NADH-Cytochrome b_5 Reductase Activity in Lymphoid Cell Lines

EXPRESSION OF THE DEFECT IN EPSTEIN-BARR VIRUS TRANSFORMED LYMPHOBLASTOID CELL LINES FROM PATIENTS WITH RECESSIVE CONGENITAL METHEMOGLOBINEMIA

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ABSTRACT Recessive congenital methemoglobinemia (RCM) is due to the homozygous deficiency of NADH-cytochrome b_5 reductase (EC 1.6.2.2.). In type I disease, in which the patients are only methemoglobinemic, the enzyme defect is fully expressed in the erythrocytes, whereas the leukocytes are much less affected. In type II disease, in which the patients are, in addition, mentally retarded, the defect is generalized to all the tissues including cultured fibroblasts. In the present study we have investigated Epstein-Barr virus (EBV) transformed lymphoid cell lines (LCL) derived from patients with both types of cytochrome b_5 reductase deficiency and from nondeficient individuals. The total cytochrome b_5 reductase activity of the control LCL was found to be similar whatever the LCL origin, except for one lymphoma line (Daudi). The enzyme from the control LCL (C 252/B 95) was found to be immunologically related to the human soluble erythrocyte cytochrome b_5 reductase, indicating that it is the product of the same gene: the DIA_1 (diaphorase) locus. The LCL derived from one patient with the type I disease and two patients with the type II disease were investigated. In the former the defect was expressed to a lesser degree than in the cases with mental retardation in which the defect was much pronounced, and involved both the mitochondrial and the microsomal fractions. This indicates that all the subcellular forms of the cytochrome b_5 reductase are under the same genetic control. Altogether, these data show that the LCL are a favorable material for studying both types of cytochrome b_5 reductase deficiency and for in-
vestigating in depth the molecular aspects of this metabolic disease.

INTRODUCTION

Recessive congenital methemoglobinemia (RCM)[1] is due to the homozygous deficiency of erythrocyte NADH-diaphorase (1). This enzyme was found to be ubiquitously distributed (2) and to represent a soluble form of cytochrome b_5 reductase (3). The identity between the microsomal form of cytochrome b_5 reductase and the erythrocyte soluble form so-called "methemoglobin reductase," was assessed by immunological methods (4–6). Two clinical forms of RCM have been defined (7): (*a*) a benign form, called type I in which the erythrocytes are mainly affected, whereas the leukocytes are normal or much less affected; (*b*) a severe form with mental retardation, called type II, in which the enzyme defect is generalized to all the tissues: liver, muscle, brain, leukocytes, fibroblasts (7, 8). These two different forms are probably due to different mutations at the same locus DIA_1, which has been assigned to chromosome 22 (9, 10). To further understand the molecular pathology of cytochrome b_5 reductase deficiency, continuous lymphoblastoid cell lines (LCL) were established by Epstein-Barr virus (EBV) infection of peripheral lymphocytes from one patient with type I RCM, and two patients with type II RCM. The aim of this work was to study the molecular forms of cytochrome b_5 reductase in control

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[1] *Abbreviations used in this paper*: EBV, Epstein-Barr virus; LCL, lymphoid cell lines; RCM, recessive congenital methemoglobinemia.

lymphoblastoid cell lines, and to see if the mutations affecting the DIA_1 gene were expressed in EBV transformed LCL in both forms of disease.

METHODS

LCL and culture conditions

Three types of control LCL were investigated: (a) LCL established by in vitro EBV infection (11) of normal cord blood human lymphocytes isolated on Ficoll-Hypaque (Radiopaque Media, Winthrop Laboratories, New York). Six different lines, designated C 252/B 95, C 18/M 81, C 18/B 95, C 34/M 81, C 62/M 81, C 62/B 95 were studied. (b) Lymphoblastoid cell lines spontaneously obtained from patients with nasopharyngeal carcinoma. These cell lines, containing the EBV genome (Ly 11, Ly 26, Ly 28, Ly 64, and Ly 72) had been established by cultivation of nasopharyngeal carcinoma tumor as reported before (12). (c) Lymphoma cell lines from Burkitt's tumors: two of them containing the EBV genome (RAJI and DAUDI) the third one (BJAB) being free of EBV DNA.

