PRESENCE OF RED CELL TYPE NADH-METHEMOGLOBIN REDUCTASE (NADH-DIAPHORASE) IN HUMAN NON ERYTHROID CELLS

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SUMMARY - On the basis of immunologic and electrophoretic studies evidence is given that the NADH diaphorase which promotes methemoglobin reduction in the red cell is also present in cells which do not contain hemoglobin : leukocytes, platelets, brain, placenta, muscle and liver. This enzyme is not found in human cultured fibroblasts.

INTRODUCTION - In the red blood cell (RBC) hemoglobin is maintained in a reduced state mainly by the action of methemoglobin reductase (MetHb-R), an enzyme which uses NADH as a source of electrons (1). In vitro this enzyme is able to reduce several artificial dyes such as 2.4.dichlorophenol indophenol (DCIP) (2) and therefore belongs to the ill-defined group of diaphorases. Whereas methemoglobin is in the test tube a poor substrate for the enzyme as compared to DCIP (2), this NADH-diaphorase plays a physiological role of methemoglobin-reductase since its deficiency induces congenital methemoglobinemia (3). By use of a specific staining method (4) this enzyme has been electrophoretically characterized in the RBC from normal subjects and methemoglobinemic patients (1, 4, 5). Extension of this method to leukocytes and platelets has shown an electrophoretic similarity between MetHb-R in RBC and the other blood cells (6, 7). Moreover in one case of congenital methemoglobinemia associated to severe mental retardation MetHb-R has been found to be markedly reduced not only in RBC but also in white blood cells (WBC) (8)*. Therefore the possible existence

* This work is part of a Doctoral Thesis to be submitted.

* Since completion of the present work we made the same observation in a second case of methemoglobinemia with mental retardation.

of the enzyme in different human tissues which do not contain hemoglobin was considered.

In the present study we bring immunologic and electrophoretic evidence for the presence in human leukocytes, platelets, brain, placenta, muscle and liver of the same MetHb-R as found in the RBC. However this enzyme was not found in human cultured fibroblasts.

EXPERIMENTAL - MetHb-R was partially purified from human red cells using a modification of the technique of West (9). A 700-fold purified enzyme (specific activity = 2 500 units**/mg protein) was obtained and subsequently used as antigenic material. Antiserum against MetHb-R was elicited in chicken by weekly injections, alternately intra-muscular and intra-venous, of this material (5-7 mg protein per injection). The inhibitory capacity of the antibody against MetHb-R from different tissues was measured after incubation for 30 min. at 37°C and 15 hours at 4°C of a given amount of enzyme with increasing amounts of chicken antiserum. After centrifugation at 12 000 x g for 15 min., the residual activity was both estimated and submitted to electrophoresis.

Starch gel electrophoresis was performed as previously described (4). Preliminary experiments had shown to us the electrophoretic identity of NADH-diaphorase and MetHb-R in the red cells (11).

RESULTS AND DISCUSSION - As shown on Figure 1, the inactivation curve obtained with the red cell enzyme was identical to that found with crude homogenates of WBC, platelets, liver, muscle, brain, placenta. In the four last tissues an additional slope was also observed indicating the presence of at least another reacting antigen with lesser affinity. In contrast the slope obtained with two different cultured lines of human embryo fibroblasts was completely different from that given by the red cell enzyme.

These results were further confirmed by electrophoretic studies. Two electrophoretically distinct bands (A and B,

** One unit is the amount of enzyme which reduces 1 mμmole of methemoglobin-ferrocyanide complex per minute (10).

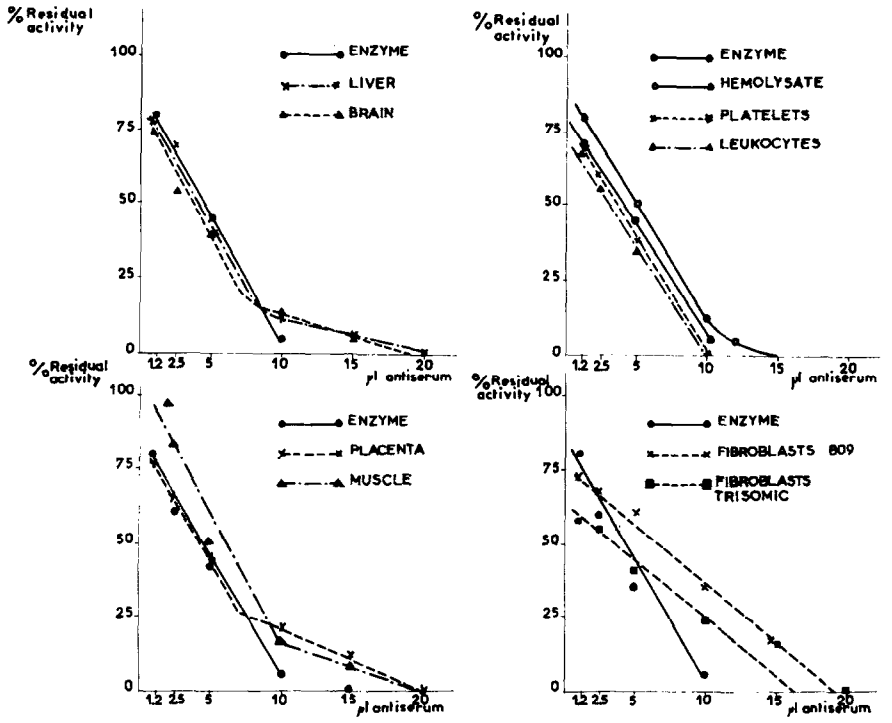

Fig. 1 - Inactivation curves of MetHb-R from various human tissues by chicken antiserum prepared against human red cell methemoglobin-reductase.
The 30 000 x g supernatant of homogenates prepared in 1 volume of cold 0.06 M Tris buffer pH 7.6 containing 1 mM EDTA was incubated with antiserum (see text) and subsequently assayed for residual MetHb-R activity according to Hegesh (10). The results are expressed as percentage of the activity measured in control experiments where antiserum was replaced by normal chicken serum. "Enzyme" refers to the standard curve obtained with the antigenic material used to produce the antiserum.

