Cystathionine Synthase Activity in Human Lymphocytes: Induction by Phytohemagglutinin

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Research Article

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Cystathionine Synthase Activity in Human Lymphocytes: Induction by Phytohemagglutinin

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ABSTRACT Uncultured human leukocytes contain no detectable cystathionine synthase activity. A method is described in which the addition of phytohemagglutinin (PHA) to short-term lymphocyte cultures results in a significant induction of enzymatic activity. This PHA-stimulated activity has characteristics that resemble those previously described for cystathionine synthase of normal liver and cultured fibroblasts. Lymphocyte cystathionine synthase activity is completely dependent on the presence of homocysteine and is absent or severely deficient in extracts from individuals with the syndrome of homocystinuria. This system for induction of cystathionine synthase in lymphocytes thus provides a simple in vitro technique for (*a*) diagnosing homocystinuria, (*b*) studying the mechanism of enzyme regulation and differentiation, and (*c*) examining the nutritional and hormonal control of cystathionine synthase activity both in normal subjects and homocystinuric patients.

INTRODUCTION

Cystathionine synthase (E. C. 4.2.1.21) is the enzyme in the mammalian transsulfuration pathway which catalyzes the condensation of homocysteine and serine to cystathionine, this being an intermediate step in the conversion of methionine to cysteine (1). A deficiency of this enzyme results in the clinical syndrome of homocystinuria (2), a disorder characterized genetically by autosomal recessive inheritance (3), chemically by abnormal elevations of homocystine and methionine in plasma and urine (4), and clinically by mental retardation, dislocation of the lens, thromboembolic episodes, and osteoporosis (3, 4). Studies of cystathionine synthase have been limited by the need to work with postmortem material or liver biopsy specimens since previous attempts to detect enzyme activity in human blood leukocytes, platelets, and red blood cells have been unsuccessful (2, 5). Uhlendorf and Mudd reported cystathionine synthase activity in cultured fibroblasts even though no activity was present in the original skin biopsy (6). Their finding suggested to us that cystathionine synthase activity might be induced in short-term lymphocyte cultures by the addition of the mitogenic agent phytohemagglutinin (PHA),[1] and this report describes our studies with this system.

METHODS

Establishment of short-term lymphocyte cultures. Each lymphocyte culture was initiated from either 10 or 20 ml of peripheral blood. The blood was drawn into a heparinized (40 USP U heparin/ml blood) plastic syringe and allowed to sediment at 37°C for 90 min. The plasma leukocyte suspension (2–4 ml) was then inoculated into a sterile culture bottle and brought to a final volume of 24.5 ml with minimal Eagle's media containing 10% fetal calf serum[2] (North American Biological Inc., North Miami, Fla.), 50 mM (*N*-tris[hydroxymethyl]methylglycine, pH 7.4, (Sigma Chemical Co., St. Louis, Mo.), penicillin (100 μg/ml), streptomycin (100 μg/ml), and 0.5 ml of 1:50 dilution of Phytohemagglutinin P (Difco Laboratories, Detroit, Mich.). Each culture was incubated at 37°C and left undisturbed until the time of preparation of the lymphocyte extract,

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[1] *Abbreviation used in this paper:* PHA, phytohemagglutinin.

[2] Fetal calf serum contained no detectable activity of cystathionine synthase.

which was at 144 hr for all experiments except as indicated in Fig. 2.

Preparation of lymphocyte extract. The cells from each culture were collected by centrifugation at 1000 rpm at 4°C for 10 min, washed once with 1 ml of cold normal saline, and the final pellet suspended in 0.1 ml of buffer containing Tris-HCl (pH 8.3), 30 mM; and pyridoxal phosphate, 1.3 mM. A lymphocyte extract was then prepared from this suspension by freezing and thawing the cells three times, followed by centrifugation in a Beckman Microfuge (model 205 Beckman Instruments, Inc., Fullerton, Calif.) at 12,000 rpm at 4°C for 5 min. The supernatant fraction was then used for all enzyme assays.

Determination of cystathionine synthase activity. The incubation reaction was modified after that described by Mudd, Finkelstein, Irreverre, and Laster (1) and contained in a final volume of 0.1 ml: 5–50 μl lymphocyte extract, 0.05–0.6 mg protein; Tris-HCl (pH 8.3), 150 mM; EDTA, 2.5 mM; pyridoxal phosphate, 0.65 mM; cystathionine, 0.17 mM; homocysteine, 10 mM; and serine-3-^{14}C (Schwarz Bio Research, Inc., Orangeburg, N. Y.), 6.6 nmoles containing 300–330 × 10^3 cpm. Each reaction was incubated at 37°C for 4 hr except as indicated in Fig. 3.