Cytochrome b_5 reductase deficient lines were established by EBV infection of peripheral blood lymphocytes (11) from one subject with type I disease (without mental retardation): line REN, and from two subjects with type II disease (with mental retardation): lines BEN and BOU. Preliminary reports of the case study of these three patients have been published (7).

In all cases, the cells were cultivated in RPMI 1640 containing 20% heat-inactivated fetal calf serum, 100 IU/ml of penicillin and 100 μg/ml streptomycin. The cells were collected 3 d after medium change and were still at this moment in exponential growth phase. In one experiment, cells were collected 1, 3, or 7 days after medium change. The cells collected after 7 d had already reached saturation density 2 or 3 d before. The cell suspensions were washed twice in phosphate buffered saline, and stored at $-70°C$ as frozen pellets until enzyme analysis.

Leukocyte and fibroblast preparations of normal and cytochrome b_5 reductase deficient patients

Leukocytes were obtained by sedimentation of whole blood added to polyvinyl pyrrolidone (4:1 vol) for 1 h at 37°C; the contaminating erythrocytes were removed by osmotic shock. Skin fibroblasts were cultivated in Eagle's minimal essential medium containing 10% calf serum. The pellets of leukocytes and fibroblasts were stored at $-70°C$ immediately after centrifugation.

Preparation of detergent-treated cell extracts

Procedure 1: total extracts. The cell pellet (containing $\sim 10^8$ cells) was suspended in 0.3 ml of 1% (wt/vol) Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) in water, vigorously stirred in a Vortex apparatus, and frozen and thawed three times. The 12,000 g supernate was used for enzyme assays and characterization.

Procedure 2: cell fractionation. The cell pellet (10^8 cells) was homogenized with a Teflon Potter homogenizer in 3 vol of 0.25 M saccharose in a 1-mM Tris–HCl buffer (pH 7.0), 1 mM EDTA, 1 mM phenyl methyl sulfonyl fluoride and centrifuged at 12,000 g for 15 min to obtain a pellet containing

nuclear, mitochondrial, and lysosomal fractions. All experiments were carried out at 4°C. The supernate was transferred into microtubes (170 μl) and centrifuged in a Beckman Airfuge (Beckman Instruments, Inc, Fullerton, Calif.) at 105,000 g for 10 min at 4°C, to obtain a microsomal pellet. The 12,000 g and microsomal pellets were suspended, respectively, in 0.5 and 0.1 ml 1% Triton X-100 and frozen and thawed three times. The enzymatic activity of these extracts was determined as described below and the protein concentration assayed according to Lowry et al. (13).

Enzyme assays

NADH-cytochrome b_5 reductase is capable of reducing different types of electron acceptors: natural, seminatural, and artifical (xenobiotic) substrates. Therefore five types of assay were performed.

Assay 1. With soluble cytochrome b_5 as electron acceptor, assay 1 was prepared by trypsin digestion of rabbit liver microsomes according to Omura and Takesue (14). The technique used was that described by Leroux et al. (6). Due to pseudo first-order kinetics of the reaction, the velocity was defined by the apparent first-order rate constant k per minute. Results were expressed as k per milligram protein.

Assay 2. With the ferrocyanide-methemoglobin complex as electron acceptor, assay 2 was prepared as described by Hegesh et al. (15). The activity was expressed as micromoles or nanomoles of substrate reduced per minute and per milligram protein.

Assay 3. The NADH-diaphorase method of Scott and Mc Graw (16) using 2,6-dichlorophenol indophenol as final electron acceptor.

Assay 4. Another diaphorase assay in which the reduction of 2,6-dichlorophenol indophenol is coupled to that of 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT, Sigma Chemical Co.) (17).

Assay 5. This explored the NADH-ferricyanide reductase activity of the enzyme and was performed as described by Zamudio and Canessa (18).

Among these different methods that of Hegesh et al. (15), i.e. assay 2, was the simplest and the most accurate, and therefore used in most of the experiments.

Immunological methods

Chicken anti-human erythrocyte cytochrome b_5 reductase was prepared as described previously (2). The 12,000-g supernate of the C 252/B 95 control line prepared as described in procedure 1 was preincubated with increasing amounts of antibody. The incubation mixture was brought with saline up to a final volume of 80 μl and left for 30 min at 4°C. Then polyethylene glycol was added (5% final) and the mixture was incubated during 10 min. The residual activity of the 30,000-g supernates was assayed according to the method of Hegesh et al. (15). The antiserum was titrated with a human erythrocyte cytochrome b_5 reductase crude preparation freed from hemoglobin by DEAE cellulose treatment (2, 6). Control experiments were performed using serum from a nonimmunized chicken.

