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Properties of Fetal and Adult Red Blood Cell Arginase: A Possible Prenatal Diagnostic Test for Arginase Deficiency

E. B. SPECTOR,[1] M. KIERNAN,[1] B. BERNARD,[2] AND S. D. CEDERBAUM[1]

SUMMARY

Prenatal diagnosis of inborn errors of metabolism has been possible only if the enzyme affected is expressed in amniotic fluid cells grown in culture. Arginase is essentially undetectable in normal human fibroblasts, amniotic fluid, and amniotic fluid cells but is present in high amounts in red blood cells. It is absent in the red blood cells of patients with liver arginase deficiency. The properties of the enzyme in the red cells of healthy children and adults were compared to those of the enzyme obtained from cord blood red cells of 13–20-week fetuses obtained at hysterotomy. The activities, heavy metal requirements, heat stability, pH optimum, kinetic properties, and reaction with anti-arginase antibody were examined. Both enzyme species were either identical or substantially similar by all criteria. The adult and fetal enzymes are, therefore, probably determined by the same structural gene. Fetal red cells obtained during amniocentesis and amnioscopy should then be a suitable tissue to use to make the prenatal diagnosis of arginase deficiency.

INTRODUCTION

The urea cycle is the major pathway for detoxification of ammonia in mammals. Arginase (E.C.3.5.3.1), the fifth enzyme in the pathway, catalyzes the hydrolysis of

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[1] Mental Retardation Research Center and Departments of Psychiatry and Pediatrics, University of California, Los Angeles, School of Medicine.

[2] Department of Pediatrics, University of Southern California Medical School, Los Angeles.

arginine to urea and ornithine. Deficiency of this enzyme, inherited in an autosomal recessive manner, has been reported in nine patients of different ethnic origin [1–6]. Arginase deficiency is characterized by severe mental and neurological deterioration after apparently normal development for the first few years of life [1–6]. Two patients have been found to have reduced arginase activity in liver, in red and white cells, and in stratum corneum [3, 6].

Prenatal diagnosis of arginase deficiency has not been possible, since the enzyme activity is very low or undetectable in amniotic fluid and amniotic fluid cells grown in tissue culture. In the past few years, however, it has become possible to obtain fetal red blood cells largely free of maternal cells by amnioscopy and amniocentesis [7, 8]. This technique has been used successfully in the prenatal diagnosis of inherited hemoglobinopathies [9].

Red blood cells from young children and adults have a high arginase content [1–6]. In addition, the kinetic and immunologic properties of human red blood cell arginase are identical with those of liver arginase [10, 11], a finding consonant with their coordinate absence in inherited arginase deficiency [6]. We have studied the biochemical and immunologic properties of arginase in red cells of cord blood from 13- to 20-week fetuses obtained at hysterotomy. The fetal and adult arginases have very similar properties; hence, fetal red blood cells would be suitable cells to use for the prenatal diagnosis of arginase deficiency.

MATERIALS AND METHODS

Preparation of Red Blood Cell Hemolysates

Cord blood from abortuses was obtained at the time of hysterotomy performed for medical indications, following a procedure approved by the Los Angeles County-USC Human Subjects Protection Committee. The samples were collected in heparinized containers and stored at 4°C for 24–48 hrs. Approximately 1.5 ml of blood was obtained from each abortus. The samples were spun at 320 *g* for 5 min, the plasma was removed, and the red cells were washed twice with 2 vol 0.85% NaCl. The supernatant was removed and the red cells resuspended in 1 ml 10 mM Tris-HCl buffer, pH 7.4, and frozen at −70°C. Red blood cell hemolysates from adults were prepared in the same manner. Two vol buffer was added before the samples were frozen.

