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THE IMMUNOLOGICAL PROPERTIES OF PYRUVATE KINASE

II. THE RELATIONSHIP OF THE HUMAN ERYTHROCYTE ISOZYME TO THE HUMAN LIVER ISOZYMES

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Summary

We have examined the hypothesis that the human erythrocyte isozyme of pyruvate kinase (EC 2.7.1.40) is a hybrid of the two isozymes present in liver. Rabbit antiserum against purified human erythrocyte pyruvate kinase inactivates the erythrocyte isozyme and the major liver isozyme from human tissue but does not inactivate the minor liver isozyme. The electrophoretic mobilities of the erythrocyte and major liver isozymes are altered by anti-erythrocyte enzyme antibody while the mobility of the minor liver isozyme is unaffected. Gel diffusion analysis indicates cross-reactivity between the erythrocyte and major liver isozyme but no cross-reactivity with the minor liver isozyme. The hybrid hypothesis would predict cross-reactivity including changes in activity and mobility of all isozymes and we conclude, therefore, that the hypothesis is incorrect.

Pyruvate kinase (ATP : pyruvate phosphotransferase, EC 2.7.1.40) exists in human and rat tissues as four isozymes which differ in their kinetic, electrophoretic or immunologic properties [1,2]. M_1 is the isozyme in muscle, L the major isozyme in liver and M_2 the minor isozyme in liver. Based on electrophoretic mobilities and inactivation by antiserum against the rat M_2 isozyme [4], two groups of investigators have proposed that the R isozyme in human erythrocytes is a hybrid of the L and M_2 isozymes [1,3—5].

Preparation of purified human R isozyme and of monospecific antiserum against the R isozyme are described elsewhere [6,7]. The antiserum was partially purified by $(NH_4)_2SO_4$ fractionation and the globulin fraction used after heat inactivation and absorption with human erythrocytes as described previously [7].

Rabbit anti-human muscle pyruvate kinase (anti-M_1) was prepared by injecting 1 mg of purified M_1 emulsified in 0.5 ml of complete Freund's ad-

juvant at several subcutaneous sites. 10 months later the rabbit was reinjected with 0.7 mg of purified M_1 in adjuvant as before. The serum used was obtained 15 days after the second injection. The antiserum neutralized the M_1 but not the R isozyme and formed a single line of identity between the purified enzyme used for immunization and crude muscle extract [8].

Human liver obtained at autopsy was rinsed in saline and homogenized in 3 volumes of cold extraction buffer (0.5 M sucrose, 25 mM Tris, 2.5 mM $MgCl_2$, 2.5 mM EDTA (dipotassium salt), pH 7.4) for 1 min at low speed in a Waring blender. The supernatant fluid was fractionated by $(NH_4)_2SO_4$ precipitation at 4°C after centrifugation for 1 h at 20 000 × *g*; the L isozyme was obtained between 20 and 40% saturation and the M_2 isozyme from 50 to 75% saturation.

The R isozyme was prepared from washed human erythrocytes as described previously [9].

The various isozymes were checked for contamination with the other isozymes by thin-layer polyacrylamide gel electrophoresis [4]. Fig. 1 shows that each preparation gave only the distinct band typical for the respective isozyme. The human R isozyme gives two bands in this electrophoretic system [10].

For inactivation studies, the antibody was serially diluted with 0.1 M triethanolamine · HCl, pH 7.4. Equal volumes of enzyme solution in the same buffer at a final concentration of 0.13 units/ml for the L and R isozymes and 0.23 units/ml for the M_2 isozyme were then added to each dilution. The activity differences were calculated to give equal molar concentrations of the isozymes assuming that the human isozymes have the same specific activity ratios as the rat L and M_2 isozymes [2,3,5]. After incubation for 5 min at 37°C, a sample was removed to determine enzyme activity. Controls used isozyme incubated with buffer in place of antibody; control globulin preparations from non-pyruvate kinase-immunized rabbits have no inhibitory activity in this assay [7]. Pyruvate kinase activity was measured by the coupled assay with lactic dehydrogenase [1].

The anti-R antibody inactivates the R and the L isozymes but not the M_2 isozyme (Fig. 2). The anti-M_1 antibody inactivates M_2 but does not inactivate the R or L isozymes (not shown). We obtained comparable results with rat L, R and M_2 isozymes using the anti-human R and M_1 antisera. These results are not consistent with the hypothesis that the R isozyme is a hybrid of the L and M_2 isozymes.

We examined interactions with determinants other than those related to the active sites by electrophoresis of the isozymes in the presence and absence of antibody. Interaction between isozyme and specific antibody molecules alters the electrophoretic mobility of the isozyme. The results obtained using anti-R and anti-M_1 antibody are shown in Fig. 3. Again the results are inconsistent with the hybrid hypothesis; anti-R interacted with the R and L isozymes while anti-M_1 interacted with M_2 and M_1.