Thin-layer chromatography for separating cystathionine and serine. In order to separate the radioactive cystathione formed in the reaction from the radioactive serine added as substrate, an ascending thin-layer chromatographic system was developed. After incubation, a 50 μl portion of the reaction mixture was combined with a carrier mixture of cystathionine and serine. The sample was then spotted across one-third of a cellulose thin-layer chromatogram 20 × 20 cm (J. T. Baker Chemical Co., Phillipsburg, N. J.) and developed at room temperature overnight in solvent containing isopropanol: formic acid: water (80:6:20). The chromatogram was air-dried and divided into consecutive areas (each 1 cm in height) from the origin to the front. Each of these areas was then scraped into separate counting

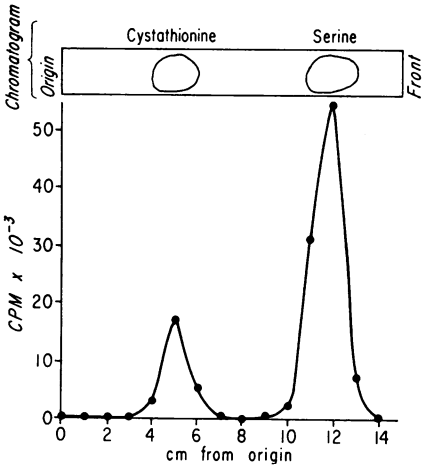

FIGURE 1 Thin-layer chromatography for separation of cystathionine and serine. The details of this procedure are described in Methods. The marker spots for cystathionine and serine were identified by spraying with ninhydrin. In most chromatograms cystathionine and serine were the only peaks of radioactivity identified. However, in about 20% of normal cultures, a small peak of unidentified radioactivity (< 0.5% total cpm) was observed at 8 cm from the origin.

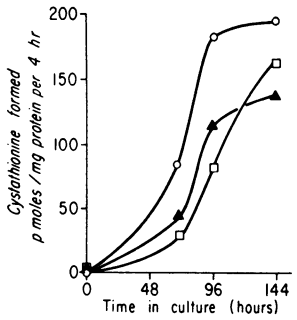

FIGURE 2 Induction of cystathionine synthase by PHA in three normal lymphocyte cultures. Each culture was initiated from 20 ml peripheral blood obtained from three different normal individuals. At 0, 72, 96, and 144 hr, 6-ml portions were removed from each culture (total volume of starting culture, 24.5 ml) and the lymphocyte extract that was prepared from each portion was assayed for cystathionine synthase and protein. Enzyme activity on the ordinate is expressed as the amount of cystathionine formed in picomoles/milligram protein in each portion per 4 hr.

vials to which 5 ml of 0.4% solution of 2,5-diphenyloxazole (PPO) in toluene were added. The samples were assessed for radioactivity in a liquid scintillation counter in which the efficiency for ^{14}C averaged 50–60%. As shown in Fig. 1, this chromatographic system allows a clear-cut separation of cystathionine and serine. The amount of cystathionine formed was quantitated by summing the total radioactivity under the cystathionine peak and subtracting from this value the lowest value of radioactivity observed between the peaks of cystathionine and serine. This blank value averaged about 300 cpm, or 6 pmoles, and was equivalent to values of radioactivity obtained under the cystathionine spot when reactions were carried out using either a boiled lymphocyte extract or without the addition of homocysteine.

Protein concentration of the lymphocyte extracts was determined by the method of Lowry, Rosebrough, Farr, and Randall (7).

RESULTS

The cystathionine synthase assay developed for these studies can detect the formation of as little as 5 pmoles of cystathionine, a value twice that for background radioactivity. This assay is, therefore, about 500 times more sensitive than that previously reported (1). With this degree of sensitivity, no activity of cystathionine synthase was detectable in extracts of uncultured human leukocytes, and only a trace of activity was measured when lymphocytes were cultured 144 hr in the absence of a mitogenic agent (Table I, Experiment A). The addition of phytohemagglutinin (PHA) to the lymphocyte cultures resulted in a significant induction in enzyme activity (45-fold increase) (Table I, Experiment A). The time-course of the PHA-stimulated rise in cystathionine synthase activity is shown in Fig. 2. Cystathionine synthase activity was first detectable at 72 hr after culture and rose rapidly over the subsequent 72 hr. Since maximal activity in the short-term culture of normal

individuals occurred at 144 hr, this time point was used in all further studies. This induction of cystathionine synthase in normal lymphocytes was completely abolished by the addition to the culture at 0 time of either actinomycin D (25 μg/ml) or cycloheximide (50 μg/ml), suggesting that this phenomenon required both RNA and protein synthesis.

