N^5-METHYLTETRAHYDROFOLATE: HOMOCYSTEINE METHYLTRANSFERASE ACTIVITY IN EXTRACTS FROM NORMAL, MALIGNANT AND EMBRYONIC TISSUE CULTURE CELLS

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Received January 28,1974

SUMMARY

Malignant cells (J111, L1210, W-256) and human embryonic cells (FL) are unable to survive and grow when homocystine replaces methionine in tissue culture media containing excess vitamin B_{12} and folic acid. Extracts of these same cells when grown in media containing methionine and more than adequate vitamin B_{12} and folic acid have diminished N^5-methyltetrahydrofolate: homocysteine methyltransferase activities in the absence of added cyanocobalamin when compared with extracts of normal cells (adult rat thymus and liver fibroblasts). Extracts of human monocytic leukemia (J111) and human amnion cells (FL) have normal enzymatic activity in the presence of added cyanocobalamin whereas the rodent malignant cells (W-256 and L1210) have abnormally low activity in the absence or presence of added vitamin B_{12}.

The enzyme, N^5-methyltetrahydrofolate: homocysteine methyltransferase, catalyzes the final methyl transfer reaction converting homocysteine to methionine. This activity enables whole animals and tissue culture cells to grow on nutrients devoid of methionine but containing homocystine, folic acid and vitamin B_{12} (1,4,5,6). This enzyme has been found in all tissues of the rat except possibly the small intestine (2), in all adult human tissues surveyed (3), in normal adult mammalian tissue culture cells (4,5,6) and in certain malignant tissue culture cells (1,8,9).

Recently, we reported that three tissue culture cell lines with concurrently demonstrable oncogenicity - the Walker-256 breast carcinosarcoma of the rat (W-256), an acute lymphatic leukemia of the mouse (L1210) and a monocytic leukemia of man (J111) - can neither grow nor survive in media devoid of methionine but containing non-limiting concentrations of DL-homocystine (0.2 mM) and an excess of both vitamin B_{12} (2 mg/L) and folic

acid (10 mg/L). Normal adult rat and human fibroblasts and rat liver epithelial cells thrive under these same conditions (10).

We now report the measurement of N^5-methyltetrahydrofolate: homocysteine methyltransferase activity in extracts from these same three malignant cell lines as well as in extracts from normal adult rat fibroblasts (L 3-16), normal adult rat thymus fibroblasts and human amniotic cells (FL), all grown in medium containing methionine (0.1 mM), vitamin B_{12} (2 mg/L) and folic acid (10 mg/L). The human amniotic cells are also shown to be incapable of growing when homocystine replaces methionine in the growth medium.

MATERIALS AND METHODS

Tissue cultures of human amniotic cells (FL) (concurrently PPLO negative) were grown in a commercially available specifically modified McCoy's medium. The composition of the medium was routinely checked by amino acid analysis before use. It was devoid of bactopeptone but reinforced with 10% fetal calf serum previously dialyzed 24 hrs at 4°C against three changes of a solution containing 8 g NaCl, 0.4 g KCl, 1 g glucose and 0.35 g $NaHCO_3$ per L. In addition, the medium contained trace metals (10 ml per L of medium of a solution containing $FeSO_4$, 1 mg; $CuCl_2$, 100 µg; $ZnSO_4$, 100 µg per L), vitamin B_{12}, 2 mg/L; folic acid, 10 mg/L, and gentamycin (50 µg/ml). Except for subtractions or additions of methionine and homocystine, there were no other alterations in the medium. The two growth curves were determined simultaneously. One medium contained 0.1 mM L-methionine while the other was devoid of methionine, but instead contained 0.2 mM DL-homocystine. Total protein determinations (13) were utilized for growth rate measurements. Both cell cultures were continued for 7 days and the media were replenished on day 4.

Enzyme preparation: W 12-4 (W-256 carcinosarcoma), L1210, J111, adult rat thymus fibroblasts, L 3-16 (adult rat liver fibroblasts) and FL (human amniotic cells) were grown in McCoy's medium in 75 cm^2 Falcon flasks or in Belco roller bottles. The cells were harvested during the log phase of growth. The attached cells were removed by the use of trypsin and EDTA.

