Localization of a Lactic Dehydrogenase Isozyme in Nuclei of Young Cells in the Erythrocyte Series.* (27715)

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Multiple enzymatic forms exhibiting similar substrate specificities have been termed isozymes by Markert and Møller(1). The demonstration of the heterogeneity of lactic dehydrogenase (LDH) activity in human tissues(2) has been employed diagnostically(2-6), and recent studies suggest that isozymes may be useful in embryology(1,7), tissue culture(7), and genetics(8). Human tissues vary in distribution of total LDH activity among the 5 LDH isozymes observed in most organs(4-6). In a given tissue there are marked differences among species in number of LDH isozymes present, in distribution of total LDH activity among these isozymes, and in their electrophoretic mobilities(1,9). The mature erythrocyte is the only human cell which exhibits only 4 LDH isozymes; it lacks isozyme 5, the cathodally migrating form (10). Evidence will be presented to suggest that isozyme 5 is present in early forms of both human and guinea pig erythrocytes but is absent in the mature erythrocyte of both species. Since mature erythrocytes of both species lack a nucleus, whereas earlier forms of the erythrocyte series are nucleated, isozyme 5 appears to be associated with a cell nucleus.

Materials and methods. Four pieces of evidence relate to the hypothesis. The first is the isozyme pattern in hemolysates from 4 individuals with reticulocytosis secondary to anemia compared to that obtained from 500 individuals without reticulocytosis. Reticulocyte counts in the 4 individuals with reticulocytosis were 5%, 6%, 8%, 26%; 3 had sickle cell disease and the 4th with 6% reticulocytes in the peripheral blood had chronic lymphocytic leukemia. All had normal white blood cell counts. A fifth individual with anemia whose marrow did not produce early erythrocyte forms was also studied. In this individual the reticulocyte count was less than 1%. Hemolysates prepared in distilled water and toluene after removal of the plasma and buffy coat and after washing the packed red cells 3 times in normal saline(9) were separated by vertical starch gel electrophoresis in phosphate-citric acid buffer pH 7.0, 0.01 M (4). The hemoglobin being near its isoelectric point remained close to the origin and did not interfere with the histochemical demonstration of the LDH isozymes which migrated elsewhere (Fig. 1). A potential gradient of 6 V per cm was employed for 12 hours at 21°C. The gel was then sliced into 2 half sections and the bottom half was stained by incubation of the gel for 1 hour at 37°C in the dark with lactate as substrate, DPN as coenzyme, phenazine methosulfate as electron transporter and nitro blue tetrazolium as dye. The quantities of these reagents and appropriate controls to ensure the specificity of the method have been described(4,9).

In the second experiment guinea pigs were selected because the LDH isozyme pattern of their hemolysates most closely resembled that of man(9). Guinea pig hemolysates were examined before and after administration of phenylhydrazine. 0.5 ml of a 1% solution of phenylhydrazine was injected subcutaneously on each of 4 days and one week after the first injection the blood was studied. Before phenylhydrazine administration the guinea pig hematocrits were 45%, 43%, 45%; and the reticulocyte counts were 1.2%, 1.3%, 1.2%; after phenylhydrazine administration the hematocrits were 14%, 22%, 24% and the reticulocyte counts were 9.0%, 8.1%, 4.7%.

The third experiment involved hemolysates prepared from the cord blood of an hydropic newborn. The reticulocyte count of this blood was 12%. One portion of the packed washed red cells was treated with a hypotonic lysing solution composed of 1 g sodium citrate dihydrate, 7 ml saturated solution NaCl made up to 1 liter and adjusted to pH 5 by addition of 1N HCl to lyse mature erythrocytes but

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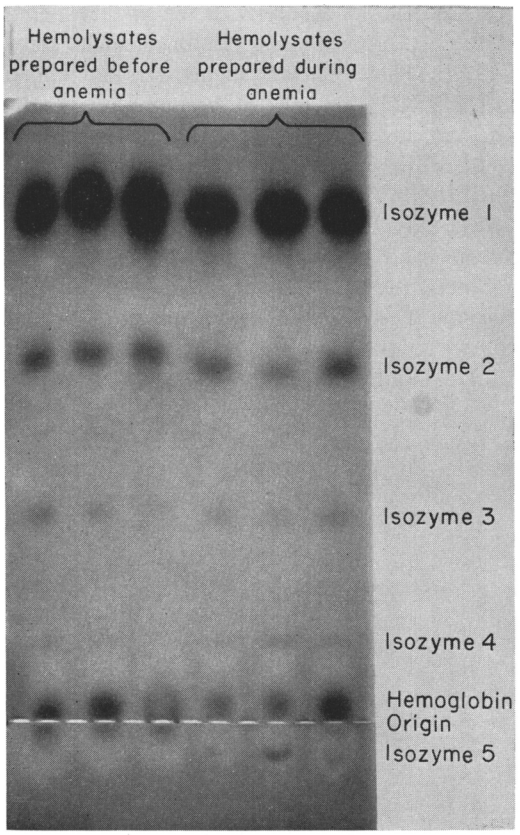