RESULTS

Cytochrome b_5 reductase activity in various human LCL

Importance of the final electron acceptors. In order to study the specificity of the enzymatic activities of

normal LCL, various substrates were tested such as: artifical electron acceptors (ferricyanide or 2,6-dichloro-phenol indophenol or MTT-2,6-dichlorophenol indo-phenol), a seminatural substrate (the ferrocyanide-methemoglobin complex of Hegesh) or the natural substrate itself (cytochrome b_5). A detergent-treated total cell extract of the control C 252/B 95 line was used. Table I shows the results obtained with these different substrates: a methemoglobin reductase or cytochrome b_5 reductase activity is clearly present in normal LCL. The apparent Michaelis constant (K_m) for the methemoglobin ferrocyanide complex obtained according to Hanes' plot is 20 μM.

Influence of phase growth on enzymatic activity. Reproducible enzyme activity was found for a same cell line (C 252/B 95) at different intervals. The relation between enzymatic activity and phase growth has been studied. Throughout the different experiments, the presence of the enzyme was monitored by the ferrocyanide-methemoglobin reductase assay (assay 2). When cells were harvested after 1, 3, or 7 d, the level of enzyme activity remained comparable (not shown).

Enzymatic activity in LCL of various origin. To determine whether the various LCL were relatively homogenous in their enzymatic expression, the ferro-cyanide-methemoglobin reductase activity (assay 2) was measured in different lymphoid lines, six of them established from normal human B lymphocytes in-fected by EBV, five others derived from patients with nasopharyngeal carcinoma, and three others derived from Burkitt's lymphoma. Except for Daudi, the values obtained with these lines did not differ significantly from those repeatedly found with the control C 252/B 95 line (Table II).

Subcellular distribution of the enzyme. It has been shown that, according to its amphipathic nature (19),

TABLE I
Results Obtained with Different Final Electron Acceptors

Acceptor	Activity
	Mean±SD
Cytochrome b_5	6.53±0.05 (5)
Ferrocyanide-methemoglobin complex	0.057±0.019 (13)
2,6-dichlorophenol indophenol	0.034±0.008 (5)
MTT-2,6-dichlorophenol indophenol	0.077±0.007 (5)
Ferricyanide	0.343±0.095 (5)

Activity was determined in the total extracts (Methods, pro-cedure 1) of control LCL (C 252/B 95), and expressed as micromoles substrate per minute per milligram protein with all the acceptors, with the exception of cytochrome b_5, for which the activity is expressed as first order rate constant (k) per milligram protein. MTT, 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide.
The number of experiments is indicated between parentheses.

TABLE II
Ferrocyanide-Methemoglobin Reductase Activity of Different Lymphoid Cell Lines

Lymphoid cell lines	Activity
	μmol/min/mg protein
Established from cord lymphocytes infected by EBV	
• C 252/B 95	0.057±0.019 (13)
• C 18/M 81	0.037
• C 18/B 95	0.039
• C 62/M 81	0.038
• C 62/B 95	0.045
• C 34/M 81	0.034
Derived from patients with nasopharyngeal carcinoma	
• Ly 11	0.041
26	0.033
28	0.042
64	0.048
72	0.046
Lymphoma lines	
• DAUDI	0.021
• RAJI	0.060
• BJAB	0.053

Enzymatic activity was determined according to the method of Hegesh (15) in total extracts (Methods, procedure 1).
The number between parentheses refers to the number of experiments.

cytochrome b_5 reductase is bound to membranes such as microsomes (20), mitochondria (21), erythro-cyte ghosts (22). It was also found as a free soluble form in the erythrocyte (3), in liver and placenta cytosol (6, 23). We estimated the distribution of the enzyme in the LCL. The ferrocyanide-methemoglobin reductase activity (assay 2) was determined in fractions obtained after homogenization of the control cells (C 252/B 95) and centrifugation, as described in methods: 71% of the total enzymatic activity was found in the 12,000-g pellet, 16% in the 105,000-g microsomal pellet, and 7% in the cytosolic super-nate (Table III).