The trypsin subsequently was inactivated by the addition of serum. The cells were centrifuged at 3000 xg for 5 minutes at 4°C. The supernatant was discarded and the cell pellet was resuspended in 10 ml of phosphate buffered saline, pH 7.4. The suspension was centrifuged as above, and the cells were resuspended in 1.6 ml of 0.05 M potassium phosphate buffer, pH 7.2, containing 0.5 mM reduced glutathione, then frozen and lyophilized to break the cells. The lyophilized preparations were resuspended in 0.8 ml of cold distilled H_2O and centrifuged at 50,000 xg for 60 minutes at 4°C. The clear supernatant fractions were removed from the preciptated cell debris. 0.1 ml of this supernatant was used as the enzyme source, and the remainder of each enzyme preparation was frozen and stored in 0.2 ml lots. The protein concentration of each extract was measured by Lowry's method (11).

Assay: The reaction mixture in 0.2 ml final volume contained the following: sodium phosphate buffer, pH 7.4, 100 mM; 2 mercaptoethanol, 125 mM; S-adenosyl methionine, 250 μM; DL-homocysteine, 250 μM (freshly prepared from DL-homocysteine thiolactone); N^5-^{14}C-methyltetrahydrofolate, 600 μM with a specific activity of 650 cpm/nmole; with and without cyanocobalamin, 50 μM and 100 μl of enzyme. The reaction mixture was incubated under nitrogen at 37°C for 60 minutes. The reaction was stopped by the addition of 0.6 ml of isopropanol. After 30 minutes at 4°C, the protein precipitate was removed by centrifugation and the supernatant (isopropanol extract) was recovered for the determination of methionine formation.

The ^{14}C-CH_3-labeled methionine synthesized was measured using our cyanogen bromide assay (11) which we have found to be more specific than the Dowex-1-Cl column technique but comparable and more rapid than chromatographic methods (3,12).

To minimize exposure to cyanogen bromide, all the following operations were carried out in a well ventilated fume hood. 0.1 ml of supernatant (isopropanol extract) was transferred to a 12 ml screw-top centrifuge tube and 0.25 ml of freshly-prepared 5% cyanogen bromide in 0.1 N acetic acid was

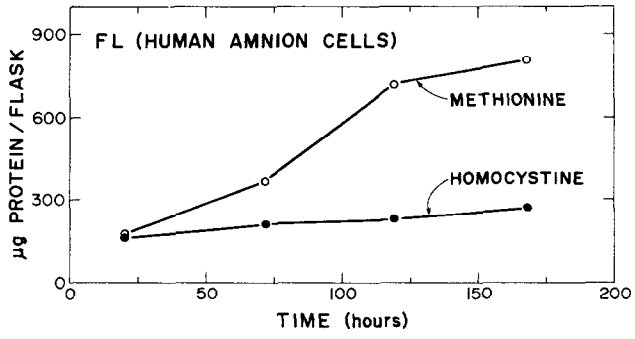

Figure 1. Growth of FL (human amnion cells) in culture. Human amnion cells were grown in two modified McCoy's media, each containing folic acid (10 mg/L), vitamin B_{12} (2 mg/L) and 10% dialyzed fetal calf serum. One medium contained L methionine (15 mg/L); the other medium contained DL homocystine (30 mg/L) and no methionine.

added. After the tubes were tightly capped with screw tops containing teflon-coated pressure linings, they were first heated for 5 minutes in a boiling water bath followed by 2 minutes in a water-ice bath and then allowed to come to room temperature. 1.0 ml toluene was added; the tubes were recapped, shaken vigorously and centrifuged. 0.5 ml of the toluene layer was transferred to a scintillation vial containing 10 ml of toluene-based scintillation fluid and counted in a Beckman LS-100 scintillation counter. A control of the assay procedure was carried out in an identical manner on aliquots of the supernatant (isopropanol extract) in which the heating step was carried out in 0.1 N acetic acid to which no cyanogen bromide was added.

