Multiple Enzymatic Defects in Mitochondria in Hematological Cells of Patients with Primary Sideroblastic Anemia

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ABSTRACT Activities of mitochondrial enzymes in blood cells from 69 patients with primary sideroblastic anemia were determined to elucidate the pathogenesis of the disease. In erythroblasts of patients with primary acquired type the activities of both δ-aminolevulinic acid synthetase and mitochondrial serine protease were inevitably decreased. The susceptibility to the protease of apo-δ-aminolevulinic acid synthetase prepared from erythroblasts of patients with this type was within the normal range, in contrast to that of pyridoxine-responsive anemia. The activities of mitochondrial enzymes such as cytochrome oxidase, serine protease, and oligomycin-sensitive ATPase, except citrate synthetase, were usually decreased in mature granulocytes of the patients. Patients with hereditary sideroblastic anemia also had decreased δ-aminolevulinic acid synthetase activity in erythroblasts, and decreased serine protease activity in both erythroblasts and mature granulocytes. Mature granulocytes obtained from patients with pyridoxine-responsive anemia before therapy had decreased cytochrome oxidase activity, however, the activity increased to a normal level when the patients were in remission. The activities of other mitochondrial enzymes in mature granulocytes were within normal range in these patients before pyridoxine therapy. The activities of these mitochondrial enzymes in lymphocytes were within normal range in all groups of patients with primary sideroblastic anemia.

We suggest that patients with primary acquired, and possibly also those with hereditary sideroblastic anemia have impaired mitochondrial function in both erythroblasts and granulocytes. That only anemia is observed in these patients is because a functional abnormality of mitochondria in erythroblasts is most important because of the role of mitochondria in the formation of heme in erythrocyte development. In contrast to these two types of sideroblastic anemia, only δ-aminolevulinic acid synthetase in both erythroblasts and granulocytes seems to be impaired in patients with pyridoxine-responsive anemia.

INTRODUCTION

Patients with primary sideroblastic anemia show hypochromic or dimorphic anemia, elevated serum iron with high saturation of total iron binding capacity, and erythroid hyperplasia in the bone marrow with the presence of a large number of ring sideroblasts. Ferrokinetic studies usually show accelerated plasma iron disappearance with impaired iron incorporation into erythrocytes. Almost all features characteristic of this disease can be ascribed to a disturbance of heme synthesis in erythroblasts (1, 2). In fact, the activity of δ-aminolevulinic acid (ALA)[1] synthetase, the rate-limiting enzyme in heme synthesis is inevitably decreased in erythroblasts of patients with primary sideroblastic anemia (2).

In the course of investigation of the regulation of ALA synthetase activity in erythroblasts, a new serine protease was found on the inner mitochondrial membrane of bone marrow cells including both erythroblasts and granulocytes (3). An inverse relationship was observed between ALA synthetase activity and this serine protease activity in erythroblasts (3). Therefore, the protease activity in erythroblasts of patients with primary sideroblastic anemia was measured. Unexpectedly, not only ALA synthetase activity but also the serine protease activity was inevitably decreased in erythroblasts of patients with primary acquired sideroblastic anemia. Also the protease activity in mature granulocytes obtained from patients with primary acquired sideroblastic anemia was decreased.

In this paper we studied several mitochondrial en-

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[1] *Abbreviations used in this paper*: ALA, δ-aminolevulinic acid.

zyme activities in erythroblasts, granulocytes, and lymphocytes obtained from patients with primary sideroblastic anemia. We found that multiple enzymatic defects in mitochondria in both erythroblasts and granulocytes are characteristic to primary acquired sideroblastic anemia and possibly to hereditary sideroblastic anemia. On the other hand, only ALA synthetase in both erythroblasts and granulocytes seems to be impaired in patients with pyridoxine-responsive anemia.

