Reversal of UDP-galactose 4-epimerase deficiency of human leukocytes in culture

(gene expression/ enzyme deficiency/ lymphocyte transformation)

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ABSTRACT Stimulation with phytohemagglutinin of the leukocytes from six of the seven known individuals with UDP-galactose 4-epimerase (= UDP-glucose 4-epimerase; EC 5.1.3.2) deficiency consistently resulted in the appearance of epimerase activity in the cultured cells. A long-term lymphoblast culture derived from one proband also contained an active epimerase enzyme. A comparison of the properties of this enzyme with those of epimerase produced by control lymphoblast lines revealed comparable Km values for UDP-galactose and NAD and identical behavior on polyacrylamide electrophoresis. However, a difference in the NAD requirement for heat stability at 40° provided some evidence for a structural defect in this enzyme. Possible explanations for the appearance of UDP-galactose 4-epimerase activity in stimulated lymphocytes include an increased rate of synthesis of a mutant enzyme and a derepression of an epimerase locus during lymphocyte transformation.

The establishment of long-term lymphoblast lines from individuals with inborn errors of metabolism has proved to be a useful technique for the study of specific metabolic defects in cell culture (1, 2). These cells tend to maintain their individual genetic characteristics (3) and have a normal diploid chromosome complement. The initial intent of the present investigation was to study the effect of UDP-galactose 4-epimerase (= UDP-glucose 4-epimerase; EC 5.1.3.2) deficiency on the structure and metabolism of human lymphoblast cells in continuous culture. This enzyme defect (4), inherited as an autosomal recessive disorder (5), has been discovered in the peripheral blood cells of seven probands in three families and is characterized by elevated levels of erythrocyte galactose 1-phosphate (B. Steinmann et al., in preparation). Although there do not appear to be any associated clinical abnormalities in these individuals, deficiency of UDP-galactose 4-epimerase, which catalyzes the reaction UDP-galactose = UDP-glucose, is known to result in marked abnormalities of cell wall synthesis in bacteria (6, 7) and has been postulated to be of serious consequence for mammalian cells (8).

We found, however, that a lymphoblast line established from a human homozygote for this disorder contained an active epimerase enzyme which was not present in her peripheral blood lymphocytes. This called into question the role of lymphocyte transformation in the expression of epimerase activity. Therefore, we undertook further studies into the effect of the mitogen phytohemagglutinin (PHA) on the appearance of epimerase activity in deficient leukocytes and on the specific activity of epimerase in isolated normal lymphocytes. In addition, properties of the enzyme produced by the long-term lymphoblast line from a proband were compared with those of epimerase produced by control lines in an attempt to further elucidate the genetic nature of this defect.

EXPERIMENTAL

PHA-Stimulation of Leukocytes and Isolated Lymphocytes. Leukocytes were obtained from heparinized venous blood by gravity sedimentation for 90 min at room temperature and were washed once in Hank’s balanced salt solution and once in RPMI 1640 medium (Flow Laboratories). Lymphocytes were isolated from heparinized venous blood on a Ficoll–Hypaque gradient (9) and washed in the same manner. All cultures contained 10^6 mononuclear cells per ml of complete medium, which consisted of RPMI 1640 supplemented with 20% fetal calf serum (GIBCO), 2 mM L-glutamine (Flow Laboratories), 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 0.25 μg/ml of Fungizone (GIBCO). Cultures containing 10–15 ml of cell suspension were incubated with a predetermined optimal stimulatory dose of 1 μg/ml of PHA (Burroughs Wellcome purified, lot HA17) in upright 25 cm^2 flasks (Falcon Plastics) at 37° and in a 5% CO_2 environment. After thorough cell suspension, triplicate 0.2 ml aliquots were taken from each culture at 0 time and after 72 hr and were incubated in a microculture plate (Falcon Plastics) with 1 μCi of [3H]thymidine (Schwarz/Mann, 1.9 Ci/mm) at 37° for 6 hr. Each microculture was precipitated onto Whatman GF 83 glass filter paper, washed with saline, and transferred to a counting vial containing 10 ml of Insta-Gel (Packard). Radioactivity counting was performed on a Packard Tri-Carb scintillation counter with an efficiency of 40%.