Enzyme Assay

Arginase activity was measured by an adaptation of the method of Schimke [12]. The blood sample was diluted with 3 vol 0.1 M Tris-glycine buffer, pH 9.5. A sample, 0.05 ml, was added to 0.10 ml 0.05 M $MnCl_2$ and heated at 55° for 5 min. The samples were cooled to room temperature, and a mixture of 10 μmoles nonradioactive arginine, pH 9.5, and 0.1 μCi guanidino[^{14}C]arginine (Amersham-Searle, Arlington Heights, Ill.; 53 μCi/μmole) in 0.1 ml of the Tris-glycine buffer was added. The reaction was allowed to proceed at 37°C for 30 min and was terminated by placing the tubes in a boiling water bath for 5 min. Potassium phosphate buffer, 0.2 M, pH 6.6, 1 ml, was added to bring the pH of the reaction mixture to 7.0–7.4. Jack bean urease (Sigma, St. Louis, Mo.; 11 U/ml; 1 U = 1 mg ammonia produced/5 min at 30°C), 0.1 ml, was added and the reaction tubes covered with rubber stoppers holding center wells (Kontes, San Leandro, Calif.) containing filter paper (Whatman, #1) soaked in 0.15 ml of 8% NaOH. After 90 min incubation at 37°C, with shaking, the radioactivity trapped on the filter paper was counted in 15 ml of scintillation fluid containing 12.0 g 2,5-diphenyloxazide (PPO) and 0.3 g p-bis(2-[5-phenyloxazoly])-benzene (POPOP) in 1 liter absolute methanol and 2 liters

toluene. The amount of radioactivity in the cups was determined in a Beckman LS 230 scintillation counter with a 95% counting efficiency. One U enzyme is equal to 1 μmole arginine cleaved/30 min.

pH Optimum

Hemolysate, diluted with water, 0.025 ml, was added to 0.05 ml 0.05 M $MnCl_2$ and incubated at 55°C for 5 min. The activated hemolysate was brought to 0.239 ml, with 0.2 M Tris-HCl buffers from pH 2.0–11.0. The reaction was initiated by adding 11 μl of 1 M nonradioactive arginine in Tris-glycine buffer, pH 9.5, containing 0.10 μCi guanidino[^{14}C]arginine. The assay was completed as described above.

Cation Activation

Diluted hemolysate, 0.05 ml, was added to 0.10 ml of either 0.05 M $MnCl_2$, $CoCl_2$, $CaCl_2$, or $MgCl_2$ and incubated for 30 min at 55°C. The arginase activity was determined as described above.

The activity of arginase at different concentrations of $MnCl_2$ and $CoCl_2$ was also determined. $MnCl_2$ and $CoCl_2$ were added to final concentrations of 1, 2, 10, 20, 100, and 200 mM. The blood was incubated for 5 min at 55°C and the arginase activity determined by the standard method.

Kinetics

The apparent K_m for arginine and V_{max} of arginase in the fetal and adult red blood cells was determined using the standard arginase assay. The final concentration of arginine (nonradioactive plus radioactive) was 0.5, 1, 2, 5, 10, 15, 20, 40, and 100 mM.

The apparent K_i for lysine and ornithine was determined by adding lysine at a final concentration of 50 mM or ornithine at a final concentration of 20 mM to the same concentrations of arginine used for the K_m determinations.

Heat Stability

Diluted hemolysate was heated at 68°C without additional $MnCl_2$ or at 81°C in the presence of 0.02 M $MnCl_2$. Aliquots were taken at various times during a 30-min period. The remaining arginase activity was determined as described above.

Immunoprecipitation

Rabbit anti-rat liver arginase was obtained as a gift from Dr. R. Palacios of the Institute of Biomedical Investigation in Mexico City. Immunized or unimmunized rabbit serum was brought to a concentration of 60% ammonium sulfate, the precipitate collected by centrifugation at 30,000 *g* for 30 min, and the pellet resuspended in 0.01 M Tris-HCl, pH 7.4, at a concentration of 0.6 mg protein/ml. Enough hemolysate to contain approximately 0.2 U of arginase activity was added to various amounts of anti-arginase antibody. The protein concentration was kept constant in each tube by adding IgG from unimmunized rabbits. The mixture was incubated at 37°C for 30 min and then at 4°C overnight. Goat anti-rabbit immunoglobulin (Calbiochem-Behring, La Jolla, Calif.), 2.5 U, was added to precipitate all the immunoglobulin and kept at 4°C for 4 more hrs. The entire mixture was spun at 30,000 *g* for 30 min, and the activity remaining in the supernatant determined as described above.