METHODS

Patients. 61 cases with primary acquired sideroblastic anemia, 6 cases with hereditary sideroblastic anemia, and 2 cases with pyridoxine-responsive anemia were employed in this report. Samples from these patients were collected from 34 hospitals in various parts of Japan between June 1972 and September 1979. These patients were diagnosed on the basis of hypochromic or dimorphic anemia, hyperferremia with increased saturation of iron binding capacity, erythroid hyperplasia in the bone marrow, the presence of a large number of ring sideroblasts (above 30%), and the absence of causative diseases such as leukemia, chronic inflammation, or malignancy. Diagnosis of six cases with hereditary sideroblastic anemia was based on family history, male sex, and early occurrence of anemia. Every patient with these two types of sideroblastic anemia was treated unsuccessfully with pharmacological doses of pyridoxine. Two patients with pyridoxine-responsive anemia (classical form) have been described (4).

Cell separation. The erythroblast-rich fraction was prepared from aspirated bone marrow cells as described (4). Cellular components of bone marrow aspirate that were suspended in 20 ml of saline were filtered through four layers of gauze, and the filtrate was layered on a separating medium consisting of two parts (10 ml each), which were made by mixing appropriate amount of Lymphoprep (Nyegaad & Co. AS. Oslo, Norway) and saline. Specific gravity of each part was 1.067 (upper) and 1.073 (lower). After centrifugation at 170 *g* for 30 min at 4°C the cell layers formed between the sample and upper part, and between upper and lower parts were collected to obtain the erythroblast-rich fraction and the immature granulocyte-rich fraction, respectively.

Mature granulocytes and lymphocytes were prepared from the peripheral blood. Heparinized peripheral blood (15–20 ml) was diluted with equal volumes of saline. It was layered on the top of equal volumes of Lymphoprep, and centrifuged at 400 *g* for 30 min at 18°C. The layer formed between the sample and Lymphoprep was collected to obtain lymphocytes. The precipitate that contained both erythrocytes and mature granulocytes was mixed with equal volumes of plasma, and was layered on the top of 8 ml of Lymphoprep. It was left at room temperature for 20–30 min, when almost all erythrocytes precipitated to the bottom and the layer was formed between plasma and Lymphoprep. This layer was collected to obtain mature granulocytes.

Enzyme assays. ALA synthetase activity was measured as described (2). Cellular components of bone marrow aspirates (5×10^6–5×10^7 of erythroblasts) were hemolyzed with 4 vol of water for 10 min. The hemolysate was restored isotonicity by adding 11.5% KCl solution, and was centrifuged at 10,000 *g* for 10 min. The precipitate was washed once by centrifugation, suspended in 10 ml of 1.15% KCl solution containing 0.01 M potassium phosphate buffer, pH 7.2, and sonicated for 1 min in ice water (20 kcycle/s, 120 W, model UR-150p, Tomy, Tokyo). From the precipitate that was obtained by centrifuging the sonicated solution at 20,000 *g* for 20 min ALA synthetase was extracted with 1 ml of 0.2% sodium deoxycholate (2). ALA synthetase activity was determined using [^{14}C]succinyl-CoA as the precursor, and [^{14}C]-ALA formed was isolated by Dowex (Dow Corning Corp., Midland, Mich.) 50 × 8 W column chromatography to be counted by a liquid scintillation counter as described (2). 1 U was defined as the enzyme activity producing 1 nmol of ALA in 30 min under the conditions described (2).

The assay of the protease activity was performed as described (4). Blood cells (5×10^6–1×10^7) suspended in 3 ml of 0.85% NaCl containing 0.01 M potassium phosphate buffer, pH 7.5, were sonicated at 50 W for 15 s in a 50-ml centrifuge tube (Branson Sonic Power Co. Danbury, Conn.; model W 185), and then centrifuged at 20,000 *g* for 15 min. From the precipitate, the protease was extracted with 1 ml of 0.5 M potassium phosphate buffer, pH 7.0, for 30 min at 37°C, and the activity was measured using apo-ornithine transaminase as substrate (3, 4). 1 U was defined as the amount of protease inactivating 50% of apo-ornithine transaminase in 30 min under the conditions described (3, 4).