Immunological studies. The immunological prop-erties of the cytochrome b_5 reductase of lymphoid cells were compared to those of the erythrocyte enzyme. Fig. 1 shows the immunoinactivation curve obtained after incubation of a detergent-treated lym-phoblast extract with different concentrations of an antiserum directed against human erythrocyte cyto-chrome b_5 reductase. The curve indicates that the cytochrome b_5 reductase of the LCL is inhibited by the antibody and therefore is immunologically related to the erythrocyte enzyme. However, the immunoinacti-vation curve slope of the lymphoblast cell extract is not identical to that of the erythrocyte enzyme. This

TABLE III

Subcellular Distribution of Ferrocyanide-Methemoglobin
Reductase of Normal LCL

Subcellular fractions	Total activity	
	μmol/min	%
Homogenate	0.191	100
12,000 g pellet	0.135	71
105,000 g microsomal pellet	0.030	16
105,000 g cytosolic supernate	0.013	7

The ferrocyanide-methemoglobin reductase activity was
measured according to the method of Hegesh (15) in the
12,000-g pellet, the 105,000-g pellet, and supernate of
homogenized LCL (C 252/B 95).
The subcellular fractionation and extraction was performed
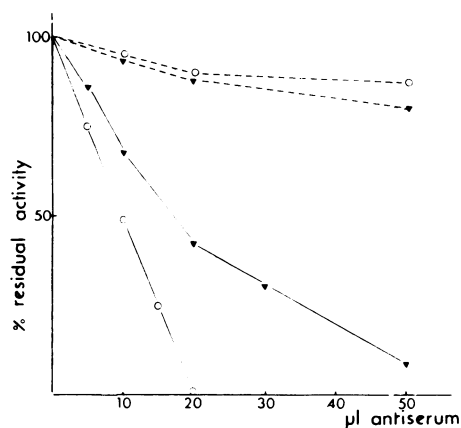as described in Methods (procedure 2).

FIGURE 1 Immunoinactivation of LCL enzyme by antiserum
prepared against human erythrocyte cytochrome b_5 reduc-
tase. The results are expressed as percentage of the activity
measured in control experiments according to the method of
Hegesh (15). Chicken antiserum directed against human
erythrocyte cytochrome b_5 reductase was preincubated with:
O —— O, partially purified erythrocyte enzyme, ▼ —— ▼,
12,000-g supernate from normal lymphoblastoid cells (C 252/
B 95) extracted with 1% Triton X-100. Normal chicken serum
was incubated with identical preparations (dotted lines).
Other conditions are described in Methods.

may be due to a different accessibility for the anti-
body to the membrane bound enzyme of the lymphoid
cells as compared to the soluble erythrocyte enzyme
or to the difference in specific activity and in enzyme
concentrations of the two preparations. Control serum
of nonimmunized chicken had nearly no inhibitory
effect. These results indicates the immunological
relationship of the reductase from the LCL with the
erythrocyte enzyme.

Cytochrome b_5 reductase activity in LCL derived from subjects with congenital methemoglobinemia

*Enzyme activities in total extract of LCL from
patients with type I and type II RCM.* The methemo-
globin reductase and cytochrome b_5 reductase activities
were determined in the lymphoblastoid cells derived
from one subject with type I RCM (case REN) and from
two subjects with type II RCM (cases BOU and BEN)
and compared to the control line C 252/B 95 (Table
IV). In the LCL from type I RCM, the methemoglobin
reductase and cytochrome b_5 reductase activities rep-
resent, respectively, 49 and 53% of those from the
controls. In the LCL from type II RCM, the enzymatic
values were 5–9% of those from the control line.
Therefore, a cytochrome b_5 reductase deficiency is
clearly demonstrable in the LCL derived from patients
with type I RCM. This deficiency is considerably more
pronounced in the cells from the two patients with
type II RCM. It is noteworthy that the values of methe-
moglobin reductase and of cytochrome b_5 reductase
are in good agreement.

