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Heat-Labile Enzymes in Circulating Erythrocytes of a Progeria Family

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INTRODUCTION

Progeria is a rare disease of unknown etiology that is manifested during infancy as severe stunting of growth followed by premature and accelerated aging [1, 2]. Death usually occurs in the teens as a result of occlusive vascular disease. Although claims have been made for autosomal recessive inheritance [3], most cases appear sporadically [1, 2] suggesting a dominant mutation related to increased paternal age [4]. In vitro studies demonstrated a diminished growth capacity of cultured skin fibroblasts [5, 6], and more recently, evidence has been marshalled for a widespread cellular disturbance affecting diverse gene products [7–10], including an increased heat-labile fraction of various enzymes [8-11]. Since only cultured fibroblasts were used in these studies, it is possible that the tissue culture milieu, based upon the needs of normal cells, predisposed them to changes in progeric protein metabolism [11].

The purpose of this study is (1) to see if cells freshly obtained from a progeria patient contain altered proteins and (2) to clarify the genetics of this disorder. In this paper, we demonstrate that circulating erythrocytes from a child with progeria contain an increased heat-labile fraction of two genetically distinct enzymes, glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD), while her parents have values intermediate to those of their daughter and controls consistent with an autosomal recessive mode of inheritance in this family.

MATERIALS AND METHODS

Patients

The proband (B. S.), a 5-year-old female with classical progeria, was the product of a single uncomplicated pregnancy. A subsequent pregnancy ended in a spontaneous first trimester abortion. The 31-year-old mother (A. S.) and the 43-year-old father (B. S.) were clinically normal. This Caucasian family had no history of consanguinity or any progeria-like disorder and had not been studied before.

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Controls

Four healthy adults age 25–65 years with no known metabolic diseases and no family history of disorders associated with shortening of the lifespan were used as normal controls. Abnormal control subjects included a 78-year-old female with myelofibrosis, an 8-year-old with a fever of unknown origin plus lymphadenopathy, and a 9-year-old with Hodgkin's disease. These three chronically ill individuals were selected for the effect their illnesses could have on erythrocyte turnover, and consequently, the content of heat-labile enzymes. All controls were Caucasian.

Laboratory Studies

Blood was obtained by venipuncture without anticoagulants and immediately defibrinated at room temperature by gentle rotary shaking in a flask containing glass beads. The supernatant blood was decanted and centrifuged at 200 *g* removing the buffy coat and uppermost layer of packed erythrocytes followed by careful mixing and recentrifugation. This procedure was repeated three times until the final leukocyte count was less than 0.01% of the erythrocyte count using a hemocytometer. Erythrocytes were fractionated by a slight modification of the method of Murphy [12] which yields good separation of young cells without the use of foreign media [13]. Erythrocytes were suspended in homologous serum and adjusted to a hematocrit of 85%. An aliquot of this whole erythrocyte population (WEP) was removed and held on ice while the rest was added to the 6.5 cm mark of a 7.8 × 1.0 cm Sorvall tube (about 5 ml).

Cells were then centrifuged at 27,000 *g* in a Sorvall SS-34 rotor and RC-2B centrifuge for 1 hr at 30°C. The efficacy of fractionation was confirmed on two control subjects during diagnostic workup. Blood collected 8 days after ^{59}Fe injection and fractionation as above contained about 80% of the label in the top 0.5 ml of the gradient and less than 10% of the radioactivity in the bottom 0.5 ml. All procedures from this stage until enzyme assay were then carried out at 0°–4°C. The top 0.1–0.2 ml of the gradient (young cells) and the WEP removed before fractionation were rinsed twice in 5 mM Tris buffer (pH 7.4) containing 150 mM NaCl, then hemolyzed in 10 vol distilled water containing 1mM EDTA, 1 mM dithiothreitol, 1 mM ϵ-amino caproic acid, and 20 μM NADP. The hemolysate was cleared by centrifugation at 30,000 *g* for 30 min in a Beckman L3-50 ultracentrifuge.