Cytochrome oxidase activity was measured by the method of Smith (5). Blood cells (5–8×10^6) suspended in 1 ml of saline were sonicated at 50 W for 15 s in a 10-ml centrifuge tube to be used as the enzyme solution. Reaction mixtures contained reduced cytochrome *c*, 90 nmol; potassium phosphate buffer, pH 6.0, 225 μmol; and enzyme solution, 0.2 ml in a final vol of 3 ml. The control tube contained 1 mM potassium cyanide in addition to the above components. Reactions were started by adding the enzyme solution, and initial reaction rates were determined by measuring continuously the decrease of absorbance at 550 nm at 25°C. 1 U was defined as the enzyme activity oxidizing 1 μmol reduced cytochrome *c*/min under the conditions described above. The reaction was linear up to 3×10^6 of blood cells/3 ml of incubation mixture, and the difference in duplicate analysis was always within 10%.

Oligomycin-sensitive ATPase activity was measured by the method of Kagawa (6). The enzyme solution was prepared by sonicating cells (1–1.5×10^7) suspended in 1 ml of 0.85% NaCl that contained 50 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$ at 50 W for 15 s in a 10-ml centrifuge tube. Reaction mixtures contained ATP, 3 μmol; $MgCl_2$, 1.5 μmol; phosphoenol pyruvate, 2.5 μmol; pyruvate kinase, 16 μg; Tris-HCl (pH 7.4), 50 μmol; 2,4-dinitrophenol, 50 nmol; oligomycin A, 5 μg; and enzyme solution, 0.2 ml in a final vol of 0.5 ml. After incubation at 30°C for 15 min, reactions were terminated by the addition of 0.1 ml of 50% trichloroacetic acid. After centrifugation inorganic phosphate was determined in the supernate by the method of Lohmann and Jendrassik (7). Oligomycin-sensitive ATPase activity was determined by calculating ATPase activity measured with and without oligomycin. 1 U was defined as the enzyme activity releasing 1 μmol inorganic phosphate/min under the conditions described above. Reactions were linear up to at least 4×10^6 blood cells/0.5 ml of incubation mixture, and up to 25 min of incubation time. Differences in duplicate analysis were always within 10%.

Citrate synthetase activity was measured according to the method of Ochoa (8). Blood cells (5–7×10^6) suspended in 1 ml of saline were sonicated at 50 W for 15 s in a 10-ml centrifuge tube and 0.2 ml was used as the enzyme solution. The reaction mixture contained sodium malate, 10 μmol; NAD, 0.3 μmol; acetyl-CoA, 0.21 μmol; malate dehydrogenase from pig heart (Boehringer Mannheim GmbH, Mannheim, West Germany), 0.5 μg; Tris-HCl (pH 8.0), 75 μg; and enzyme solution, 0.2 ml in a final vol of 1.5 ml. Tubes containing the reaction mixture without sodium malate were

employed as the control. The enzyme activity was measured by monitoring continuously, at 340 nm, NADH produced at 25°C. Reactions were linear up to 3×10^6 blood cells/1.5 ml of incubation mixture, and differences in duplicate analysis were always within 5%. 1 U was defined as the enzyme activity producing 1 μmol NADH/min under the conditions described above.

RESULTS

Cell separation. The erythroblast-rich fraction obtained from bone marrow aspirate contained erythroblasts (20–50%), lymphocytes (40–70%), and immature granulocytes (5–15%). The immature granulocyte-rich fraction consisted chiefly of immature granulocytes (85–90%) with a small number of lymphocytes (5–10%). Preparations of mature granulocytes obtained from peripheral blood contained mainly mature granulocytes (90–97%), and those of lymphocytes chiefly lymphocytes (85–95%).

ALA synthetase activity in erythroblasts. 61 cases with primary acquired type, 6 cases with hereditary type, and 2 cases with pyridoxine-responsive type were employed for the measurement of the ALA synthetase activity in erythroblasts. Every case showed decreased ALA synthetase activity in erythroblasts as shown in Fig. 1.

Protease activity. The protease activity assayed by the method described above is considered to indicate the activity of the new serine protease described (3, 4) from the following reasons. Firstly, the activity assayed by this method was completely inhibited by the addition of a small amount of elastatinal (100 μg/ml), the inhibitor of both pancreas elastase and this protease (3, 9). Secondly, the enzyme solution used in these experiments showed no detectable elastolytic activity. Thirdly, elastase from swine pancreas (Worthington Biochemical Corp., Freehold, N. J.) exhibited a poor ability to inactivate apo-ornithine transaminase as compared with that of this new protease (17% of the protease).