*Comparison of the enzymatic activities of the LCL
with those of leukocytes and fibroblasts from patients
with type I and type II RCM.* Table IV compares the
total activity obtained in LCL, leukocytes, and fibro-
blasts from patients with type I and type II RCM. In the

LCL from a patient (REN) with type I RCM, the level
of methemoglobin reductase (28 nmol/min per mg pro-
tein) was found lower than that of the control value
of LCL (57 ± 19 nmol/min per mg protein). However, in
the leukocytes of this patient, the methemoglobin re-
ductase level was subnormal (54 nmol/min per mg pro-
tein) as compared to the control value of methemoglo-
bin reductase of leukocytes (70 ± 20 nmol/min per mg
protein). Therefore, the enzymatic defect of type I RCM
was more expressed in the lymphoid cells as compared
to the other cells. In type II RCM (BOU and BEN),
the enzyme deficiency was major in the three types
of cells, regardless of the substrate used. However,
the defect was less pronounced in the LCL than in
leukocytes and fibroblasts and some cytochrome b_5
reductase activity was detectable in the LCL from
the type II RCM (Table IV).

*Subcellular distribution of the residual enzyme ac-
tivity.* As recently reported (21), the molecular iden-
tity of microsomal and mitochondrial cytochrome b_5
reductase of rat liver has been established on enzymatic
and immunochemical grounds. The study of the sub-
cellular distribution of the residual enzyme activity of
LCL from type I and type II RCM may provide a
genetic verification of this hypothesis. The methemo-
globin reductase activity was determined in the dif-
ferent fractions: 12,000-g pellet, 105,000-g pellet and
supernate of the homogenized LCL. In the LCL from
type I RCM (REN) and from type II RCM (BEN and
BOU), the enzymatic defect is clearly expressed (Table

TABLE IV
Enzymatic Activities in Lymphoblastoid Cells Compared with Those of Leukocytes and Fibroblasts
from Patients with Type I and Type II RCM and from Control Subjects

		Specific activity					
		Methemoglobin reductase nmol/min/mg			Cytochrome b_5 reductase k/mg*		
		LCL	Leukocytes	Fibroblasts	LCL	Leukocytes	Fibroblasts
Control		57.4	70.1	70	6.5	3.2	4.0
REN	Type I RCM	28.2	54.3	ND	3.4	ND	ND
BOU	Type II RCM	3.5	1.0	1.5	0.3	0	0
BEN	Type II RCM	5.1	2.5	3.4	0.5	0	0

The ferrocyanide-methemoglobin reductase (15) and cytochrome b_5 reductase (6) activities were measured in the total extracts prepared as described under material and methods (procedure 1).
Control LCL: C252/B95.
* Activity expressed as first order rate (k) per milligram protein.

V) in the three subcellular fractions investigated (12,000-g pellet, 105,000-g pellet, and supernate), as compared to the values obtained with the control LCL (C 252/B 95). The lower enzymatic activity found in the total extracts of the LCL of the type II RCM (Table IV) seems to be essentially due to a decrease of activity in the 12,000-g pellet fraction of these cells (Table V).

DISCUSSION

NADH-cytochrome b_5 reductase (EC 1.6.2.2.) is an amphipathic protein that is tightly bound to different membrane systems such as endoplasmic reticulum, mitochondria, and erythrocyte ghosts (19–22). It is monomeric and is encoded by a single locus-DIA_1-assigned to chromosome 22 (9, 10). In the erythrocyte it has been shown that the enzyme that acts as

TABLE V
Subcellular Distribution of the Residual Methemoglobin Reductase Activity of Lymphoblastoid Cell Lines Derived from Subjects with Type I and Type II RCM

Cell lines	Origin	Specific activity nmol/min/mg protein		
		12,000 g pellet	105,000 g pellet	105,000 g supernate
C 252/B 95	Control	82.0	46.7	3.7
REN	Type I RCM	26.5	7.2	1.7
BEN	Type II RCM	6.4	9.3	1.6
BOU	Type II RCM	2.8	10.5	2.6

The enzyme activity was assayed by the method of Hegesh et al. (15) in fractions obtained as described in Methods (procedure 2).
Full extraction of the 12,000 g and 105,000 g pellets was ensured by Triton X-100 (1%).

a methemoglobin reductase, the so-called NADH-diaphorase (16), is actually a soluble form of NADH-cytochrome b_5 reductase (3–6). This form most probably derives from the membrane-bound enzyme by partial proteolysis and removal of the C-terminal hydrophobic segment (3, 19, 22–24).

