Chemical Co., Milwaukee, Wis. 53233).

Boron trifluoride/propanol (Applied Sciences Laboratories, State College, Pa. 16801).

3% FFAP on Chromosorb WHP, 100/120 mesh (Varian Aerograph, Walnut Creek, Calif.).

Assay Procedures

Amniotic fluid preparation: Amniotic fluids (10-15 ml) from 15- to 20-week gestations were obtained by amniocentesis and centrifuged (500 × g, 5 min).

To obtain non-cultured cells, we washed the cell pellet from the initial centrifugation with 5 ml of buffered isotonic saline and recentrifuged (500 × g, 5 min). The resulting pellet was suspended in 30 μl of water and...
Table 1. MethyImalonyl-CoA Mutase Activity In Cultured and Non-Cultured Amniotic Fluid Cells

<table>
<thead>
<tr>
<th>Category</th>
<th>Protein, µg/sample</th>
<th>cpm</th>
<th>Activity, pmol/mg protein per min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-cultured cells</td>
<td>23.8*</td>
<td>4667</td>
<td>140</td>
</tr>
<tr>
<td>(no blood)</td>
<td>(3.9–71.5)</td>
<td>(1186–10 308)</td>
<td>(46.0–295)</td>
</tr>
<tr>
<td>n = 8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-cultured cells</td>
<td>587</td>
<td>7708</td>
<td>14.6</td>
</tr>
<tr>
<td>(bloody)</td>
<td>(105–1625)</td>
<td>(132–21 169)*</td>
<td>(0.4–37.8)</td>
</tr>
<tr>
<td>n = 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult blood and cell-</td>
<td>1454</td>
<td>556</td>
<td>0.2</td>
</tr>
<tr>
<td>free amniotic fluid</td>
<td>(1013–1825)</td>
<td>(329–960)</td>
<td>(0.1–0.3)</td>
</tr>
<tr>
<td>n = 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cultured cells</td>
<td>28.6</td>
<td>11 701</td>
<td>329</td>
</tr>
<tr>
<td>n = 8</td>
<td>(15.0–50.0)</td>
<td>(697–33 746)</td>
<td>(18.0–1045)</td>
</tr>
</tbody>
</table>

* n is number of amniotic fluid specimens tested.
* Mean plus range in parentheses.
* Next higher value after 132 was 1084 cpm.

freeze-thawed five times in an acetone/solid CO₂ bath. The cell homogenate plus 7.4 nmol of adenosylcobalamin was incubated at 37 °C for 5 min in the dark and frozen until assay.

To obtain cultured cells, we resuspended the cell pellet resulting from the initial centrifugation in 5 ml of Ham’s F-12 media containing fetal calf serum (30 ml/dl), and 100 units of penicillin and 100 µg of streptomycin per milliliter. The suspension was seeded into a 25 cm² culture flask and allowed to grow until the cells were confluent, usually four to six weeks. At confluence the cells were rinsed with 3 ml of buffered isotonic saline and harvested with 1 ml of trypsin solution (2.5 g/liter). After centrifugation (500 × g, 5 min) lysates were prepared by freeze-thawing as described above.

At the time of assay the lysates from both cultured and non-cultured amniotic fluid cells were centrifuged (10 °C, 2000 × g, 10 min). The resulting supernate was used to quantitate the mutase activity as described previously (12). The reaction mixture contained 500 pmol of [14C]-methylmalonyl-CoA (sp. acty., 45.7 Ci/mol), 20 µl of cell lysate, 1 µmol of phosphate buffer, pH 7.4, 7.4 nmol of adenosylcobalamin. The final volume was 50 µl. Incubations were for 20 min at 37 °C. Incorporation of radiolabel into succinate was the index to mutase activity. Protein was measured by the method of Lowry et al. (13).

Many of the amniotic fluids were contaminated with maternal blood components. To establish that mutase activity was minimal in these components, we added fresh blood from control subjects to cell-free amniotic fluid until it was visibly more opaque than the amniotic specimens obtained from the pregnant women. This mixture was then centrifuged, rinsed, freeze-thawed, and processed the same as the non-cultured amniotic cells. As noted in Table 1 the protein content of these control amniotic fluids was more than twice that of the bloody specimens obtained by amniocentesis.