RESULTS

The striking impairment of growth of certain malignant cells as compared with normal cells when either homocystine or homocysteine was substituted for methionine in a medium rich in folic acid and cyanocobalamin, has already been reported (10). When human amniotic cells were tested in this system, they also failed to grow when homocystine replaced methionine in the folic acid- and cyanocobalamin-rich medium. The results are presented in Figure 1. Table 1 shows the specific activity of N^5-methyltetrahydrofolate: homocysteine methyltransferase in extracts of normal, malignant and embryonic cells. Extracts of the two normal adult rat cells studied (liver and thymus

TABLE 1

Measurement of N^5-Methyltetrahydrofolate-homocysteine Methyltransferase Activity in Extracts of Cells Grown in Tissue Culture Containing Methionine, Folic Acid and B_{12}

Cell Type	Ability to replace methionine in tissue culture*	Protein concentration of Extracts mg/ml	nmoles methionine formed per mg/protein per hour	
Normal Adult Rat Cells			$+B_{12}$**	$-B_{12}$
L3-16	yes	5.1	32	23
Thymus Fibroblasts	yes	2.2	34	26
Malignant Cells				
L1210	no	2.6	1.5	0.6
W256 (W12-4)	no	1.4	7.7	3.3
J111	no	4.0	27	7.8
Embryonic Cells				
Human Amniotic	no	5.0	38	8.6

*with homocysteine, folic acid, and vitamin B_{12}.

**cyanocobalamin

The reaction mixtures contained 20 μmoles sodium phosphate buffer pH 7.0, 50 nmoles S-adenosylmethionine, 50 nmoles DL homocysteine, 120 nmoles DL [methyl - ^{14}C]5-methyltetrahydrofolate (sp. act. 650 cpm per nanomole), 25 μmoles mercaptoethanol, 50 nmoles cyanocobalamin) and 0.1 ml of appropriate cell supernatant in a final volume of 0.2 ml. Any deletions or additions are indicated in the tables. The mixtures were incubated and assayed as described in the text (Methods).

fibroblasts) demonstrate high specific activities of N^5-methyltetrahydrofolate: homocysteine methyltransferase in the presence and absence of added cyanocobalamin (32 and 34 units respectively when vitamin B_{12} was added). In the absence of added vitamin B_{12} the specific activities were 23 and 26 units/mg protein. Thus 72% and 78% of the enzyme as measured in the absence of added cyanocobalamin exists in the holoenzyme form in these cell extracts. Measurements of enzymatic activity in the absence of added cyanocobalamin have been taken as a measure of holoenzyme content (15). Extracts of the rodent cancers, W 12-4 and L1210 have specific activities below 8 units/mg protein (7.7 and 1.5 respectively) in the presence of exogenous cyanocobalamin and below 4 units/mg protein (3.3 and 0.6 respectively) in the absence of added vitamin B_{12}. Thus only 40% of their respective enzymes exist in the holoenzyme form; the remainder is present as apoenzyme. Extracts of the human monocytic leukemia cell, J111, differ from the two rodent malignant cell extracts in that when the assay is carried out with added vitamin B_{12}, the specific activity of the enzyme is in the normal tissue range (27 units/mg protein). However in the absence of added vitamin B_{12}, J111 extracts show only 28% of their measurable total enzyme activity as the holoenzyme. Similarly, human amnion cell extracts have high enzyme specific activity (38 units/mg protein) when measured with added cyanocobalamin and like the human monocytic leukemia cell extracts, the assay without added cyanocobalamin reveals a relatively low specific activity (22% holoenzyme).

The normal cell extracts have a higher percentage (about 75%) of their total enzyme in the holoenzyme form whereas all the malignant cell and embryonic cell extracts have less than 45% of their total enzyme in this form. In addition none of the malignant or embryonic cell extracts have holoenzyme activity above 9 units/mg of protein whereas the normal cell extracts have holoenzyme activities above 22 units/mg protein.

It has been suggested (16) that during the course of the enzymatic reaction, the methyl group of N^5-methyltetrahydrofolate can be transferred

to 2-mercaptoethanol but it has been also stated that the formation of S-^{14}C-methylmercaptoethanol is negligible in the presence of 0.25 mM homocysteine (17). Although our reaction mixtures contained β-mercaptoethanol, no significant radioactivity was recovered in the toluene layer (in which S-methylmercaptoethanol is soluble) when aliquots were carried through the assay procedure in the absence of cyanogen bromide.

DISCUSSION

All cells were grown in a medium which had an excess concentration of cyanocobalamin and folic acid and an optimal concentration of methionine. Since all the cells grew very rapidly, their need for vitamin B_{12}, folic acid and methionine as well as the other constituents was amply satisfied.

The two normal adult cell lines were able to thrive in a medium devoid of methionine but supplied with 0.2 mM DL-homocystine or 0.1 mM L-homocysteine, vitamin B_{12} (2 mg/L) and folic acid (10 mg/L). None of the malignant cells, L1210, W-256, J111 nor the human amnion cells were able to survive and grow under the same conditions (10).