Assays for G6PD and 6PGD heat-lability were then carried out on hemolysates as described for cultured fibroblasts [11] with minor modifications. Exactly 650 μl of cleared hemolysate were placed in a 5 ml glass centrifuge tube using a set of matched tubes throughout all experiments. Hemolysates were incubated in a continuously stirred water bath at 51°C. At intervals, 50 μl were withdrawn into tubes and chilled in an ice bath before assay. Unheated (zero time) aliquots held on ice, were assayed at the beginning and end of the assay period and showed identical activities during this interval of about 30–40 min. Each cuvette contained 0.99 ml of assay mix plus 10 μl of hemolysate, and the rate of increase of absorbance at 340 nm was measured. Zero time samples were run in triplicate, whereas heated samples were measured singly. Assays were carried out on a Gilford spectrophotometer Model 2400 (Gilford Instrument Laboratories, Oberlin, Ohio). It was established under the conditions used for G6PD that the increase in 340 nm absorbance was related entirely to G6PD activity and not to 6PGD. An international unit (IU) is the amount of each enzyme required to convert one μmole of NADP to NADPH per min.

RESULTS

Routine blood tests showed normal hemoglobin, hematocrit, reticulocyte fractions, white count, and differential in the progeria child and her parents. Similarly, starch and polyacrylamide gel electrophoresis of G6PD from erythrocytes showed normal banding patterns for wild type enzyme (type B). Electrophoretic patterns of 6PGD were also indistinguishable from normal controls. As in earlier reports [8, 11], the

heat-labile fraction of G6PD and 6PGD was elevated in fibroblasts from the patient as well as both parents; these data will be presented in detail elsewhere.

No significant differences were found between the family and controls in total enzyme activities. Young erythrocytes from all subjects contained higher G6PD activity than WEP: 2.67 ± 0.10 vs. 2.22 ± 0.11 (means ± SEM, IU/ml packed cells). Although this ratio of 1.20 is identical to some published values using a similar method of density gradient separation [14], it is somewhat lower than results obtained using exogenous media [16]. However, this criterion in conjunction with data on ^{59}Fe distribution in the gradient indicates that substantial enrichment for young cells was achieved. On the other hand, the ratio of 6PGD activity in young cells (1.23 ± 0.09 IU/ml packed cells) vs. WEP (1.27 ± 0.15) was close to unity. This difference in ratios between G6PD and 6PGD is not understood but may reflect the more rapid loss of 6PGD activity that occurs in relatively young cells in lighter gradient fractions [16].

Results of heat-lability studies on WEP are shown in figure 1. The child showed the highest heat-labile fraction of G6PD (fig. 1*A*) and 6PGD (fig. 1*B*) followed by values in the parents which were intermediate to those of their daughter and controls. Results from abnormal controls were indistinguishable from those of normal subjects, so the control data were combined. Similarly, no consistent differences in heat-lability were detected as a function of the chronologic age within controls. The second linear phase which represented the stable component of enzyme was not significantly different in the family and controls; when subjected to linear regression analysis, the slopes of these lines for all subjects combined gave a calculated mean half-life (± SD) of 32.54 ± 2.44 min for G6PD and 22.80 ± 0.92 min for 6PGD. In addition, equal part mixtures of hemolysates from progeria and normal WEP gave approximately intermediate fractions of heat-labile G6PD: proband, 18.70 ± 1.67%; control, 2.24 ± 1.48%; mix, 12.20 ± 1.02%. This finding suggests, although it does not prove, that progeric hemolysates did not contain proteolytic or other detrimental factors or were not lacking in essential stabilizing factors.

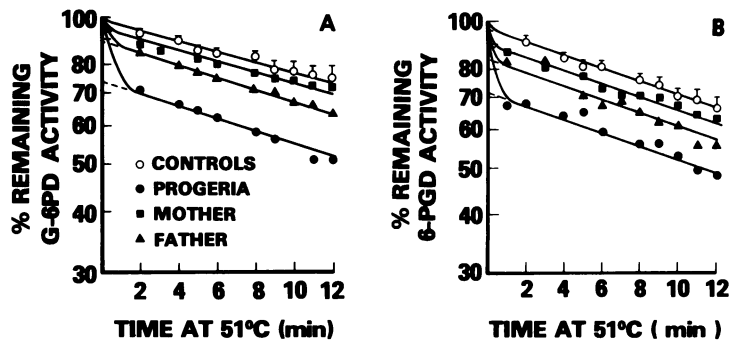

FIG. 1.—Heat-lability of (*A*) glucose-6-phosphate dehydrogenase and (*B*) 6-phosphogluconate dehydrogenase in hemolysates of whole erythrocyte populations. An initial rapid loss of activity followed by a linear exponential decay was always seen for both enzymes in the progeria family. The heat-labile fraction is estimated by extrapolating the linear portion of the curve back to zero time, determining the ordinate intercept and then subtracting this value from 100% [11]. Control values represent the mean ± SEM of experiments on nine separate blood samples from six subjects while other data points are single representative experiments in the progeria family.