Organic acid assay: To a 2-ml sample of cell-free amniotic fluid or urine we added 1 g of NaCl crystals and 10 µg of ethylmalonic acid, the latter serving as the internal standard. The samples were adjusted to pH 1 with dilute sulfuric acid, extracted three times with 3-ml portions of ether, and taken to dryness under a gentle stream of air. Propanol derivatives of the organic acids were prepared by addition of 0.2 ml of boron trifluoride/propanol to the dried samples and heating in a boiling water bath for 10 min. Five milliliters of water was added to the derivatized sample. The derivatized organic acids were extracted from the water phase with 1 ml of “spectral grade” hexane. The hexane phase was injected directly into a column of 3% FFAP on Chromosorb WHP, 100/120 mesh, in a Varian 2100 Gas Chromatograph. For the first 10 min the column was maintained in isothermal mode at 60 °C. The chart strip remained turned off during the isothermal period (see Figures 1A and 1B). The temperature program was then initiated at 6 °C per minute to a maximum of 150 °C. Under these conditions, lactate, pyruvate, methylmalonate, ethylmalonate, fumarate, and succinate were well separated. It was not possible to quantitate lactic and pyruvic acids because of their lability, but the peak heights of the other organic acids were directly proportional to the amount in the standard.

Results

Measurement of methylmalonate: Twenty-four specimens of amniotic fluid were obtained from pregnant women at high risk because of advanced maternal age. One amniotic fluid was obtained from a woman with a fetus at high risk for methylmalonic acidemia. When tested for methylmalonate, none of these amniotic fluids exhibited a measurable peak in the region of methylmalonate. Gas chromatography of derivatized organic acid standards gave readily detectable peaks of methylmalonate that were clearly distinguishable from the peaks of the other organic acids (Figure 1A). The methylmalonate peak height in Figure 1A is equivalent to a concentration of 10 mg/liter. Published data on methylmalonate in amniotic fluid from mothers of affected fetuses (7–9) range from 2.2–10 mg/liter. Even
methylmalonate concentrations of less than 2 mg/liter are readily detectable by our method.

This gas-chromatographic method has several distinct advantages when compared to the traditional colorimetric assay of Giorgio and Plaut (14). It requires a smaller sample, is more sensitive, detects smaller amounts of methylmalonate, and the method is more specific for methylmalonate.

Figure 1B shows a chromatogram of amniotic fluid from the woman with a fetus at high risk for methylmalonic acidemia. No measurable methylmalonate was found. This chromatogram is typical of that obtained for normal control amniotic fluids.

**Measurement of mutase activity:** Mutase activity in liver tissue is very stable, because it is present as the holoenzyme. However, most of the mutase enzyme in fibroblast lysates is present in the apoenzyme form (12) and consequently it is not stable for more than a few days in the frozen state. In a series of early experiments with seven amniotic fluids that were not stabilized immediately with the mutase coenzyme, adenosylcobalamin, incorporation of radioactivity was low (<300 cpm). These lysates were stored for an average of eight days (range, 1–14 days). However, by adding adenosylcobalamin immediately to the lysate, the mutase was stabilized and maintained its activity after storage. Eighteen days was the longest any specimen was stored frozen.

Table 1 lists the mutase activity from 14 non-cultured and eight cultured amniotic fluid cells. The non-cultured cells that were not contaminated with blood had only 42% of the activity found for the cultured amniotic cells, because they contained more non-viable cells as determined by their inability to extrude trypan blue. As a result they contributed protein but no mutase activity. Despite the lower radioactivity (cpm), the specific activity of the mutase enzyme in the non-cultured cells was adequate for an accurate prenatal diagnosis, especially when combined with data on methylmalonate concentration in amniotic fluid.

When the amniotic fluid was bloody, it was necessary to know if the blood components contributed to the mutase activity. Therefore mutase activity was measured in whole blood, in cell-free amniotic fluid, and in a mixture of blood from adults and cell-free amniotic fluid. The activities of all three preparations were low, the highest being that in the combination of cell-free amniotic fluid plus adult whole blood (Table 1). Because estimation of mutase activity is based on the total protein in a specimen, contamination with blood will lower the specific activity but not the amount of radiolabel incorporated into succinate. As can be seen in Table 1, bloody, non-cultured amniotic fluid cells gave higher mean counts per minute (7708 compared to 4687), but gave only 10% of the specific activity (14.6 compared with 140.0 pmol of [14C]succinate per milligram per minute). These data present a problem of interpretation when bloody amniotic-fluid specimens are obtained. In marginal cases, mutase activity must be interpreted on the basis of both the counts per minute and specific activity. In the cases in which the specific activity is low because of contamination with blood, data on the methylmalonate concentration must be used to determine if the fetus is affected.
One example in which methylmalonate concentrations in amniotic fluid have to be known is when a falsely positive mutase result is obtained. One example is illustrated in Table 1. The lowest count per minute obtained for bloody, non-cultured amniotic fluid cells was 132. Mutase activity was calculated as 0.4 pmol/mg of protein per minute. Had this specimen been evaluated on the basis of specific activity and count alone, the diagnosis would have been erroneous. However, because amniotic fluid methylmalonate was absent, the results from this uncultured cell preparation could be interpreted as a false positive. Thirteen of the 14 specimens of non-cultured amniotic fluid cells displayed readily detectable mutase activity.