FIG. 1. Photograph of a starch gel stained for LDH activity. The 3 hemolysates on left were prepared from guinea pig erythrocytes prior to administration of phenylhydrazine; 3 on right prepared after phenylhydrazine administration and during period of anemia and reticulocytosis. Each hemolysate was adjusted to the same LDH activity of 60,000 units/ml prior to application to gel. Note presence of isozyme 5 in the 3 hemolysates from the anemic guinea pigs.

to spare the more resistant nucleated erythrocytes and reticulocytes. After centrifugation the sediment was enriched with these young cells. The other portion of the erythrocytes was treated as usual to produce hemolysate.

The fourth experiment was designed to obtain nuclei from duck erythrocytes. Duck erythrocytes washed 4 times in normal saline were lysed with distilled water and centrifuged to yield a pellet which microscopically contained mostly nuclei as indicated by Wright's stain in addition to a few ghosts and unruptured erythrocytes. The nuclear pellet was washed 5 times in normal saline and then subjected to ultrasonic disruption followed by centrifugation. The supernatant and normal duck hemolysate were then assayed spectrophotometrically for LDH activity and a sample of each containing 3000 units/ml was applied to starch gels. Nuclei of chicken erythrocytes were similarly prepared.

Results. In studies of human hemolysates LDH isozyme 5, the cathodally migrating band, appeared in the hemolysates of the 4 individuals with reticulocytosis and in the cord blood with 12% reticulocytosis from the hydropic newborn. In hemolysates from 500 individuals without reticulocytosis isozyme 5 was not observed, nor was it seen in hemolysate from an anemic individual whose reticulocyte count was less than 1%.

Hemolysates prepared from 3 guinea pigs prior to phenylhydrazine administration do not exhibit LDH isozyme 5 (Fig. 1). In hemolysates prepared from these 3 guinea pigs during the period of anemia and reticulocytosis following phenylhydrazine administration LDH isozyme 5 is observed. Each of the 6 samples contained 60,000 LDH units/ml prior to application to the gel.

The results of the third experiment on hemolysates prepared from cord blood with 12% reticulocytes are shown in Fig. 2. Hemolysate from packed erythrocytes treated with lysing solution to remove differentially the older cells and to spare the younger cells reveals a greater intensity of staining of LDH isozyme 5 than does hemolysate prepared from untreated erythrocytes of the same cord blood. Both samples contained 20,000 units/ml of total LDH activity prior to application on the gel.

The results of the fourth experiment are shown in Fig. 3. The isozyme pattern of the nuclear extract of duck erythrocytes exhibits greatest LDH activity in the cathodal region as compared to the isozyme pattern of the supernatant material after removal of nuclei in the precipitate. Both samples contained 3000 units/ml of LDH activity prior to application on the gel. Nuclear preparations of chicken erythrocytes exhibited most LDH activity in the cathodal band, whereas the supernatant material revealed more activity in the anodal bands.