Hemoglobin Determinations

The amount of hemoglobin (Hb) contained in each sample was determined as methemoglobin by adding 0.02 ml of the sample to 5.0 ml Drabkins reagent [13] (1.0 g sodium bicarbonate, 0.05 g potassium cyanide, and 0.02 g potassium ferricyanide). The optical density at 540 nm was determined.

RESULTS

Enzyme Activities

A total of 15 cord blood samples from 13–20-week abortuses and five samples from normal adults were collected. The activity of arginase in the fetal blood, 357.7 ± 175.9 U/g Hb, (134.0–638.0 U/g Hb) was significantly lower than that of the adult blood, 848.7 ± 455.5 U/g Hb, (517.2–1,615.2 U/g Hb), ($P < .01$).

Activation by Cations

The arginase in both the fetal and the adult blood was activated maximally by Mn^{2+}. Activation by Co^{2+} was only 27 ± 2% and 22 ± 4% of that seen with manganese in the adult and fetal red blood cells, respectively. Mg^{2+} gave 15 ± 2% and 11 ± 3% activation in the adult and fetal red blood cells, while Ca^{2+} gave less than 1% of the activity seen with Mn^{2+} (table 1).

The final concentration of Mn^{2+} and Co^{2+} that gave maximal activation was less than 10 mM. There is essentially no difference between the fetal and adult activity curves for Mn^{2+} and Co^{2+} activation (data not shown).

pH Optimum

The adult and fetal arginases exhibited the same pH optimum (fig. 1). Maximum enzyme activity was reached at pH 9.5.

Kinetics

The K_m was determined utilizing the direct linear plot described by Cornish-Bowden (table 2) [14]. The average apparent K_m for arginine of fetal arginase is 9.0 mM and that of adult arginase, 10.1 mM. A linear regression analysis of the Lineweaver-Burk transformation of the data yields an average apparent K_m for arginase of 3.5 mM for adult red blood cells and 3.6 mM for fetal red blood cells (table 2). By both methods, the K_i for lysine and ornithine was in the same range as the K_m for arginine in both the fetal and adult red blood cells (table 2).

Heat Stability

The fetal and adult red blood cell arginases in the hemolysates showed similar patterns of inactivation at 68°C or at 81°C in the presence of 20 mM $MnCl_2$ (figs. 2*A*

TABLE 1

ACTIVATION OF ARGINASE IN ADULT AND FETAL RED BLOOD CELLS BY CATIONS RELATIVE TO Mn^{2+}

SAMPLE	ACTIVITY (%) RELATIVE TO Mn^{2+} AT pH 9.5			
	Mn^{2+}	Ca^{2+}	Mg^{2+}	Co^{2+}
Adult (no. = 5)	100	0.7 ± 0.9*	14.7 ± 2.2	27.0 ± 1.9
Fetal (no. = 3)	100	< 0.01	10.8 ± 3.2	21.7 ± 4.5

* Each sample was assayed in triplicate and data for all samples averaged and reported as mean ± 1 SD. No detectable activity was found in the absence of any cation.

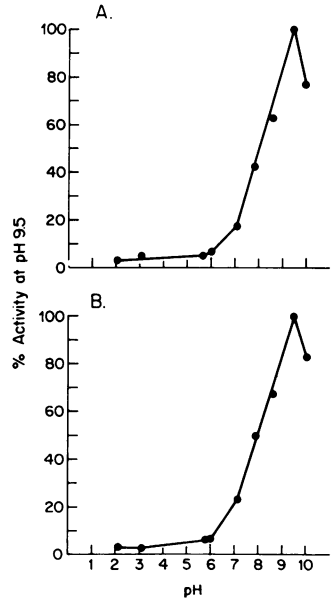

FIG. 1.—Fetal and adult red blood cell arginase activity at different pH's. Arginase activity in (*A*) adult and (*B*) fetal blood determined as described in MATERIALS AND METHODS. Data represent the average of three replicates of one adult and one fetal sample. Two additional adult and fetal samples were studied with similar results.

and 2*B*). The heat denaturation curve for both the fetal and adult enzymes are biphasic at 68°C without $MnCl_2$. Under these conditions, 40% of the total arginase activity was destroyed within 2 min. After the rapid loss of this unstable component, the rate of decay of the residual enzymes was similar, with the rate of decay in the fetal samples being slightly faster. The heat denaturation curve is not biphasic at 81°C in the presence of Mn^{2+}. Both enzymes undergo exponential decay with 50% of the activity being destroyed after 5 min of heating.