The compiled data on heat-labile fractions are shown in figures 2*A* and 2*B*. In WEP, significantly increased values were seen for heat-lability of both enzymes in the progeric child with intermediate levels in her parents. Young erythrocytes of controls showed somewhat lower heat-labile fractions than WEP for both enzymes but corresponding values were still significantly elevated in the progeric child and again intermediate in both parents.

DISCUSSION

Two clear findings emerge from these studies. First, an increased fraction of heat-labile G6PD and 6PGD was detected in freshly obtained progeric blood cells. The presence of altered proteins in erythrocytes and cultured skin fibroblasts implies that the progeric defect affects genetic expression within cells both in vivo and in vitro. Second, the pattern of heat-labile enzyme fractions in this family was consistent with autosomal recessive inheritance. This is of immediate relevance in genetic counseling since the risk that future progeny might be affected would be one in four. In contrast, an etiology based on fresh dominant mutation [4] would carry an extremely low risk, perhaps as low as 10^{-6}.

Since several enzymes in the circulating erythrocyte show progressive deterioration of various physiochemical properties, it is possible that G6PD and 6PGD molecules of the progeric family are older due to slow erythrocyte turnover. However, two findings militate against this idea. First, all three members of the family had normal blood indices and reticulocyte fractions. Second, G6PD and 6PGD activities in young cells and WEP were similar in the family and controls; activity of several enzymes including G6PD and 6PGD provides an excellent index of erythrocyte age [14–16].

The possibility of simultaneous primary mutations in the structural genes for G6PD and 6PGD is also unlikely. While both WEP and young erythrocytes of the progeric family showed an increased fraction of heat-labile enzymes, the second phase of decay, which represents the normal majority of enzyme molecules, was indistinguishable from controls. In fact, inherited changes in the primary sequence of amino acids are usually associated with increased thermolability of various enzymes, including G6PD [8, 17], but in such instances, the entire population of enzyme molecules is labile rather than a subfraction. Thus, the present results, taken together with those in cultured fibroblasts [7–11], indicate that a unique defect exists in progeria cells.

The most likely common denominator for a multiplicity of altered proteins in this family is a single gene mutation affecting a critical step of macromolecular turnover [2, 8]. For example, a defective enzyme involved in protein synthesis could lead to the production of a mixture of normal and abnormal proteins; the latter proteins would be heterogeneous since inaccurate synthesis would create a random array of amino acid substitutions. Such a mechanism would also explain why our electrophoretic studies were negative since multiply affected proteins would be dispersed into many positions in the gel. Alternatively, a mechanism which produces diverse post-translational changes could lead to the same picture. It is also possible that a defect exists in proteolysis, a normal process whereby proteins that are synthesized incorrectly or otherwise modified with time, are selectively degraded [18]. Since synthesis and