The PHA-stimulated cystathionine synthase activity was completely dependent on the addition of homocysteine and was destroyed when a boiled extract was used as a source of enzyme (Table I, Experiment B). The omission of pyridoxal phosphate from the incubation reaction did not greatly alter the activity of a crude lymphocyte extract (Table I, Experiment A). The values for enzyme activity, under the conditions of assay, were not maximal, since the usual serine concentration was not saturating (Table I, Experiment C). The reaction rate was constant for as long as 6 hr and was proportional to protein concentration up to 0.6 mg (Fig. 3).

TABLE I

Factors Influencing Cystathionine Synthase Activity in Extracts of Human Lymphocytes

Conditions	Cystathionine formed
	pmoles/4 hr
Experiment A	
Complete system, 0.37 mg protein	271
Extract changed to uncultured leukocytes, 0.98 mg protein	0
Extract changed to lymphocytes cultured in absence of PHA, 0.50 mg protein	6.4
Experiment B, 0.29 mg protein	
Complete system	227
Without homocysteine	0
Without pyridoxal phosphate	200
Boiled extract	0
Experiment C, 0.25 mg protein	
Complete system (Serine, 6.6×10^{-5} M)	200
Serine changed to 8.0×10^{-6} M	33
Serine changed to 1.1×10^{-4} M	330
Serine changed to 2.6×10^{-4} M	590

The complete system in each Experiment consisted of a reaction mixture incubated for 4 hr at 37°C containing in 0.1 ml: lymphocyte extract (protein concentration indicated) prepared from a 144 hr culture and additional components as described in methods. In Experiment A, 30 ml of peripheral blood from a normal individual was divided into three equal parts, one portion used as a source of the uncultured leukocyte extract, another portion placed in culture with PHA, and the third portion placed in culture without PHA. The lymphocyte extracts used in Experiments B and C were obtained from two different cultures, each initiated from 20 ml of peripheral blood of a normal individual.

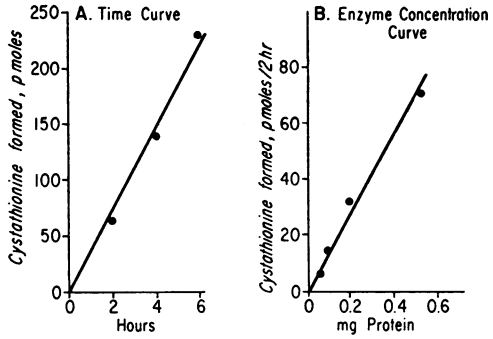

FIGURE 3. *A.* Rate of formation of cystathionine by lymphocyte extract. Each reaction was incubated for the indicated time at 37°C and contained in 0.1 ml: lymphocyte extract (0.30 mg protein) and additional components described in Methods. The lymphocyte extract was prepared from a 144 hr culture that was initiated from 10 ml of peripheral blood obtained from a normal individual. *B.* Relation between the concentration of lymphocyte extract and the rate of formation of cystathionine. Each reaction was incubated for 2 hr at 37°C and contained in 0.1 ml: lymphocyte extract (protein concentration as indicated) and additional components described in Methods. The lymphocyte extract was prepared from a 144 hr culture initiated from 10 ml of peripheral blood obtained from a normal individual.

A comparison of cystathionine synthase activity of three unrelated patients with homocystinuria (each proved by classic clinical manifestations and by demonstration of elevated levels of homocystine and methionine in plasma and urine)[3] and 23 normal subjects (consisting of 10 males and 13 females, ranging in age from 15 to 55) is presented in Table II. Although the range of activity for normal subjects in lymphocytes is wide (90.8–888.6 pmoles cystathionine formed/mg protein per 4 hr), there is no overlap with the values of the three homozygotes with homocystinuria. Two of these homocystinurics (J. W., 20 yr old male, nonresponder to pyridoxine, and R. S., 20 yr old male, responder to pyridoxine) had no detectable activity, while the other patient (L. G., 15 yr old female, pyridoxine responder) consistently showed 1-2% normal activity. None of these homocystinurics were taking pyridoxine at the time of these enzyme studies.

DISCUSSION

A simple method is described for the induction of cystathionine synthase activity by PHA in short-term cultures of human lymphocytes. The induced enzyme has characteristics similar to that previously described for cystathionine synthase from human liver and cultured fibroblasts (1, 6). Enzyme activity is completely dependent on the presence of homocysteine, it is destroyed by boiling an extract of normal human lymphocytes, and

[3] Scott, C. R. 1971. Personal communication.