Reaction with Antibody

Both the fetal and adult red blood cell arginase reacted with antibody directed against rat liver arginase (figs. 3*A* and 3*B*). An average of 31.1 mU and 30.6 mU of activity were neutralized by 35 μg of antibody in adult and fetal red blood cell hemolysates, respectively.

DISCUSSION

Fetal and adult red blood cell arginases have very similar properties. Both require a divalent cation, preferentially Mn^{2+}, for maximum activity and have a pH optimum of 9.5. Both the fetal and adult enzymes showed similar heat denaturation curves when heated at 68°C without Mn^{2+} or at 81°C in the presence of Mn^{2+}. The affinity for the substrate, arginine, and for the inhibitors, lysine and ornithine, was also similar for both enzymes. Additionally, both fetal and adult arginase reacted similarly with antibody to rat liver arginase. Cabello et al. [10, 11] have demonstrated that partially

TABLE 2

KINETIC PROPERTIES OF ARGINASE FROM ADULT AND FETAL RED BLOOD CELLS

SAMPLE	DIRECT LINEAR PLOT			LINEWEAVER-BURK PLOT		
	K_m* (mM)	K_i(Orn)† (mM)	K_i(Lys)‡ (mM)	K_m* (mM)	K_i(Orn)† (mM)	K_i(Lys)‡ (mM)
Fetal:						
J	7.0	...	...	1.6	...	...
C	7.9	...	...	4.7	...	...
W	8.2	2.0	...	2.7	1.0	7.7
B	12.9	19.6	4.7	5.3	2.8	1.6
Adult:						
F	9.5	12.3	19.3	3.1	2.9	4.2
P	11.9	3.0	15.8	4.3	1.2	7.7
M	9.0	17.9	8.8	3.2	1.6	7.6

* Concentration of arginine was varied from 0.5–100 mM.
† Concentration of ornithine was 20 mM and of arginine, 0.5–100 mM.
‡ Concentration of lysine was 50 mM and of arginine, 0.5–100 mM.

purified human red blood cell and liver arginase from adults have similar kinetic properties and react similarily with their antibodies against human liver and human erythrocyte arginases. Preliminary results in this laboratory indicate that fetal and adult liver arginases are identical with red blood cell arginase [15]. These data support the assumption that fetal and adult arginases in red blood cells and liver are encoded in the same gene and are confirmed by the coordinate absence of arginase in both tissues in inherited arginase deficiency [6].

The fetal red blood cell arginase differed from adult arginase only in its total activity. The decreased activity did not correlate with the length of time that a sample was stored before reaching us. Blood samples from adults stored the same way did not show decreased activity. Herzfeld et al. [16] reported that the arginase activity in liver from 16–20-week human fetuses is approximately 50% of that found in adult liver. Greengard [17] examined 23 liver enzymes participating in various biochemical pathways and found that in 18 of these enzymes the ratio of the activity/gram wet weight of fetal to adult enzyme was less than 0.80. The mechanism(s) leading to this reduction in enzyme activity are not known. The reduced arginase activity/mg Hb in the fetal red blood cells could be due to the presence of a higher proportion of other proteins to arginase than in adult red blood cells. Arginase would then account for a smaller percentage of the total red cell proteins and have a reduced specific activity. A second gene coding for the fetal form of the enzyme need not be invoked to explain the reduction in enzyme activity found in the fetal red blood cell.