Another situation in which determination of methylmalonate in the amniotic fluid is crucial is when dealing with the vitamin B₁₂-responsive variant of methylmalonic acidemia. Mutase activity in lysates from these patients will be normal after adenosylcobalamin is added (12), because the apoenzyme is normal. Mutase activity will be normal in cells that are either cultured or not cultured. Therefore, to detect the mutant fetus the clinician must rely on finding amniotic fluid methylmalonate. A definitive diagnosis in the responsive mutant can only be made after vitamin B₁₂ coenzyme synthesis is determined in the fibroblasts (2).

Discussion

For the physician attempting to make a prenatal diagnosis, time is crucial. If a correct diagnosis requires enzyme analysis from cultured amniotic fluid cells, six weeks may elapse between when the initial amniocentesis is performed and when the final diagnosis is confirmed. This typical situation may preclude the option of termination of the pregnancy. Any modifications of techniques that can shorten the time between amniocentesis and results will allow for more carefully thought-out decisions as to the appropriate course of action.

The method reported here allows a rapid diagnosis to be made in pregnant women with a fetus at high risk for methylmalonic acidemia by using non-cultured amniotic fluid cells coupled with amniotic fluid methylmalonate concentrations. Our data indicate that non-cultured amniotic fluid cells have readily detectable mutase activity once the lysate has been stabilized with adenosylcobalamin. If the lysate is stabilized, the amniocentesis can be performed in a hospital other than the one measuring the mutase enzyme activity and can be mailed between institutions. Once the lysate is received, the mutase activity and methylmalonate concentrations can be determined within two days. This procedure shortens the time required to reach a diagnosis by almost five to six weeks as compared to the time required to assay cultured amniotic cells.

One complication of amniocentesis is that the fluid is often contaminated with blood, usually from the mother, and the possibility arises that her leukocyte mutase may contaminate the amniotic fluid cell lysate.

We have shown that when large amounts of blood from adults are added to amniotic fluid that is free of cellular material, the incorporation of radiolabel into [¹⁴C]succinate is lower than it is for amniotic fluid cell lysates of either blood-contaminated or non-blood-containing specimens. In those cases in which the lysate has a high protein content because of contamination with blood, the concentration of methylmalonate in the amniotic fluid and the actual radioactivity (counts per minute) incorporated into [¹⁴C]succinate are indices to fetal status. In those instances in which the enzyme data are not clear-cut—e.g., because of low specific mutase activity and low counts—experiments on cultured amniotic fluid cells should be performed. However, we think that the absence of methylmalonate in amniotic fluid, particularly if confirmed by a subsequent amniocentesis, is very strong evidence that the fetus does not have methylmalonic acidemia.

A second complication arises with a fetus at high risk for vitamin B₁₂-responsive methylmalonic acidemia. Because these have normal mutase apoenzyme but defective synthesis of the coenzyme, adenosylcobalamin, the enzymatic method described in this paper will not differentiate the affected from unaffected fetus on the basis of mutase activity alone. As a result, the clinician must rely on data on amniotic fluid methylmalonate concentrations and, if possible, cultured amniotic fluid cells (2, 15) to arrive at the correct diagnosis. Although only one case of methylmalonic acidemia due to a methylmalonyl-CoA racemase (EC 5.1.99.1) defect has been described (5), the present enzymatic method would not be applicable, because the L-form of the racemic substrate, [methyl-¹⁴C]methylmalonyl-CoA, would be metabolized to [¹⁴C]succinate. However, amniotic fluid from such a fetus would contain abnormal quantities of methylmalonate.

In summary, if non-cultured amniotic fluid cells have adequate mutase activity and there is no detectable amniotic fluid methylmalonate, the diagnosis of a normal fetus can be made with a high degree of certainty. Attempts should be made to confirm these results by enzyme analysis of cultured amniotic fluid cells. However, time constraints as well as failure to culture the cells successfully under some circumstances may make it necessary to rely on the data obtained from non-cultured cells and amniotic fluid. With proper stabilization of the lysate apoenzyme, specimens can be mailed between the referring institution and a core laboratory that is experienced in performing these techniques.

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References

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