Because the erythroblast-rich fraction contained a small number of immature granulocytes and lymphocytes, the protease activity in erythroblasts was calculated considering relative contribution of different cell populations to the protease activity as described (4). In contrast to the normal protease activity in erythroblasts of patients with pyridoxine-responsive anemia, erythroblasts obtained from 18 cases with primary acquired sideroblastic anemia and those from three cases with hereditary sideroblastic anemia had decreased protease activity as shown in Fig. 2.

The protease activity in mature granulocytes from patients with primary sideroblastic anemia is shown in Fig. 3. All except three cases with primary acquired sideroblastic anemia had decreased protease activity in mature granulocytes. Mature granulocytes obtained from three cases with hereditary sideroblastic anemia

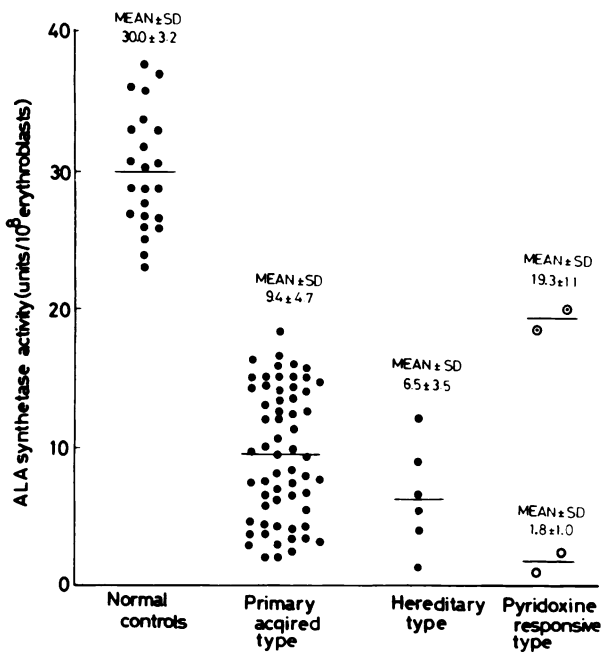

FIGURE 1 ALA synthetase activity in erythroblasts of patients with primary sideroblastic anemia. In the column of pyridoxine-responsive anemia the symbol (O) shows the activity before therapy, and ⊙, that after treatment with pharmacological doses of pyridoxal phosphate. Horizontal bars present the mean values.

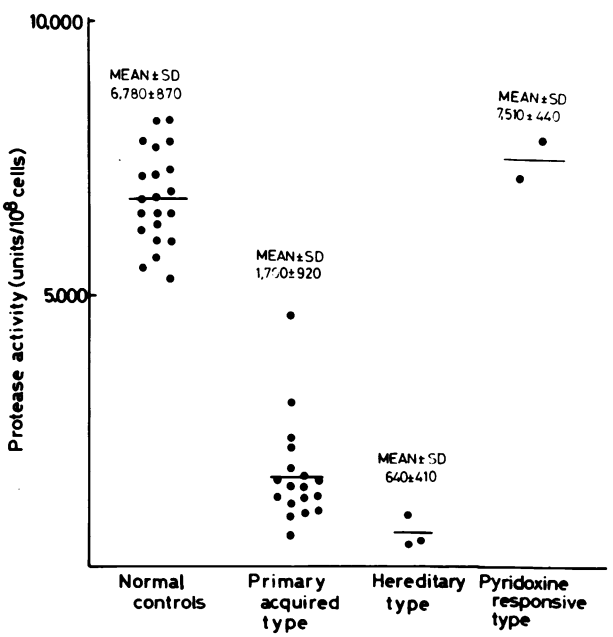

FIGURE 2 The activity of the new serine protease in erythroblasts of patients with primary sideroblastic anemia. Horizontal bars present the mean values.