TABLE II

Cystathionine Synthase Activity in PHA-Stimulated Lymphocytes of Normals and Homocystinurics

Phenotype	Cystathionine formed
	pmoles/mg protein per 4 hr
Normals (23)	406.4 (range 90.8–888.6)
Homocystinurics (3)	
J. W.	0, 0
L. G.	3.6, 4.4, 6.0
R. S.	0, 0

Each reaction was incubated for 4 hr at 37°C and contained in 0.1 ml: lymphocyte extract (protein concentration varied between 0.35 and 0.62 mg) and additional components described in Methods. Each lymphocyte extract was prepared from a 144 hr culture that was initiated from 10 ml of peripheral blood. The number in parentheses refers to the number of individuals studied in each phenotypic category. Addition of the extract prepared from cultured cells of R. S. did not inhibit the cystathionine synthase activity of an extract prepared from a normal culture.

it is either absent or severely deficient in the extracts of cultured lymphocytes from patients with the syndrome of homocystinuria.

In general, mammalian cells in culture tend to either retain or lose the enzyme activities and other specialized functions of their in vivo tissue of origin (8). Very few mammalian cells have been reported to acquire new properties when cultured in vitro. Yet, the studies reported here provide evidence for the apparent acquisition of an enzyme activity by the lymphocytes during short-term culture. Uhlendorf and Mudd observed a similar phenomenon for this same enzyme when they reported that the activity of cystathionine synthase was not demonstrable in human skin but was easily detectable in fibroblasts cultivated from the skin (6). An understanding of the molecular basis of this unique aspect of the regulation of cystathionine synthase activity in cultures of lymphocytes and fibroblasts would surely advance the understanding of the mechanisms controlling mammalian differentiation.

The finding that patients with the syndrome of homocystinuria show absent or deficient induction of cystathionine synthase activity by PHA in short-term lymphocyte cultures indicates that this system can be used for tissue diagnosis of homozygotes with this disorder. Whether heterozygotes can be reliably identified by this method is currently under investigation in our laboratory.

This method may also be useful in studying the hormonal and nutritional control of cystathionine synthase in both normal subjects and mutants with homocystinuria. Of particular interest is the pyridoxine response that occurs in some, but not all homocystinuric patients given large doses of vitamin B_6 (9). On the basis of a limited number of enzyme studies in liver biopsies of two pyridoxine responders, Mudd has suggested that the observed pyridoxine-induced reductions in urinary and plasma concentrations of homocystine can be accounted for by a small enhancement (1–3%) of residual cystathionine synthase activity in the homocystinuric liver (9). Since repeated samples of small amounts of peripheral blood are more readily available than repeated liver biopsies, the lymphocyte culture technique for studying cystathionine synthase may provide a system for the further study of the mechanism of this pyridoxine response.

ACKNOWLEDGMENT

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REFERENCES

1. Mudd, H. S., J. D. Finklestein, F. Irreverre, and L. L. Laster. 1965. Transsulfuration in mammals. *J. Biol. Chem.* **240**: 4382.
2. Mudd, H. S., J. D. Finklestein, F. Irreverre, and L. L. Laster. 1964. Homocystinuria: an enzymatic defect. *Science (Washington)*. **143**: 1443.
3. Schimke, R. N., V. A. McKusick, and R. G. Weilbaecher. 1967. Homocystinuria. *In* Amino Acid Metabolism and Genetic Variation. W. L. Nyhan, editor. McGraw-Hill Book Company, Inc., New York. 297.
4. Laster, L., G. L. Spaeth, S. H. Mudd, and J. D. Finklestein. 1965. Homocystinuria due to cystathionine synthase deficiency. *Ann. Intern. Med.* **63**: 1117.
5. Manowitz, P., J. Racevskis, and D. G. Gilmour. 1970. A modified assay for cystathionine synthase. *Clin. Chim. Acta.* **28**: 269.
6. Uhlendorf, B. W., and S. H. Mudd. 1968. Cystathionine synthase in tissue culture derived from human skin: enzyme defect in homocytinuria. *Science (Washington)*. **160**: 1007.
7. Lowry, C. H., N. J. Rosebrough, A. C. Farr, and R. J. Randall. 1951. Protein measurement with the phenol reagent. *J. Biol. Chem.* **193**: 265.
8. Krooth, R. S., G. A. Darlington, and A. A. Velasquez. 1968. The genetics of cultured mammalian cells. *Annu. Rev. Genet.* **2**: 141.
9. Mudd, S. H., W. A. Edwards, P. M. Loeb, M. S. Brown, and L. Laster. 1970. Homocystinuria due to cystathionine synthase deficiency: the effect of pyridoxine. *J. Clin. Invest.* **49**: 1762.