Long-Term Lymphoblast Cultures. Long-term lymphoblast cell lines were initiated by a modification of the method of Beratis and Hirschhorn (10). Peripheral blood leukocytes obtained by gravity sedimentation of 10 ml of heparinized venous blood were washed once in RPMI 1640 medium and cultured in triplicate in upright 25 cm^2 Falcon flasks containing 9 ml of cell suspension in complete RPMI medium, 0.1 ml of PHA (Difco-M), and 1 ml of cell lysate from previously established cultures. Cells were incubated at 37° in a 5% CO_2 environment and maintained by weekly removal of 5 ml of supernatant fluid and addition of an equal volume of fresh medium free of PHA. The establishment of a permanent cell line usually occurred between 40 and 60 days after the initiation of the culture and was detected by clumping of the cells on the bottom of the flask and by a pH drop in the medium. The cells were subsequently transferred to 75 cm^2 Falcon flasks and were maintained in log phase growth by weekly removal of three quarters of the total volume of suspended cells and replacement of an equal volume of fresh complete medium.

Partial Purification of Lymphoblast Epimerase. Cells

Abbreviation: PHA, phytohemagglutinin.

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harvested from lymphoblast cultures were stored in 0.01 M glycine–OH buffer, pH 9.0, at −20° for a maximum of 3 weeks before use. Cell lysates, obtained by freezing and thawing a total of three times and centrifuging at 40,000 × g for 10 min, were dialyzed overnight against 5 mM triethanolamine buffer containing 5 mM EDTA, 5 mM mercaptoethanol, 10 μM NAD, and 0.15 M KCl, pH 8.85. Dialyzed samples were loaded onto a DEAE-Sephadex A-50 column, 2.8 cm in diameter and 20 cm in length, which had been equilibrated with the same buffer. Epimerase was eluted with a linear gradient of KCl in the triethanolamine buffer system (2 liters, from 0.15 to 0.5 M KCl) at a flow rate of 30 ml/hr. Fractions containing epimerase activity were pooled and concentrated on an Amicon B 15 filter. Concentrated enzyme preparations were further dialyzed against 0.01 M glycine–OH, pH 9.0, containing 10 μM NAD and were stored at −20° until use.

Assays of Epimerase Activity. Peripheral blood leukocytes, lymphocytes, and cultured lymphoblasts were prepared and assayed for epimerase activity as described previously (5). All leukocytes and lymphocytes were washed in decreasing concentrations of NaCl (0.85–0.17%) to remove contaminating erythrocytes.

Partially purified epimerase enzyme from the DEAE-Sephadex column was assayed by a one-step procedure in which the basic assay mixture contained 1 mM NAD, 100 mM glycine–OH, pH 9.0, 1 mM mercaptoethanol, 0.014 units of UDP-glucose dehydrogenase (Sigma), and the enzyme preparation to be tested, in a final volume of 1 ml. After controlling for nonspecific reduction of NAD, 1 mM UDP-galactose was added and the reaction was followed at 340 nm. One unit, U, equals 1 μmol/min.

Polyacrylamide Disc Gel Electrophoresis. Gels were prepared in glass tubes 0.5 cm in internal diameter and 12 cm in length. A 5 cm running gel of 10% acrylamide and 0.14% bisacrylamide in Tris-HCl buffer, pH 8.7, was overlaid with a 2.5% acrylamide and 0.63% bisacrylamide spacer gel 2 cm in length. The running buffer contained 0.05 M Tris-0.38 M glycine, pH 8.3. Each sample containing approximately 75 μg of protein in 0.01 M glycine–OH buffer, pH 9.0, containing sucrose was layered onto the spacer gel. Electrophoreses were run at 4° at a current of about 2.5 mA per gel and a constant voltage of 180 V for 3 hr. Gels to be stained for protein were fixed overnight in 15% trichloroacetic acid and stained with 0.25% Coomassie blue at 60°. Acetic acid (10%) was used for destaining. Epimerase was eluted from 3 mm unstained gel slices by immersing each segment in 0.8 ml of a solution containing 0.1 M glycine–OH, pH 9.0, 1.0 mM NAD, and 1 mM mercaptoethanol for 24–48 hr at 4°. Activity was assayed on the addition of 1 mM UDP-galactose and 0.014 units of UDP-glucose dehydrogenase to this mixture.