RCM is due to a defect of this enzyme. In our laboratory 23 cases of RCM have been observed in the past 10 years. 14 individuals exhibited a benign clinical pattern, in which the only symptom was cyanosis due to methemoglobinemia. The level of methemoglobin was easily controlled by methylene blue, ascorbic acid, or riboflavin (25, 26). In this well-tolerated disease we found that the enzyme defect was expressed in the erythrocytes, whereas the leukocytes of these patients exhibited either normal or subnormal levels. We called it type I RCM (7). In contrast, nine patients exhibited a completely different picture in which methemoglobinemia was associated with severe mental retardation and early neurologic abnormalities with bilateral athetosis (7). Control of methemoglobinemia was without effect on the clinical course. In this lethal disease, the enzyme defect was fully expressed not only in the erythrocytes but also in leukocytes, fibroblasts, liver, muscle, and brain (7, 8, 27). This indicates that the severe form of RCM is a systemic disease due to generalized deficiency of cytochrome b_5 reductase. We called this form type II RCM (7). On these grounds, prenatal diagnosis was successfully performed by analyzing the cytochrome b_5 reductase of cultured amniotic cells from two fetuses at risk for the type II deficiency (28). Different mutations at the DIA_1 locus might account for the two forms. However, at the present time, there is no explanation at the molecular level for this phenotypic heterogeneity. One of the reasons for our lack of knowledge in this respect is the scarcity of the material available from patients with RCM. In the erythrocytes, the usual source of enzyme, the residual activity

is very low and extremely unstable, especially in the type II form (27 and unpublished observations). Fibroblasts were cultured from skin biopsy specimens obtained in some of our patients with RCM (7, 8). However, this material was still not enough to permit a comparative study of the type I and the type II defects. We therefore decided to establish stable LCL from these patients by EBV transformation of their peripheral lymphocytes. The advantages of such lines are well known: rapid and easy growth in suspension yielding considerable amount of cellular material, indefinite life in vitro of cells retaining their original phenotype and genotype over long periods in culture (29). These cells have been used successfully for genetic studies of human defects (29, 30).

To ascertain the usefulness of this material for studying both types of RCM, it was necessary to estimate and characterize the cytochrome b_5 reductase of LCL derived from nondeficient subjects. In six different lines established by in vitro EBV infection, in five spontaneous lines derived from nasopharyngeal carcinoma and in three spontaneous lines derived from Burkitt's tumors, the enzyme activity was found to be similar. We also checked that the enzyme activity did not vary and was independent of the growth characteristics of the cells at the time of harvesting (1, 3, or 7 d of culture). On the other hand, neither the presence of the EBV genome nor the malignant origin of some cells did affect the cytochrome b_5 reductase expression of the LCL (Table II). However, one exception was the Daudi cell line in which only half of the "normal" cytochrome b_5 reductase activity was found. This is reminiscent of the partial defect of N-acetyl hexosaminidase observed in the same cell line (31). In our study we also established, as a preliminary, that the cytochrome b_5 reductase of the LCL is immunologically related to the erythrocyte enzyme. Its subcellular distribution, investigated in one of the control LCL (C 252/B 95), showed that the enzyme is present in the mitochondrial, the microsomal, and the cytosolic fractions. This is consistent with the recently reported existence of cytochrome b_5 reductase in the mitochondrial (21) and cytosolic (23) fractions of liver. It should be noted that ~90% of the enzymatic activity of the LCL is bound to the subcellular organelles. This binding requires the use of a detergent to unmask the hidden amphipathic enzyme (19, 22).

Altogether these results indicate that LCL established from deficient patients should be suitable for studying the two types of RCM. Using natural (cytochrome b_5) and seminatural (ferrocyanide-methemoglobin complex) substrates, we demonstrated the existence of a partial defect of cytochrome b_5 reductase in the LCL from a patient with type I RCM (case REN), and a more pronounced defect in the cells derived from two patients with type II RCM (cases BOU and BEN). In both types of cells the enzyme deficiency predominated in the mitochondrial and microsomal fractions, the deficiency being more important in the type II cells. These results confirm that all the subcellular forms of cytochrome b_5 reductase are under the control of a single gene: the DIA$_1$ locus.