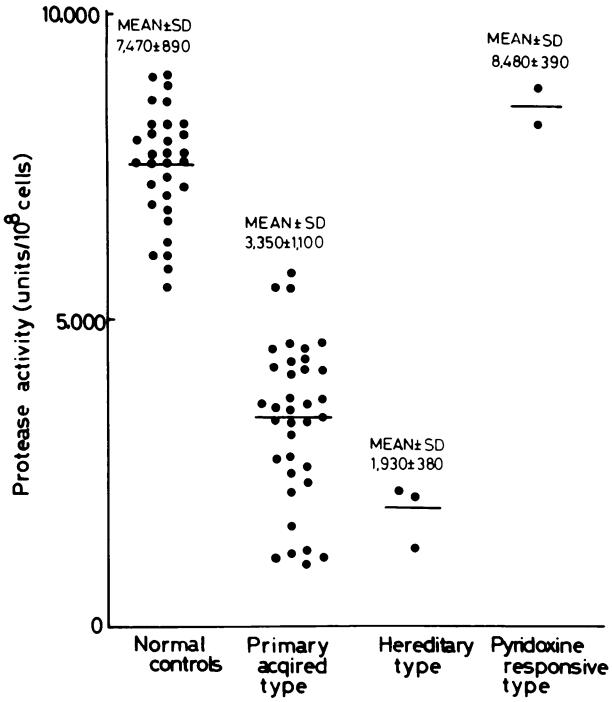

FIGURE 3 The new serine protease activity in mature granulocytes of patients with primary sideroblastic anemia. Horizontal bars present the mean values.

also showed decreased protease activity. On the contrary, mature granulocytes obtained from two cases with pyridoxine-responsive anemia before therapy revealed the protease activity within normal range.

Inactivation of apo-ALA synthetase by the protease. Degrees of inactivation of the apo-ALA synthetase by the protease that is considered to be engaged in the regulation of ALA synthetase levels in mitochondria in erythroblasts were examined by the method described (4). Results are shown in Table I. In contrast to the apo-ALA synthetase prepared from erythroblasts of patients with pyridoxine-responsive anemia, degrees of inactivation by the protease of apo-ALA synthetase prepared from patients with primary acquired sideroblastic anemia were almost the same as those of normal controls.

Cytochrome oxidase activity. Both mature granulocytes and lymphocytes in the peripheral blood were employed for the determination of cytochrome oxidase activity. Addition of catalase (10 nM) from bovine liver (Boehringer Mannheim GmbH) to the reaction mixture had no effect on the oxidation of cytochrome *c* by the enzyme solution, therefore, cytochrome *c* peroxidase does not play any roles in the oxidation of cytochrome *c* by the reaction system employed here. Furthermore, the reaction mixture containing 1 mM potassium cyanide showed only a little oxidation of reduced cytochrome *c*, and that with 1 mM

TABLE I
Susceptibility of apo-ALA Synthetase to Protease

Patients prepared apo-ALA synthetase	Susceptibility to protease
	U*
Normal controls (n = 15)	0.94±0.18‡
Pyridoxine-responsive anemia (n = 2)	4.65±0.58
Primary acquired sideroblastic anemia (n = 6)	0.83±0.12

Apo-ALA synthetase solutions were incubated at 37°C for 10 min in the presence of 120 U of protease to measure the degree of inactivation by protease as described (4).
* 1 U of susceptibility to protease was defined as the degree of inactivation losing one-half of the enzyme activity in 30 min under the conditions described (4).
‡ Mean±SD.

potassium cyanide containing oxidized cytochrome *c* instead of the reduced form revealed no significant reduction of cytochrome *c* as judged by the changes in the absorbance at 550 nm. These results indicate that the decrease in absorbance at 550 nm under the conditions described in the Methods shows the activity of cytochrome oxidase. Fig. 4 shows the cytochrome oxidase activity in both mature granulocytes and lymphocytes obtained from patients with primary sideroblastic anemia. Every case with primary acquired sideroblastic anemia had decreased cytochrome oxidase activity in mature granulocytes. Also mature granulocytes obtained from two cases with pyridoxine-responsive anemia before treatment showed decreased activity, however, the activity increased to the normal level when the patients went into remission after treatment with pharmacological doses of pyridoxal phosphate (Fig. 4). Lymphocytes obtained from both patients with primary acquired type and those with pyridoxine-responsive anemia revealed the cytochrome oxidase activity within normal range.