Our prior study and the present work show that certain malignant and embryonic cells in tissue culture are unable to utilize homocystine in place of methionine. The diminished activity of N^5-methyltetrahydrofolate: homocysteine methyltransferase in these malignant and embryonic cell extracts (measured without added cyanocobalamin) suggests a possible explanation of their inability to grow when homocystine replaces methionine in the medium.

This defect may be similar to one of the already documented inborn errors of metabolism characterized by homocystinuria (4,15,18). Our evidence in two cell types suggests that there is a failure to transport vitamin B_{12} into the cell. This impaired transport is not due to a deficiency of serum B_{12} transport proteins since: (A) normal cells grew well in media containing this same serum when homocystine was substituted for methionine; (B) extracts of these normal cells contained most of their enzyme in the holoenzyme form. Since the addition of cyanocobalamin to these extracts increased the

enzymatic activity there was no apparent defect in the conversion of cyanocobalamin to active coenzyme. However even though the J111 and human amnion cells were also bathed in a medium rich in cyanocobalamin their extracts demonstrated a reduced holoenzyme content. Nonetheless, these same extracts contained ample total enzyme (apoenzyme + holoenzyme) since full activity was restored upon the addition of cyanocobalamin to the enzyme assay mixture. The L1210 and W-256 cells may have any one of several defects since their extracts demonstrate very little holoenzyme activity in the enzyme assay and a limited capacity to form holoenzyme on the addition of cyanocobalamin.

ACKNOWLEDGMENT

This work was supported by the Andrew Norman Foundation, the Julius and Dorothy Fried Foundation and USPHS Grants 5-T01-GM 00463-13 and CA 13196.

REFERENCES

1. Bennet, M.A., J. Biol. Chem. 187, 751 (1950).

2. Finkelstein, J.D., Kyle, W.E. and B.J. Harris, Arch. Biochem. Biophys. 146, 84 (1971).

3. Mudd, S.H., Uhlendorf, B.W., Hinds, K.R. and H.L. Levy, Biochem. Med. 4, 193 (1970).

4. Mudd, S.H., Levy, H.L. and R.H. Abeles, Biochem. Biophys. Res. Commun. 35, 121 (1969).

5. Mudd, S.H., Levy, H.L. and G. Morrow, Biochem. Med. 4, 215 (1970).

6. Kamely, D., Littlefield, J.W. and R.W. Erbe, Proc. Nat. Acad. Sci., U.S.A. 70, 2585 (1973).

7. Mangum, J.H. and J.A. North, Biochem. Biophys. Res. Commun. 32, 105 (1968).

8. Mangum, J.H., Murray, B.K. and J.M. North, Biochemistry 8, 3986 (1969).

9. Kerwar, S.S., Spears, C., McAnslaw, B. and H. Weissbach, Arch. Biochem. Biophys. 142, 231 (1971).

10. Halpern, B., Clark, B.R., Halpern, R.M. and R.A. Smith, Proc. Nat. Acad. Sci, U.S.A. (in press).

11. Clark, B.R., Ashe, H., Halpern, R.M. and R.A. Smith, Anal. Biochem. (in press).

12. Weissbach, H., Peterkofsky, A., Redfield, B.G. and H. Dickerman, J. Biol. Chem. 238, 3318 (1963).

13. Oyama, V.I. and H. Eagle, Proc. Soc. Exp. Biol. Med. 91, 305 (1956).

14. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and R.J. Randall, J. Biol. Chem. 193, 265 (1951).

15. Mahoney, M.J., Rosenberg, L.E., Mudd, S.H. and B.W. Uhlendorf, Biochem. Biophys. Res. Commun. 44, 375 (1971).

16. Elford, H.L., Karzen, H.M., Rosenthal, S., Smith, L.C. and J.M. Buchanan in Transmethylation and Methionine Biosynthesis (S.K. Shapiro and F. Schlenk, eds.), University of Chicago Press, Chicago, Illinois, 1965, pg. 157.

17. Taylor, R.T. and H. Weissbach, J. Biol. Chem. 242, 1502 (1967).

18. Mudd, S.H., Uhlendorf, B.W., Freeman, J.M., Finkelstein, J.D. and V.E. Shih, Biochem. Biophys. Res. Commun. 46, 905 (1972).

19. Mudd, S.H., Uhlendorf, B.W. and K.R. Hinds, Biochem. Med. 4, 215 (1970).