Fig. 4 (top) shows the results of gel diffusion analysis using anti-R and anti-M_1 antibodies. The anti-R antibody cross-reacts with the L but not with the M_2 or M_1 isozymes. Anti-M_1 antibody, however, does cross-react with M_2 indicating that the M_2 sample contained immunologically active enzyme even

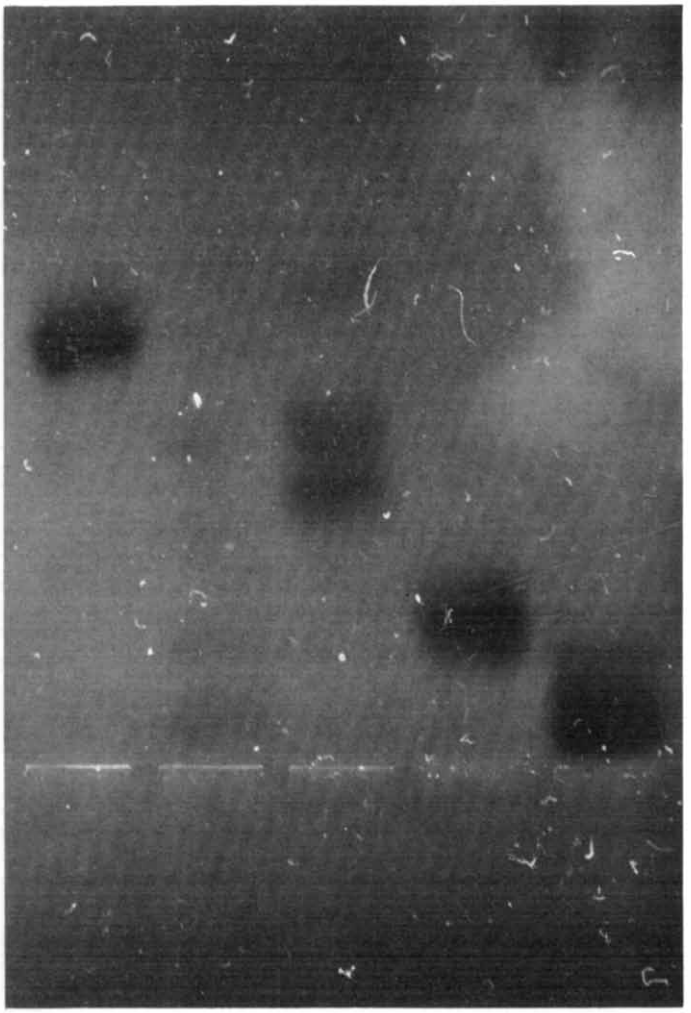

Fig. 1. Thin-layer polyacrylamide gel electrophoresis of the human pyruvate kinase isozymes. Electrophoresis was carried out at pH 8.2 with anode at the top, cathode at the bottom. The origin is detectable as the thin white line close to the bottom of the photograph. After electrophoresis the isozymes were detected by agar overlay containing the components of the linked lactic dehydrogenase reaction. NADH oxidation is indicated by the dark bands. From left to right the slots contained the L isozyme, a kidney homogenate which is not relevant to the discussion, the R isozyme, the M_2 isozyme and the M_1 isozyme. The faint dark band in the R preparation with mobility faster than the L isozyme is due to hemoglobin.

though it failed to react with anti-R. These results are consistent with those obtained by enzyme inactivation and by electrophoresis in the presence and absence of specific antibody; they are not consistent with the hybrid hypothesis.

The cross-reaction between the R isozyme and the L isozyme is complicated by the second precipitin band in the R preparation. This second inner band is partially reactive with but not identical to the L isozyme as indicated by the spur extending beyond the inner R band and in turn intersecting the

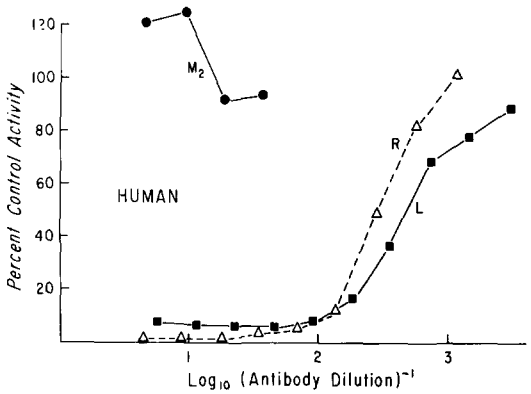

Fig. 2. Inactivation of the human pyruvate kinase isozymes by rabbit anti-R antibody. Activity remaining after 5 min incubation is expressed as a percentage of the control containing no antibody. The experimental conditions are given in the text.

major R precipitin band. The latter interaction is also one of only partial identity indicating that the anti-R antibody detects determinants not found in the L isozyme.