Oligomycin-sensitive ATPase activity. Every case with the primary acquired type had decreased oligomycin-sensitive ATPase activity in mature granulocytes as shown in Fig. 5. In contrast, the activity in mature granulocytes obtained from two cases with pyridoxine-responsive anemia before treatment was within the normal range. The oligomycin-sensitive ATPase activity in lymphocytes obtained from both patients with primary acquired type and those with pyridoxine-responsive anemia before treatment was within the normal range.

Citrate synthetase activity. Citrate synthetase ac-

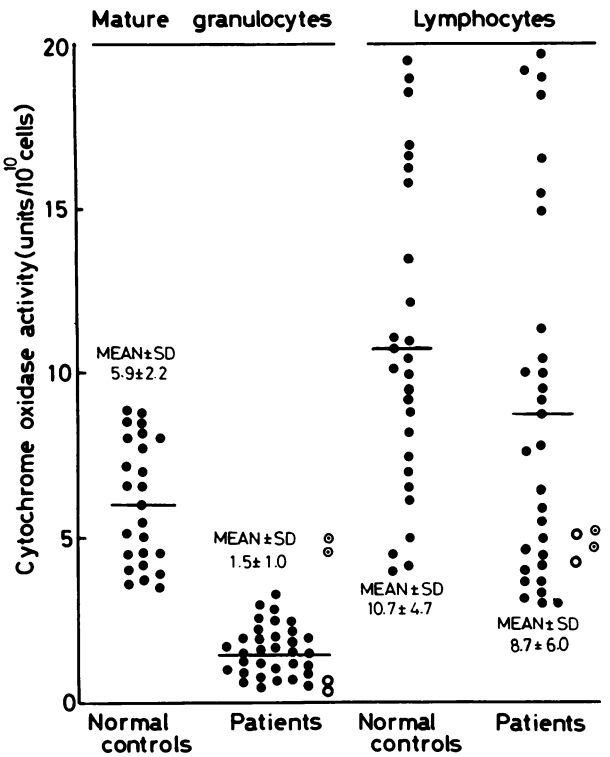

FIGURE 4 Cytochrome oxidase activity in both mature granulocytes and lymphocytes obtained from patients with primary sideroblastic anemia. In the column of patients, the symbol (●) shows the value of patients with primary acquired sideroblastic anemia, O, that of patients with pyridoxine-responsive anemia before therapy, and ⊙, that of patients with pyridoxine-responsive anemia in remission by the treatment with pharmacological doses of pyridoxal phosphate. Each horizontal bar presents the mean value (horizontal bars in the column of patients indicate the mean values of patients with primary acquired type, and Mean±SD in the column of patients shows that of patients with primary acquired type.).

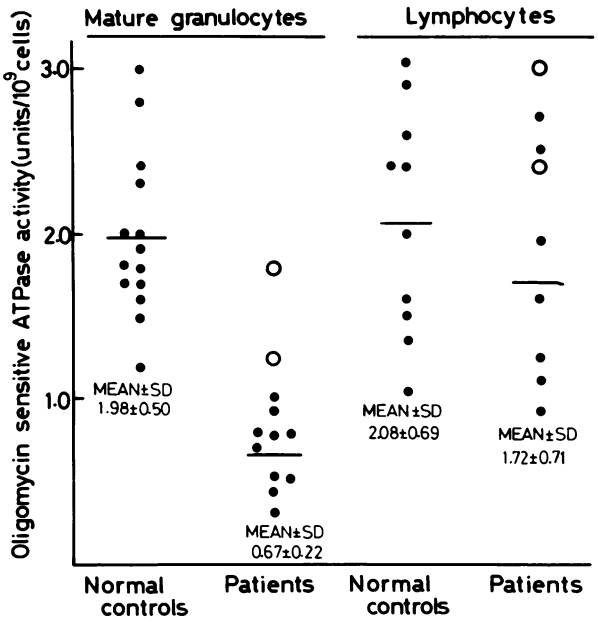

FIGURE 5 Oligomycin-sensitive ATPase activity in both mature granulocytes and lymphocytes obtained from patients with primary sideroblastic anemia. In the column of patients the symbol (●) shows the value of patients with primary acquired type, and O, that of patients with pyridoxine-responsive anemia before therapy. Horizontal bars and Mean±SD indicate the same as those in Fig. 4.