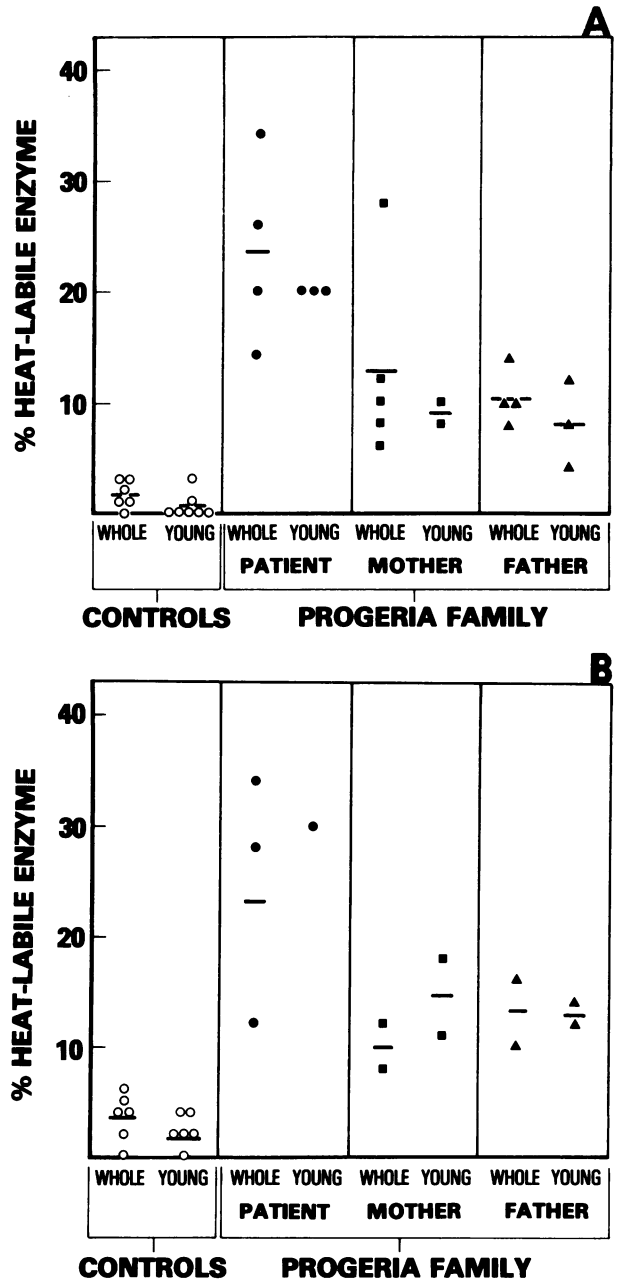

FIG. 2.—Percentage of heat-labile (A) glucose-6-phosphate dehydrogenase and (B) 6-phosphogluconate dehydrogenase in erythrocyte hemolysates. In each experiment, the heat-labile fraction was determined as in figure 1 on unfractionated whole erythrocyte populations or fractionated young cells. Each symbol within a column represents the result of individual experiments on blood samples drawn from different control subjects, or repeated blood samples drawn from the progeria family at intervals of 3–6 months. The significance of differences in mean values (horizontal bars) between controls and each member of the family are: *G6PD*: $P < .005$ for WEP, and $P < .001$ for young cells; *6PGD*: $P < .005$ for WEP, and $P < .001$ for young cells.

degradation are probably linked, defects could coexist such that progeria cells first generate then tolerate more defective proteins than normal cells.

There is also evidence that DNA repair is abnormal in progeria fibroblasts [19], but this is controversial [20, 21]. Such a defect could lead to a general increase in mutagenesis, as in fact occurs in repair-defective bacteria [22] and human fibroblasts [23], and thereby account for the widespread abnormality of genetic expression in progeric cells. However, enzymes for DNA repair may be affected as a consequence of abnormal protein turnover which now warrants careful investigation.

Although the results suggest that a diagnostic blood test is now available for progeria, it is already evident that an increased fraction of heat-labile enzymes will not be specific for this disorder. Similar results have now been found in Werner syndrome, a related but genetically distinct disorder, both in skin fibroblasts [8, 24, 25] and circulating erythrocytes (Goldstein and Moerman, in preparation). In fact, it is likely that heterogeneity exists within the clinical phenotype which characterizes progeria. Therefore, caution is needed before asserting that an increased fraction of heat-labile enzymes will become a specific diagnostic tool for progeria. In any case, this relatively simple test on blood and cultured cells should be useful in the early diagnosis of progeria, Werner syndrome, and other disorders of premature aging [2] after birth and in utero.

SUMMARY

Cultured skin fibroblasts from subjects with progeria contain an increased fraction of heat-labile enzymes and other altered proteins. To determine whether freshly obtained cells are similarly affected, erythrocytes from a progeric female and her clinically normal parents were analyzed for heat-lability of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Hemolysates of the child's whole erythrocyte populations and young erythrocytes isolated by equilibrium density centrifugation contained significantly higher heat-labile fractions of both enzymes compared to control hemolysates. Values in both parents were intermediate to those of their daughter and controls, consistent with autosomal recessive inheritance in this family. The primary source of these multiple protein defects is unknown but may reside in a mutant gene producing abnormal protein turnover or defective DNA repair. An increased fraction of thermolabile enzymes in circulating erythrocytes should be useful in identifying persons at risk for progeria and other disorders of premature aging.