The enzymatic defect observed in LCL correlates with the activities measured in a mixed population of circulating leukocytes from patients with both types of RCM (Table IV). However, in type I RCM, the enzyme activity was significantly lower in LCL than in leukocytes. We have no explanation for this phenomenon, which could be accounted for by either differential stability of the DIA$_1$ product, or by differential expression of the diaphorase isozymes (32). In the LCL from type II RCM, the enzyme defect was much more pronounced than in type I cells (Table IV), as already found with other nucleated cells (7, 8, 27, 28). Of special interest is the fact that the LCL from type II RCM, in contrast to leukocytes and fibroblasts, expressed some residual activity that can be useful to further characterize the mutated reductase. The lines derived from patients with type I and type II RCM should prove useful as a model for investigating, at cellular and molecular levels, the biological consequences of defects involving the cytochrome b_5 reductase gene.

ACKNOWLEDGMENTS

REFERENCES

1. Scott, E. M., and I. V. Griffith. 1959. The enzymatic defect in hereditary methemoglobinemia:diaphorase. *Biochim. Biophys. Acta.* **34:** 584–586.
2. Leroux, A., and J. C. Kaplan. 1972. Presence of red cell type NADH-methemoglobin reductase (NADH-diaphorase) in human non erythroid cells. *Biochem. Biophys. Res. Commun.* **49:** 945–950.
3. Hultquist, D. E., and P. G. Passon. 1971. Catalysis of methaemoglobin reduction by erythrocyte cytochrome b_5 and cytochrome b_5 reductase *Nature (Lond.)* **229:** 252–254.
4. Kuma, F., R. A. Prough, and B. S. S. Masters. 1976. Studies on methemoglobin reductase. Immunochemical similarity of soluble methemoglobin reductase and cytochrome b_5 of human erythrocytes with NADH-cytochrome b_5 reductase and cytochrome b_5 of rat liver microsomes. *Arch. Biochem. Biophys.* **172:** 600–607.
5. Goto-Tamura, R., Y. Takesue, and S. Takesue. 1976. Immunological similarity between NADH-cytochrome b_5 reductase of erythrocytes and liver microsomes. *Biochim. Biophys. Acta.* **423:** 293–302.

6. Leroux, A., L. Torlinski, and J. C. Kaplan. 1977. Soluble and microsomal form of NADH-cytochrome b_5 reductase from human placenta. Similarity with NADH-methemoglobin reductase from human erythrocytes. *Biochim. Biophys. Acta.* **481:** 50–62.

7. Kaplan, J. C., A. Leroux, and P. Beauvais. 1979. Formes cliniques et biologiques du déficit en cytochrome b_5 réductase. *C. R. Soc. Biol.* **173:** 368–379.

8. Leroux, A., C. Junien, J. C. Kaplan, and J. Bamberger. 1975. Generalized deficiency of cytochrome b_5 reductase in congenital methaemoglobinaemia with mental retardation. *Nature (Lond.).* **258:** 619–620.

9. Fisher, R. A., S. Povey, M. Bobrow, E. Solomon, Y. Boyd, and B. Carritt. 1977. Assignment of the DIA_1 locus to chromosome 22. *Ann. Hum. Genet.* **41:** 151–155.

10. Junien, C., M. Vibert, D. Weil, Nguyen Van Cong, and J. C. Kaplan. 1978. Assignment of NADH-cytochrome b_5 reductase (Dia_1 locus) to human chromosome 22. *Hum. Genet.* **42:** 233–239.

11. Yata, J., C. Desgranges, T. Nakagawa, M. C. Favre, and G. De Thé. 1975. Lymphoblastoid transformation and kinetics of appearance of viral nuclear antigen (EBNA) in cord-blood lymphocytes infected by Epstein-Barr virus (EBV). *Int. J. Cancer.* **15:** 377–384.

12. De Thé, G., H. C. Ho, H. C. Kwan, C. Desgranges, and M. C. Favre. 1970. Nasopharyngeal carcinoma (NPC). I-Types of cultures derived from tumour biopsies and non-tumorous tissues of Chinese patients with special reference to lymphoblastoid transformation. *Int. J. Cancer.* **6:** 189–206.

13. Lowry, O. H., N. Y. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.

14. Omura, T., and S. Takesue. 1970. A new method for simultaneous purification of cytochrome b_5 and NADPH-cytochrome c reductase from rat liver microsomes. *J. Biochem. (Tokyo).* **67:** 249–257.

15. Hegesh, E., N. Calmanovici, and M. Avron. 1968. New method for determining ferrihemoglobin reductase (NADH-methemoglobin reductase) in erythrocytes. *J. Lab. Clin. Med.* **72:** 339–344.