tivity was measured in both mature granulocytes and lymphocytes in the peripheral blood obtained from both patients with primary acquired type and those with pyridoxine-responsive anemia. Tubes containing the reaction mixture except both sodium malate and NAD hydrolyzed negligible amount of NADH (150 nmol) as measured by the changes in absorbance at 340 nm. Fig. 6 shows the citrate synthetase activity in both mature granulocytes and lymphocytes of the patients. Although some variations were observed, the citrate synthetase activity in both mature granulocytes and lymphocytes obtained from patients with primary acquired type was essentially within normal range. Also the activity in both mature granulocytes and lymphocytes obtained from two patients with pyridoxine-responsive anemia was within the normal range as shown in this figure.

Changes of the enzyme activities by the treatment. These enzyme activities in mitochondria were measured before and after the treatment with pharmacological doses of pyridoxal phosphate (100–200 mg/d, orally, 3–5 wk) in several patients with primary acquired sideroblastic anemia. No significant alterations of the activities were brought about by the treatment.

DISCUSSION

Primary sideroblastic anemia can be classified into three types; primary acquired, hereditary, and pyridoxine-responsive. Besides these primary sideroblastic anemias, secondary types such as those induced by drugs or alcohol, and those accompanying various diseases are recognized. Among primary sideroblastic anemias the most frequent type is the primary acquired type. Almost all the symptoms of this disorder can be ascribed to the impaired heme synthesis resulting from the decreased ALA synthetase activity in erythroblasts (1, 2). In fact, every patient with primary sideroblastic anemia, including that with primary acquired type, had decreased ALA synthetase activity in erythroblasts as shown in Fig. 1. The decrease of this enzyme activity in erythroblasts of the patients is considered not to be secondary to iron deposition in mitochondria but to be the primary defect in erythroblasts (1, 2).

Recently, we found a new serine protease in mitochondria in both erythroblasts and granulocytes (3). The protease in erythroblasts is considered to be en-

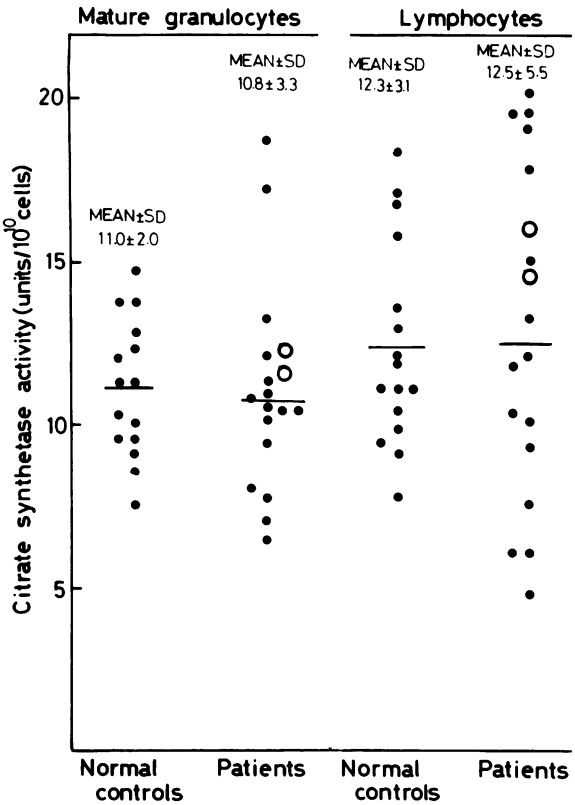

FIGURE 6 Citrate synthetase activity in both mature granulocytes and lymphocytes obtained from patients with primary sideroblastic anemia. In the column of patients, the symbol (●) shows the value of patients with primary acquired type, and O, that of patients with pyridoxine-responsive anemia before therapy. Horizontal bars and Mean±SD indicate the same as those in Fig. 4.

gaged in the regulation of ALA synthetase levels in mitochondria. However, protease activity is decreased in erythroblasts of patients with primary acquired sideroblastic anemia. Furthermore, the results that the susceptibility to the protease of apo-ALA synthetase prepared from erythroblasts of patients with primary acquired sideroblastic anemia was within normal range in contrast to that prepared from pyridoxine-responsive anemia suggest that the cause of the decrease of ALA synthetase activity in erythroblasts differs in these two types of sideroblastic anemia.