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REFERENCES

1. DEBUSK FL: The Hutchinson-Gilford progeria syndrome. *J Pediatr* 80:697–724, 1972
2. GOLDSTEIN S: Human genetic disorders which feature accelerated aging, in *The Genetics of Aging*, edited by SCHNEIDER EL, New York, Plenum Press, 1978, in press

3. McKusick VA: *Mendelian Inheritance in Man. Catalogs of Autosomal Dominant. Autosomal Recessive and X-Linked Phenotypes*. Baltimore, Johns Hopkins Univ. Press, 1975, p 548
4. Jones KL, Smith DW, Harvey MA, Hall BD, Quan L: Older paternal age and fresh gene mutation: data on additional disorders. *J Pediatr* 86:84–88, 1975
5. Goldstein S: Lifespan of cultured cells in progeria. *Lancet* 1:424, 1969
6. Danes BS: Progeria: a cell culture study on aging. *J Clin Invest* 50:2000–2003, 1971
7. Goldstein S, Niewiarowski S, Singal DP: Pathological implications of cell aging in vitro. *Fed Proc* 34:56–63, 1975
8. Goldstein S, Moerman EJ: Defective proteins in normal and abnormal human fibroblasts during aging in vitro. *Interdiscip Top Gerontol* 10:24–43, 1976
9. Goldstein S, Niewiarowski S: Increased procoagulant activity in cultured fibroblasts from progeria and Werner's syndromes of premature aging. *Nature* 260:711–713, 1976
10. Rosenbloom AL, Goldstein S, Yip CC: Insulin binding to cultured human fibroblasts increases with normal and precocious aging. *Science* 193:412–415, 1976
11. Goldstein S, Moerman E: Heat-labile enzymes in skin fibroblasts from subjects with progeria. *N Engl J Med* 292:1305–1309, 1975
12. Murphy JR: Influence of temperature and method of centrifugation on the separation of erythrocytes. *J Lab Clin Med* 82:334–341, 1973
13. Cohen NS, Ekholm JE, Lithra MG, Hanahan DJ: Biochemical characterization of density-separated human erythrocytes. *Biochem Biophys Acta* 419:229–242, 1976
14. Marks PA, Johnson AB, Hirschberg E: Effect of age on the enzyme activity in erythrocytes. *Proc Natl Acad Sci USA* 44:529–536, 1958
15. Fornaini G, Leoncini G, Segni P, Calabria GA, Dacha M: Relationship between age and properties of human and rabbit erythrocyte glucose-6-phosphate dehydrogenase. *Eur J Biochem* 7:214–222, 1969
16. Turner BM, Fisher RA, Harris H: The age related loss of activity of four enzymes in the human erythrocyte. *Clin Chim Acta* 50:85–95, 1974
17. Beutler E: Glucose-6-phosphate dehydrogenase deficiency, in *The Metabolic Basis of Inherited Disease*, edited by Stanbury JB, Wyngaarden JB, Fredrickson DS, New York, McGraw-Hill, 1972, pp 1358–1388
18. Goldstein S, Stotland D, Cordeiro RAJ: Decreased proteolysis and increased amino acid efflux in aging human fibroblasts. *Mech Ageing Dev* 5:221–233, 1976
19. Epstein J, Williams JR, Little JB: Rate of DNA repair in progeric and normal human fibroblasts. *Biochem Biophys Res Commun* 59:850–857, 1974
20. Regan JB, Setlow RB: DNA repair in human progeroid cells. *Biochem Biophys Res Commun* 59:858–864, 1974
21. Rainbow AJ, Howes M: Decreased repair of gamma ray damaged DNA in progeria. *Biochem Biophys Res Commun* 74:714–719, 1977
22. Morse LA, Pauling C: Induction of error-prone repair as a consequence of DNA ligase deficiency in E. coli. *Proc Natl Acad Sci USA* 72:4645–4649, 1975
23. Maher VM, Ouellette LM, Curren RD, McCormick JJ: Frequency of ultraviolet light-induced mutations is higher in xeroderma pigmentosum variant cells than in normal human cells. *Nature* 261:593–595, 1976
24. Holliday R, Porterfield JS, Gibbs DD: Premature aging and occurence of altered enzyme in Werner's syndrome fibroblasts. *Nature* 248:762–763, 1974
25. Goldstein S, Moerman E: Heat-labile enzymes in Werner's syndrome fibroblasts. *Nature* 255:159, 1975