The second inner band in the R preparation is most probably due to

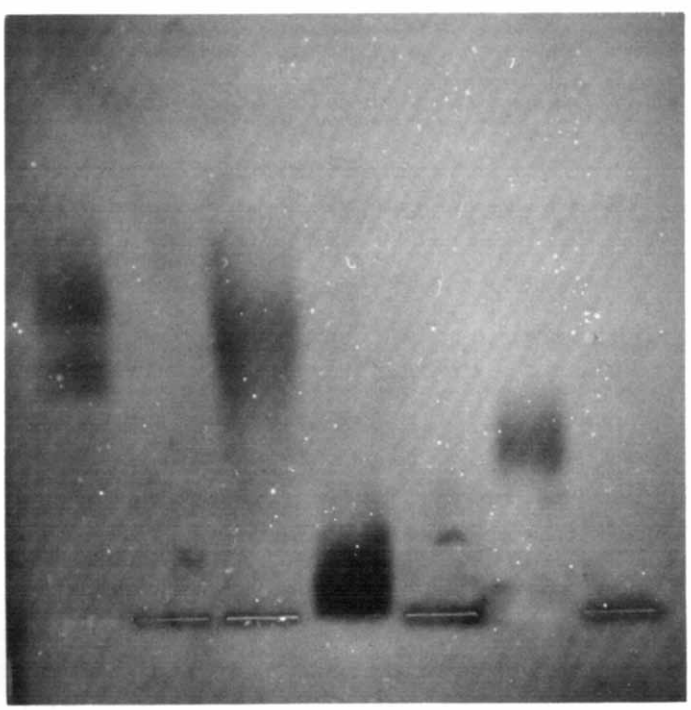

Fig. 3. The effect of specific antisera on the electrophoretic mobility of the human pyruvate kinase isozymes. Thin-layer polyacrylamide gel electrophoresis was carried out as described for Fig. 1. From left to right the slots contained: the R isozyme, R + anti-R, R + anti M_1, the M_1 isozyme, M_1 + anti-M_1, the M_2 isozyme and the M_2 + anti-M_1.

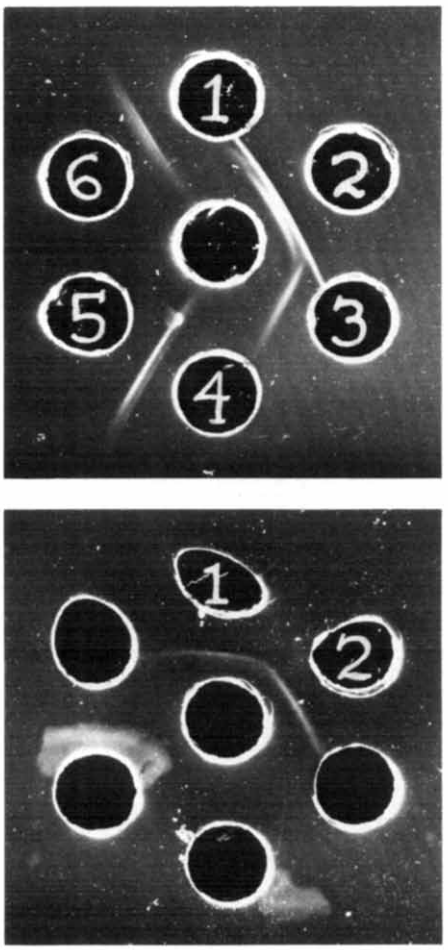

Fig. 4. Gel diffusion analysis of pyruvate kinase isozymes. Top, human isozymes: center well anti-human R; 1, the M_2 isozyme, 0.68 units/ml; 2, the R isozyme, 10.6 units/ml; 3, the L isozyme, 2.0 units/ml; 4, the M_1 isozyme, 6.9 units/ml; 5 and 6, anti-human M_1. Bottom, rat isozymes: center well anti-human R; 1, rat liver L isozyme, 30.4 units/ml; 2, rat liver M_2 isozyme, 3.1 units/ml; other wells empty.

monomers or dimers derived from the R tetramer. Thus R could be a hybrid molecule containing L subunits; or R and L may be the products of recent evolutionary divergence during which they retained some common antigenic determinants.

We have found that although our anti-human R does not neutralize rat M_2 it cross-reacts by gel diffusion with both the rat L and rat M_2 isozymes (Fig. 4, bottom) and rat R [7] indicating shared antigenic determinants. Thus antisera prepared against rat isozymes could be misleading if used to interpret the subunit relationships among the human isozymes of pyruvate kinase [1].

It could be argued that the proposed interaction of the L and M_2 subunits in forming the R isozyme alters the conformation of the M_2 subunits to such an extent that "native M_2" antigenic determinants are not recognized by anti-

bodies against the M_2 subunits present in R. We think this unlikely since we recently found that naturally occurring hybrids of the L and M_2 isozymes which occur in the kidney are partially inactivated by both anti-R and anti-M_1 sera and that both antisera alter the electrophoretic mobilities of these hybrids (Lincoln, D.R., Bigley, R.H., Rittenberg, M.B. and Black, J.A., unpublished).

We conclude that the human R isozyme is not a hybrid of the L and M_2 isozymes.

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