RESULTS

Epimerase Activity in Stimulated Lymphocytes. Epimerase activity in extracts from unstimulated peripheral blood leukocytes and isolated lymphocytes from control individuals is shown in Table 1. The presence of epimerase activity in normal polymorphonuclear cells would have made PHA-stimulation of mixed leukocytes difficult to interpret. PHA-stimulation of control lymphocytes, however, consistently resulted in an increase in the specific activity of epimerase which averaged 1.6-fold and which was statistically significant (0.005 > P > 0.001). The time course of this increase in activity is seen in Fig. 1. After an initial lag period of approximately 24 hr, both [3H]thymidine incorporation and epimerase activity increased over the subsequent 48 hr period. The addition of cycloheximide at a concentration of 50 μg/ml to a culture at 0 time caused a gradual decrease in epimerase activity and prevented any rise in [3H]thymidine incorporation. Concomitant cell counts in the presence of trypan blue indicated that 85–90% of lymphocytes remained viable under these conditions.

Epimerase activity was consistently absent from the leukocytes of all seven probands with the exception of one out of three determinations on a single individual (E.B.) when a value of 0.8 units/g was obtained (Table 1). In all six pro-

Table 1. UDPGal 4-epimerase deficiency: Comparison of epimerase activity* of unstimulated and PHA-stimulated leukocytes from probands and controls

<table>
<thead>
<tr>
<th>Leukocytes</th>
<th>Lymphocytes</th>
<th>Lymphoblasts, long-term culture</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Unstimulated</td>
<td>PHA</td>
</tr>
<tr>
<td>Probands</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E.B.</td>
<td>0.0</td>
<td>8.7</td>
</tr>
<tr>
<td>R.B.</td>
<td>0.8</td>
<td>7.1</td>
</tr>
<tr>
<td>C.K.</td>
<td>0.0</td>
<td>4.5</td>
</tr>
<tr>
<td>J.P.</td>
<td>0.0</td>
<td>1.3</td>
</tr>
<tr>
<td>M.E.</td>
<td>0.0</td>
<td>3.7</td>
</tr>
<tr>
<td>A.E.</td>
<td>0.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>x</td>
<td>2.2</td>
<td>6.2</td>
</tr>
<tr>
<td>SD</td>
<td>0.9</td>
<td>2.4</td>
</tr>
<tr>
<td>Range</td>
<td>1.0–4.6</td>
<td>3.1–10.9</td>
</tr>
<tr>
<td>(n)</td>
<td>(22)</td>
<td>(14)</td>
</tr>
</tbody>
</table>

*μmol/min · g extractable protein, at 37° and pH 9.0.

FIG. 1. Epimerase activity and thymidine incorporation of PHA-stimulated normal lymphocytes. Values for duplicate cultures are given for each time point.
band leukocyte cultures stimulated with PHA, epimerase activity was easily detected and was proportional to incubation time over a 15 min period. Similarly, epimerase activity in lysates from isolated lymphocytes was zero for two probands, although a small amount of activity was detected on one determination from the same individual who previously had demonstrated some leukocyte epimerase. PHA-stimulation of isolated lymphocytes in three cases also resulted in the appearance of epimerase activity which was similar to that found in the leukocyte extracts from the same individuals. The amount of activity obtained from both leukocyte and lymphocyte cultures correlated to some extent with the degree of lymphocyte stimulation as measured by \(^{3}H\)thymidine incorporation into DNA (Fig. 2).