Discussion. These results suggest that

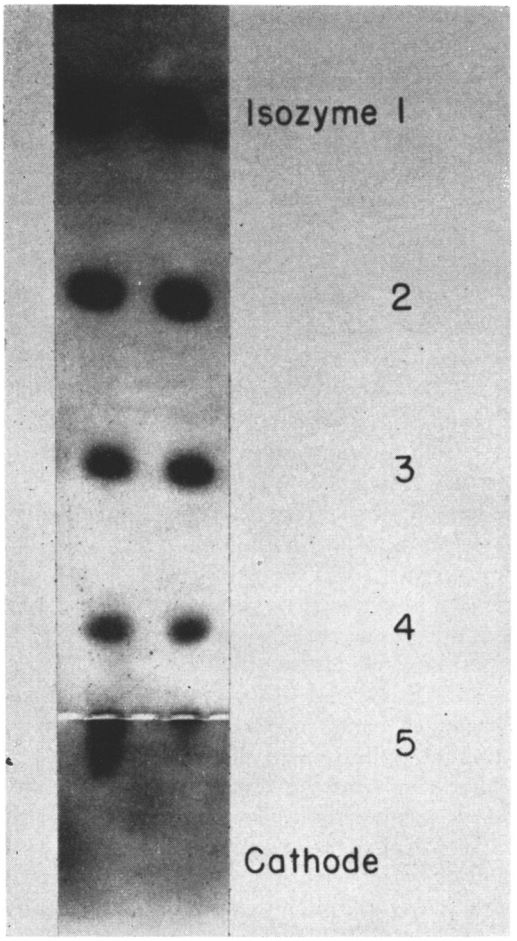

FIG. 2. Photograph of a starch gel stained for LDH activity. Hemolysate was prepared from cord blood of an hydropic newborn, with reticulocyte count of 12%. The pattern on left was obtained from a reticulocyte enriched preparation; that on right was obtained from erythrocytes prior to enrichment. Both samples were applied at 20,000 units/ml. Note that sample on left exhibits a much more intensely stained isozyme 5.

LDH isozyme 5 is present in young cells of the erythrocyte series but is absent in the mature human and guinea pig erythrocyte. The metabolism of the reticulocyte is more active than that of the mature erythrocyte (11). Several enzymes have been identified in the reticulocyte which do not appear in the mature erythrocyte; and the activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase and phosphohexose isomerase are high in young erythrocytes but diminish markedly with the *in vivo* aging of the erythrocyte(11). Embryological studies(7,12) have shown sequential alterations in the LDH isozymes of various chick, rabbit, rat and human tissues during development. The present report, however, provides the first indication that the pattern of isozymes may be altered in the adult organism during the processes involved in cell aging.

Since the only human cell lacking LDH isozyme 5 is the mature erythrocyte, which is also unique by virtue of the absence of a nucleus, and since the younger forms in the

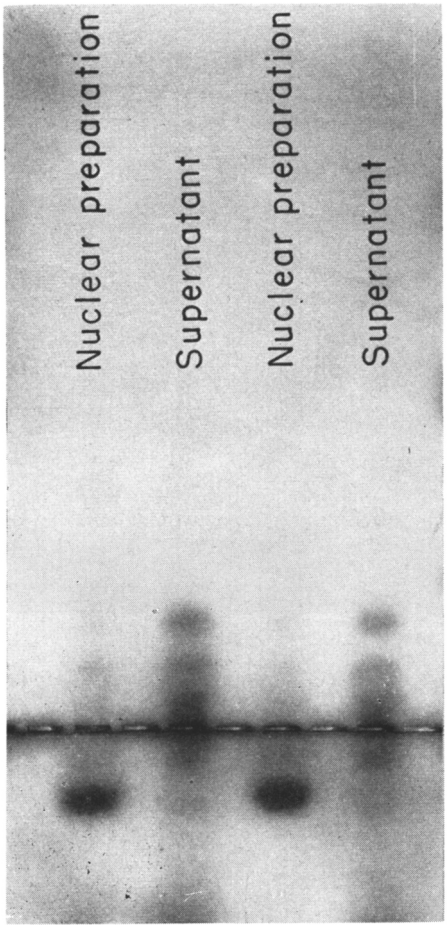

FIG. 3. Photograph of a starch gel stained for LDH isozymes in a preparation of nuclei from duck erythrocytes compared with isozyme pattern of the supernatant solution of hemolysate. Each sample contained 3000 units/ml prior to application on gel. Note in preparation from erythrocyte nuclei greater intensity of staining in the cathodal isozymes.