16. Scott, E. M., and J. C. McGraw. 1962. Purification and properties of diphosphopyridine nucleotide diaphorase of human erythrocytes. *J. Biol. Chem.* **237:** 249–252.

17. Choury, D., and J. C. Kaplan. 1980. Diaphorase P: A new foetal isozyme identified in human placenta. *Biochim. Biophys. Acta.* **613:** 18–25.

18. Zamudio, I., and M. Canessa. 1966. NADH-dehydrogenase activity of human erythrocyte membranes. *Biochim. Biophys. Acta.* **120:** 165–169.

19. Spatz, L., and P. Strittmatter. 1973. A form of reduced nicotinamide adenine dinucleotide cytochrome b_5 reductase containing both the catalytic site and an additional hydrophobic membrane-binding segment. *J. Biol. Chem.* **248:** 793–799.

20. Strittmatter, P., and S. F. Velick. 1957. The purification and properties of microsomal cytochrome b_5 reductase. *J. Biol. Chem.* **228:** 785–799.

21. Kuwahara, S., Y. Okada, and T. Omura. 1978. Evidence for molecular identity of microsomal and mitochondrial NADH-cytochrome b_5 reductases of rat liver. *J. Biochem. (Tokyo).* **83:** 1049–1059.

22. Choury, D., A. Leroux, and J. C. Kaplan. 1981. Membrane-bound cytochrome b_5 reductase (methemoglobin reductase) in human erythrocytes. Study in normal and methemoglobinemic subjects. *J. Clin. Invest.* **67:** 149–155.

23. Lostanlen, D., A. Vieira De Barros, A. Leroux, and J. C. Kaplan. 1978. Soluble NADH-cytochrome b_5 reductase from rabbit liver cytosol: partial purification and characterization. *Biochim. Biophys. Acta.* **526:** 42–51.

24. Mihara, K., R. Sato, R. Sakakibara, and H. Wada. 1978. Reduced nicotinamide adenine dinucleotide-cytochrome b_5 reductase: location of the hydrophobic, membrane-binding region at the carboxyl-terminal end and the masked amino terminus. *Biochemistry.* **17:** 2829–2834.

25. Kaplan, J. C., and M. Chirouze. 1978. Therapy of recessive congenital methaemoglobinaemia by oral riboflavine. *Lancet.* **II:** 1043–1044.

26. Schwartz, J. M., and E. R. Jaffé. 1978. Hereditary methemoglobinemia with deficiency of NADH-dehydrogenase. *In* The Metabolic Basis of Inherited Disease. J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson, editors. McGraw Hill Inc., New York. 1452–1464.

27. Kaplan, J. C., A. Leroux, S. Bakouri, J. P. Grangaud, and M. Benabadji. 1974. Le lésion enzymatique dans la méthémoglobinémie congénitale récessive avec encéphalopathie. *N. Rev. Fr. Hématol.* **14:** 755–770.

28. Junien, C., A. Leroux, D. Lostanlen, A. Reghis, J. Boué, H. Nicolas, A. Boué, and J. C. Kaplan. 1981. Antenatal diagnosis of congenital enzymopenic methaemoglobinaemia with mental retardation due to generalized cytochrome b_5 reductase deficiency. *Prenatal Diagnosis.* **1:** 17–24.

29. Povey, S., S. E. Gardiner, B., S. Mowbray, H. Harris, E. Arthur, C. M. Steel, C. Blenkinsop, and H. J. Evans. 1973. Genetic studies on human lymphocytoid lines: isozyme analysis on cell lines from 41 different individuals and on mutants produced following exposure to a chemical mutagen. *Ann. Hum. Genet.* **36:** 247–266.

30. Glade, P. R., and N. G. Beratis. 1975. Long term lymphoid cell lines in the study of human genetics. *Prog. Med. Genet.* **1:** 1–48.

31. Swallow, D. M., S. E. Gardiner, H. Harris, E. Arthur, C. M. Steel, and H. J. Evans. 1977. Lysosomal enzymes in human lymphoblastoid lines: unusual characteristics of Raji and Daudi. *Ann. Hum. Genet.* **41:** 9–16.

32. Fisher, R. A., Y. H. Edwards, W. Pritt, J. Potter, and D. A. Hopkinson. 1977. An interpretation of human diaphorase isozymes in terms of three gene loci DIA 1, DIA 2 and DIA 3. *Ann. Hum. Genet.* **41:** 139–149.