Our studies suggest that an accurate diagnosis of arginase deficiency could be made using fetal red blood cells obtained from the placenta or from a fetal blood vessel on the chorionic plate. Contamination of fetal blood with maternal blood is common in this sampling procedure [18]. The proportion of fetal blood in the sample can be determined by utilizing an acid stability test (Kleihauer-Betke [19]), or by sizing the cells using a Coulter cell counter [8]. If the proportion of maternal cells is above 10%,

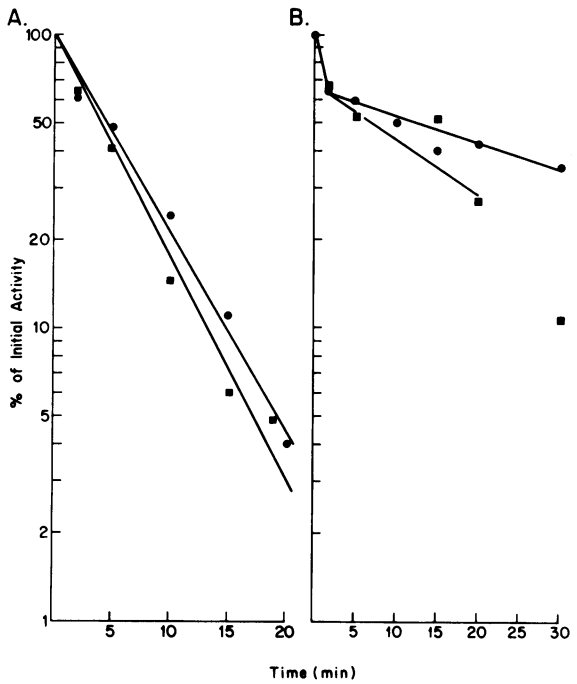

FIG. 2. —Stability of fetal (■ — ■) and adult (● — ●) red blood cell arginase to heat. *A*, Extracts at 81°C diluted in buffer containing 20 mM $MNCl_2$ and, *B*, extracts at 68°C diluted in the absence of added manganese were heated for 0–30 min. At times indicated, an aliquot was removed and the arginase activity determined. Three adult and three fetal samples were studied.

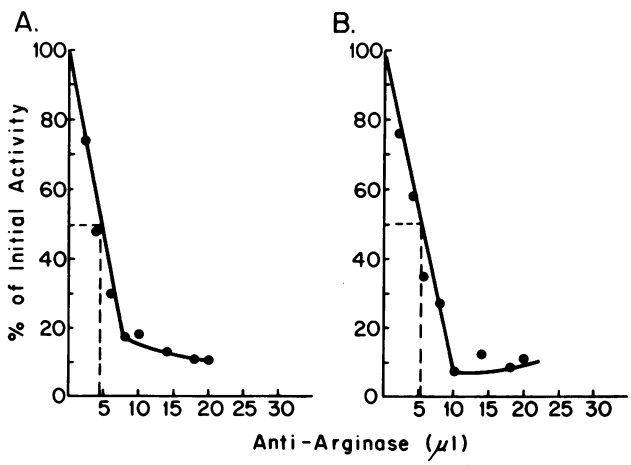

FIG. 3. —Precipitation of arginase from fetal and adult red blood cells with anti-rat liver arginase. Immunoprecipitation was carried out as described in MATERIALS AND METHODS. 100% = 83.4 mU in (*A*) fetal red blood cells and 123.4 mU in (*B*) adult red blood cells. Two additional adult and three additional fetal samples were tested with similar results.

precluding the accurate distinction of carrier status, the fetal cells can be agglutinated with anti-i antiserum, an antibody that is directed against fetal red blood cells [20]. Using this antibody, a mixture containing 5% fetal cells and 95% maternal cells can be purified to a solution containing 95% fetal cells. Alternatively, preferential lysis of adult red blood cells can be accomplished [21]. We feel that we could make an accurate diagnosis of total absence of activity on as little as 10 μl of fetal blood. Diagnosis of a heterozygous state would be very difficult, however.

Fetal red cells could also be used to diagnose other inborn errors of metabolism. They may be useful for the prenatal diagnosis of enzyme deficiencies in the glycolytic pathway leading to hemolytic anemia or in heme biosynthesis [18]. In addition, in instances where there is insufficient time to grow amniotic fluid cells, the fetal red cells may be useful as the diagnostic tissue. In all cases, there must be substantial evidence that the enzyme present in the fetal tissue is the same as that present postnatally and the range of normal values is well established. The proof of our assumption that fetal red cells can be utilized for the prenatal diagnosis of arginase deficiency will rest in the independent confirmation of the diagnosis postnatally or in the tissues of an aborted fetus.

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