Besides the characteristic anemia that is the expression of the abnormality in erythropoietic series, patients with primary acquired sideroblastic anemia often develop a decreased number of granulocytes in the peripheral blood (10, 11). In our series the majority of the patients with primary acquired type showed decreased numbers of granulocytes in the peripheral blood. As shown in Fig. 3, with three exceptions the protease activity in mature granulocytes of patients with primary acquired sideroblastic anemia was decreased. Furthermore, determination of the activities of mitochondrial enzymes such as cytochrome oxidase and oligomycin-sensitive ATPase in mature granulocytes revealed that also the activities of these mitochondrial enzymes were decreased in mature granulocytes of the patients. On the other hand, the activity of citrate synthetase that is located in mitochondria was within normal range in mature granulocytes of the patients as shown in Fig. 6. Although the reason why certain enzymes in mitochondria are impaired and others are not is remained to be clarified, these results indicate that several components in mitochondria, but not all components are impaired in both erythroblasts and granulocytes of patients with primary acquired sideroblastic anemia. Since functions of granulocytes such as phagocytosis or production of superoxide are little influenced by the manipulation impairing mitochondrial metabolism (12), we speculate that defects of several mitochondrial enzymes in mature granulocytes do not cause symptoms in patients with primary acquired sideroblastic anemia. On the contrary, mitochondria in erythroblasts are engaged in the fundamental roles of the cell, i.e., heme synthesis. Therefore, the defects in mitochondria are more apparent in erythroblasts. That activities of mitochondrial enzymes such as cytochrome oxidase, oligomycin-sensitive ATPase, and citrate synthetase are within normal range in lymphocytes of patients with primary acquired sideroblastic anemia are compatible with the clinical data that patients with this disease scarcely develop immunological abnormalities. In contrast to mitochondrial enzymes, activities of several cytosomal enzymes in erythrocytes of patients with primary acquired sideroblastic anemia were reported to be within normal range (13). Impaired synthesis of DNA and RNA, and depression of protein synthesis in erythroblasts of patients with primary acquired sideroblastic anemia was reported by Wickramasinghe and Hughes (14). They showed that ring sideroblasts at an immature stage exhibited almost normal synthesis of both nucleic acids and proteins, while at a more mature stage the synthesis of both nucleic acids and protein was diminished. Therefore, the disturbances of both nucleic acid synthesis and protein synthesis in ring sideroblasts may stem from the mitochondrial lesions. We speculate that defects in mitochondria in both erythroblasts and granulocytes are the etiology of primary acquired sideroblastic anemia.

The results that not only ALA synthetase activity in erythroblasts but also the protease activity in both erythroblasts and granulocytes was decreased in patients with hereditary sideroblastic anemia suggest that mitochondrial defects in both erythroblasts and granulocytes may also be present in the hereditary type.

Patients with pyridoxine-responsive anemia also showed decreased ALA synthetase activity in erythro-

blasts and decreased cytochrome oxidase activity in mature granulocytes. Furthermore, like ALA synthetase activity in erythroblasts, cytochrome oxidase activity in mature granulocytes increased to the normal level after the treatment with pharmacological doses of pyridoxal phosphate as shown in Fig. 4. These results seems to indicate that heme synthesis is equally impaired in both erythroblasts and granulocytes of patients with pyridoxine-responsive anemia before therapy. However, they showed normal protease activity in both erythroblasts and mature granulocytes. Also activities of mitochondrial enzymes such as oligomycin-sensitive ATPase and citrate synthetase in both mature granulocytes and lymphocytes were within normal range as shown in Figs. 5 and 6. We conclude that only ALA synthetase in both erythroblasts and granulocytes is impaired in patients with pyridoxine-responsive anemia.

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