erythrocyte series contain both LDH isozyme 5 and a nucleus or primary nuclear products, the question arises of the relationship between LDH isozyme 5 and the cell nucleus. All mature mammalian erythrocytes are anucleated and all lack a cathodal LDH isozyme, although in the rat the single LDH identified in the erythrocyte migrates toward the cathode (9). The rat, however, has a high percentage of circulating nucleated cells of the erythrocyte series. The duck and the chicken with nucleated erythrocytes contain cathodally migrating LDH isozymes(9). In the present report nuclei isolated from duck and chicken erythrocytes were shown to exhibit a greater concentration of a cathodally migrating LDH isozyme than did whole duck and chicken hemolysate. Previously it was suggested that different LDH isozymes might be associated with different intracellular organelles(3); preliminary evidence has been provided in the case of rat kidney that this may be true(13) and furthermore, LDH activity has been reported by Dounce to exist within purified cell nuclei(14). Although several groups have identified LDH activity in purified nuclear preparations, the possibility of adsorption of cytoplasmic LDH to nuclear particles has not been adequately explored. Localization of specific LDH isozymes to different intracellular organelles relates to currently entertained concepts of isozyme synthesis. Appella and Markert dissociated a purified beef LDH isozyme into 4 subunits of identical molecular weight(15). These subunits could be further divided by charge differences into 2 groups which on reassortment into all possible combinations of 4 yielded the 5 LDH isozymes commonly observed in several mammalian species(15). The fact that different organelles appear to contain specific isozymes suggests that the association of 4 subunits to form different proportions of the 5 LDH isozymes in various human tissues is not random, but rather is controlled to permit specific isozymes to become associated with specific particles within various specialized cells. The subunits may be assembled into enzymatically active tetramers in the ribosomes or in other cytoplasmic particles or even in the nucleus where one isozyme, in the case of duck and chicken erythrocytes, appears to be prominently located. Protein synthesis within the nucleus has been established(16) and recently it has been shown that nuclear ribosomes contain high levels of LDH activity(17).

Summary. 1. The mature erythrocyte is the only human cell thus far studied which lacks LDH isozyme 5, not observed in hemolysates from 500 individuals. Hemolysates from 5 patients with anemia and reticulocytosis exhibited isozyme 5. Hemolysate from an anemic patient without reticulocytosis did not contain isozyme 5. 2. Hemolysates from 3 normal guinea pigs lacked isozyme 5. After anemia induced by phenylhydrazine administration and during the period of reticulocytosis LDH isozyme 5 appeared in the guinea pig hemolysates. 3. A preparation enriched in young cells of the erythrocyte series revealed more LDH isozyme 5 than did hemolysate prepared from the untreated erythrocytes of the same blood. 4. Nuclei prepared from duck and chicken erythrocytes exhibited the majority of LDH activity in a single cathodal band in contrast to the isozyme pattern of the supernatant material. 5. In conclusion, LDH isozyme 5, absent in the anucleated mature erythrocytes of man and guinea pig, is present in the earlier forms of the erythrocyte series which are nucleated or contain primary nuclear products. In addition to those isozymic alterations previously described during embryonic development, isozymic changes also occur in the adult animal during the processes of cell aging.

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Time of Appearance of Antigenic Factors on Cattle Erythrocytes.* (27716)

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The time of appearance of antigenic factors‡ on erythrocytes (red cells) during ontogeny has long been of interest. Now, it is generally accepted that in humans most, if not all, of the blood factors are sufficiently developed in early embryonic life to be recognizable(1,2). However, the reactions are usually weaker in the embryo and in the newborn than in adults(3).

Similar results have been reported for rats (4) rabbits(5), chickens(6), pigeons and doves(7,8) and cattle(9,10). In (unpublished) studies on cattle, we were able to detect most of the blood factors on the red cells of newborn calves, although some of them were considerably weaker than those on adult red cells. For example, the H′ factor was found, both by Stormont *et al.*(10) and ourselves, as weakly reactive in embryos and newborn. It reached adult reactivity at about 6 months of age. The so-called J factor is an exception, because it has not been found on the red cells of embryos or newborn, but develops gradually within the first week *postpartum*. Since the J factor is primarily a constituent of the serum from which it may be acquired secondarily by the cells, this exceptional behavior is understandable(11,12).

Observations on the development of the H-2 red cell factors in mice indicate that they appear at about 3-8 days after birth and attain the concentrations found in adults by the 10-12th day(13,14,15). A recent report by Pizarro *et al.*(16) on the development and distribution of the histocompatibility (H-2) antigens (factors) of mice presented evidence that they were not present in tissues and on the erythrocytes of embryos or newborn, but appeared about the third day after birth and reached adult levels at about 6 days. In adults, the H-2 factors were found in most tissues except brain, but not in the same con-

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‡ The term antigenic factor or blood factor is used to denote an attribute of the erythrocyte surface responsible for the specific interaction of the erythrocyte with the antibodies in a blood typing serum. The terms antigen, antigenic determinant, haptenic group, etc., have been avoided deliberately because much confusion already exists in the literature as to their exact meanings.