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fig. 2) of NADH-diaphorase were found in brain, leukocytes, platelets and aged RBC extracts. Both bands were suppressed by incubation of the extract with antiserum prior to electrophoresis (fig. 2 and 3). This clearly indicates a common antigenic reactivity of band A and band B in brain and blood cells extracts. It also confirms our previous assumption that the anodic band seen in fresh extracts of leukocytes might be identical to that seen in aged erythrocyte extracts (6, 7). In placenta, where an additional slow-moving band is observed (band D, fig. 2), only bands A and B were sensitive to anti-

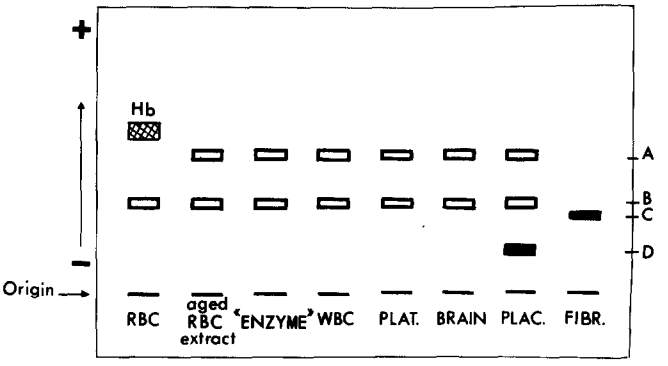

Fig. 2 - Starch gel electrophoresis of methemoglobin reductase in various human tissues. The enzyme was stained as NADH diaphorase (4).

Bands which are completely inhibited by incubation of extract with antiserum prior to electrophoresis.

Bands not inhibited by antiserum.

In all cases, control experiments showed that a serum from a normal chicken had no effect on the enzyme mobility. "Enzyme" refers to the semi-purified red cell MetHb-R used as antigen. Differences in cellular environment probably account for the discrepancy between red cells, where the A-band not seen in fresh hemolysates only appears upon aging or purification (4, 6), and the other cells where the A-band is already present in fresh homogenates.

serum inactivation. In fibroblasts extracts a single band with a different mobility (band C, fig. 2) was seen. It was not inhibited by the antiserum, thus confirming the different immunoreactivity of the fibroblast enzyme.

It is noteworthy that the red cell NADPH-dependent diaphorase, which is easily distinguishable from the NADH-diaphorase by electrophoresis (4), was totally insensitive to the antiserum.

An identical affinity of the anti-human red cell MetHb-R antiserum for the enzyme present in RBC, leukocytes, platelets, liver, muscle, brain and placenta is indicated by the identical slope of the inactivation curve. The electrophoretic data confirm this finding. Our results suggest that the enzyme asso-

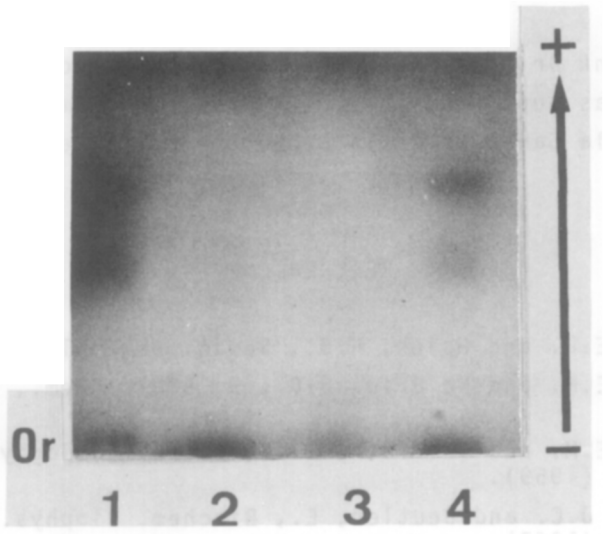

Fig. 3 - Photograph of starch gel electrophoresis of methemoglobin-reductase from WBC homogenate (30 000 g supernatant) preincubated with :

1 = 1 vol. normal chicken serum ; 2 = 1 vol. chicken antiserum ; 3 = 2 vol. chicken antiserum ; 4 = 1 vol. saline.

ciated with methemoglobin-reductase activity might be identical in the different tissues investigated, or at least might share a common antigenic determinant.

The fact that the enzyme which acts as a methemoglobin reductase in the RBC is also present in leukocytes, platelets, placenta, brain and presumably muscle and liver, raises the question of its physiological significance in cells which do not contain hemoglobin. It is likely that other heme proteins might serve as electron acceptors. In agreement with this hypothesis is the possible identity of cytochrome b5 reductase and MetHb-R in the RBC which has been recently postulated (12).

The present findings strongly support our starting hypothesis that the severe form of congenital methemoglobinemia with mental retardation is actually due to a major defect of the so-called methemoglobin-reductase in many tissues and particularly brain (8, 13).

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