A long-term lymphoblast line was established from one female proband (E.B.) using lysate from a male cell line as the source of Epstein-Barr virus. Epimerase activity in extracts of these cells, which were confirmed as having a female karyotype, fell in the lower range of values obtained from six control cell lines (from four normal individuals, 1 patient with vitamin B12-dependent methylmalonic aciduria, and 1 patient with trisomy of chromosome 10).

**Kinetic Properties of Lymphoblast Epimerase.** Partial purification of epimerase from the proband long-term lymphoblast line and from three control lines was carried out on DEAE-Sephadex A-50 columns. Elution of epimerase occurred between 0.25 and 0.3 M KCl for both proband and control enzymes. A typical purification is shown in Table 2. The resulting preparations were used for comparative studies of epimerase activity.

Neither the \(K_m\) for UDP-galactose nor the pH optimum differed significantly between proband and control enzymes (Table 3). Low concentrations of NAD were found to be essential for the stability of epimerase at 4°C. Hence, the apparent \(K_m\) for NAD as a cofactor was determined for enzyme preparations which had been dialyzed for 24 hr against buffer containing 5 \(\mu\)M NAD. In three separate experiments with control and proband enzymes treated identically, there was no difference in the apparent \(K_m\) for NAD (Table 3).

A major difference between the enzyme from the proband line and normal controls was found in studies on enzyme stability at 40°C. The proband enzyme was consistently less stable in four experiments and lost an average of 85% of its initial activity after 3 min while controls from different donors averaged only a 48% loss of activity under the same conditions. Addition of NAD effectively stabilized epimerase from both sources against heat inactivation, and two sets of experiments were performed to compare heat stability of proband and control enzymes at different NAD concentrations. For this purpose, enzymes were dialyzed for 48 hr against different NAD concentrations prior to the heat inactivation experiments. As shown in Fig. 3, proband enzyme was completely stabilized against inactivation at 40°C at an NAD concentration of 500 \(\mu\)M and partially stabilized at 50 \(\mu\)M. In contrast, over a wide range of NAD concentrations (0.5-500 \(\mu\)M), control enzyme required only about one tenth the concentration of NAD needed by the proband enzyme to achieve the same degree of heat stability. The difference in heat stability at 40°C is not explained by different proteolytic activities of the two enzyme preparations, since in both the activity was linear for up to 15 min at 37°C under optimal conditions. Thus, mixing experiments were not performed. From these studies it could be concluded that the amount of NAD required for heat stabilization differed for the two enzymes, although the NAD concentration required for optimal enzyme activity was similar.

**Gel Electrophoresis of Partially Purified Epimerase.** Polyacrylamide disc gel electrophoresis of partially purified enzyme preparations from a proband, a control, and a mixture of both are seen in Fig. 4. Epimerase activity eluted from slices of identical gels was found in the region containing two protein bands, as indicated, and had an average \(R_F\) value of 0.36. No distinction in the migration of these bands or of the corresponding epimerase activity could be detected for the proband enzyme as compared to three separate controls. In the absence of a method for catalytic staining, it could not be determined whether one or both of these bands represented epimerase.

**DISCUSSION**

The results reported in this paper indicate that lymphocytes from individuals lacking UDP-galactose-4-epimerase in peripheral blood cells are capable of producing an active epim-

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1 Blood for lymphocyte studies was drawn 4 days after assaying leukocyte epimerase. In this interval, the child was noted to have a mild temperature elevation associated with symptoms of a viral infection.
concentrations in samples of lymphoblast epimerase. Proband (12)
ficiency of the deficient enzyme. PHA-stimulation of normal lymphocytes results in a highly variable increase in the specific activity of many enzymes, but how much of this increase is attributable to new protein synthesis is not always clear (11). The coincidence of the time courses of the increase in epimerase specific activity and the rise in [3H]thymidine incorporation reported here, as well as the inhibition of both events by cycloheximide, suggest, but do not prove, that transcription and accelerated enzyme synthesis are involved. The rough correlation between the epimerase activity produced by stimulated lymphocytes from the probands and the amount of [3H]thymidine incorporated by these cells is consistent with this idea. These findings suggest two intriguing, although highly hypothetical, possibilities for the nature of this genetic defect.

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ UDPGal (μM)*</th>
<th>Apparent $K_m$ NAD (μM)†</th>
<th>pH optimum</th>
<th>Heat stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proband</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E.B.</td>
<td>18–36</td>
<td>66–133</td>
<td>9–10</td>
<td>Reduced</td>
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<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>18</td>
<td>133</td>
<td>9–10</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>17</td>
<td>89</td>
<td></td>
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</table>

* Assays were performed in the presence of 1 mM NAD.
† Enzyme preparations were dialyzed for 24 hr against 0.01 M glycine buffer, pH 9.0, containing 5 μM NAD.

The detection of epimerase activity in a long-term cell line alone could have been attributed either to the presence of the Epstein-Barr virus genome presumed to be present in these lines (11) or to a selection for normal or revertant cells during the establishment of the culture. However, the consistent induction of epimerase in PHA-stimulated proband lymphocytes after 72 hr in culture indicated that these cells had the genetic capability to synthesize an active enzyme. This is not the case for lymphocytes from individuals with two other inborn errors of metabolism, α-1,4-glucosidase deficiency (12) and cystathionine synthase deficiency (13, 14). PHA-stimulation of these cells did not result in the appearance of the deficient enzyme.

![FIG. 3. Effect of NAD on heat stability of partially purified lymphoblast epimerase. Proband enzyme (●); control enzyme (○). Enzyme samples were dialyzed 48 hr against the appropriate NAD concentrations in buffer before being heated to 40°C.](image)

![FIG. 4. Polyacrylamide gel electrophoresis of partially purified lymphoblast extracts. (1) Proband; (2) control; (3) equal mixture of both. Arrow indicates the location of epimerase activity in sliced gels.](image)
One could postulate that the structurally abnormal protein synthesized in peripheral blood cells is rapidly degraded by proteolytic enzymes. An enzyme which inactivates NAD-dependent enzymes in the absence of NAD has been found in rat intestine (15), and it is plausible that such an enzyme could be responsible for the early proteolysis of a mutant epimerase defective in the binding of NAD. The appearance of this altered epimerase in cultures of stimulated cells could result from an increase in the rate of protein synthesis which was sufficient to outpace the degradatory mechanism. We have demonstrated a less than 2-fold net increase in epimerase activity in normal lymphocytes after PHA-stimulation, but even such a modest increment could theoretically account for the appearance of mutant enzyme activity.

The possible production of an isoenzyme in stimulated cells must also be considered. Epimerase activity in the normal range has been found in cultured skin fibroblasts and in liver tissue from affected individuals (5). It is conceivable that the blastic transformation of the lymphocyte in culture is associated with a derepression of a genetic locus normally expressed in other cell types. Derepression has been suggested as an explanation for the synthesis of interferon both in lymphoblast cell lines (16) and in PHA-stimulated lymphocytes (17) and for the production of multiple types of immunoglobulins by cloned lymphocytes in long-term culture (18). Also, in the case of mal de dehydrogenase, there is evidence that a specific isoenzyme can be selectively synthesized in PHA-stimulated lymphocytes (19) although this did not involve the synthesis of a new cellular protein. Unfortunately, not much is known about the heterogeneity of epimerase in human tissues and the distinction between the production of an isoenzyme and that of an enzyme altered by mutation cannot be made at the present time.

Finally, it should be noted that leukocyte morphology and a number of functional parameters were normal in all seven individuals. If a catalytically active UDP-galactose 4-epimerase were present in undifferentiated cell precursors, this would insure the availability of UDP-galactose for the formation of glycoproteins and glycolipids during cell development, in both the presence and absence of exogenous galactose. The detection of small amounts of epimerase in the lymphocytes of one proband during a presumed viral infection provides some evidence that in vivo activation of these cells is indeed possible. This might explain the absence of any of the dire metabolic consequences of this disorder predicted by Kalckar (8) for mammalian cells and found in the less fortunate epimerase-deficient Salmonella (6, 7).

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