Vol. 102, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 16, 1981 Pages 215-218

THE EFFECT OF NITROUS OXIDE-INDUCED INACTIVATION OF VITAMIN B_{12} ON THYMIDYLATE SYNTHETASE ACTIVITY OF RAT BONE MARROW CELLS.

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Received July 28,1981

SUMMARY

Exposure to nitrous oxide (N_2O) *in vivo* is accompanied by oxidation of cob[I]alamin to the inactive cob[III]alamin [1] and to loss of methionine synthetase activity [2]. There is a steady increase in thymidylate synthetase activity in marrow collected from rats exposed to N_2O and this returns to normal on restoring the animals to an air environment.

There is impaired synthesis of deoxythymidine in patients with vitamin B_{12} deficiency and this is assessed in the deoxyuridine suppression test which is invariably abnormal in these disorders [3,4]. However, there is some uncertainty about the activity of the enzyme, thymidylate synthetase, measured directly in marrow cells from such patients. Sakamoto et al. [5] reported that the activity of thymidylate synthetase in marrow cells from patients with untreated pernicious anemia was nine-fold greater than in marrows from normal subjects. Haurani [6] used peripheral blood lymphocytes stimulated by the addition of phytohemagglutinin. With lymphocytes from patients with vitamin B_{12} deficiency he found little or no thymidylate synthetase activity, although the enzyme activity was normal in treated patients.

These studies were planned to explore the effect of inactivation of vitamin B_{12} by N_2O [1,2] on the activity of thymidylate synthetase in rats.

MATERIALS AND METHODS

Animals: Male, Sprague-Dawley, 80-100 g rats were placed in a chamber in which a mixture of N_2O (50%)/oxygen (50%) was passed and CO_2 and humidity controlled. Animals were killed by exsanguination preceded by an

0006-291X/81/170215-04$01.00/0

injection of sodium pentobarbitone, long bones removed, split with a scalpel and marrow washed into 5 ml cold phosphate buffered saline containing 0.5 ml preservative-free heparin (1000 i.u.). Control animals were left in air. A final group was returned to air for 3 days after exposure to N_2O/O_2 for 5 days.

Marrow cells: The marrow was passed through a 21 gauge needle to break up clumps. The red blood cells were lysed with cold distilled water and isotonicity restored with an equal volume of 1.8% saline. The suspension was centrifuged, supernatant discarded and cells resuspended in ice-cold 0.05M potassium phosphate buffer pH 7.4. Cells were ruptured by homogenization in a small tissue grinder followed by rapid freezing and thawing. Homogenates were centrifuged at 3000 g for 45 m at 4°C and the supernatant used in the assay.

Thymidylate synthetase assay: The method was that described by Kammen [7] measuring the release of tritium from deoxy[5-^{3}H]uridine 5'-monophosphate. Each assay contained Tris/HCl buffer pH 7.4 20μmol, formaldehyde 2 μmol, DL-tetrahydrofolate (Sigma) dissolved in 1M 2-β-mercaptoethanol and neutralized with 1N NaOH 0.2 μmol, magnesium chloride 8μmol, 2β-mercaptoethanol 40μmol, deoxy[5-^{3}H]uridine 5'-monophosphate (100 μCi/μmol) 0.04 μmol and enzyme 100 μl in a total volume of 400 μl. After incubation at 37°C for 1 h, 600 μl of a slurry of activated charcoal (100 mg/μl in 0.1 N HCl) was added and the tubes left on ice for 10 m. After centrifugation at 3000 g for 20 m at 4°C, 500 μl supernatant was counted in 5 ml NE 260 (Nuclear Enterprises Ltd) with appropriate correction for quenching. Blank tubes lacked enzyme. Proteins were estimate by Lowry's method [8].

RESULTS

The thymidylate synthetase activity of marrow cells from healthy air-breathing rats was 1.55 ± SEM 0.36 ^{3}H-released/mg protein/h. There was a steady and highly significant (P=.0007) rise in activity of thymidylate synthetase activity throughout the 5 day period of N_2O exposure. The thymidylate synthetase activity in a group of 3 animals exposed to N_2O for 5 days and then returned to air for 3 days was normal (Figure).

DISCUSSION

The increase in thymidylate synthetase activity in N_2O-induced B_{12} inactivation confirms the observations in B_{12} deficiency in man [5] but not those in the study on mitogen-stimulated lymphocytes [6]. Although lymphocytes from pernicious anemia patients usually respond well to mitogen-stimulation, this is not always the case [9] and may be the explanation for low values recorded in one study [6].

Thymidylate synthetase requires folate as a coenzyme but there is no direct requirement for cobalamin. The effect of cobalamin deficiency is,

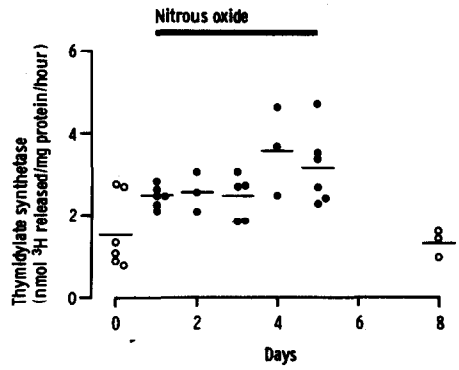

Figure. Thymidylate synthetase activity in rat bone marrow cells before (○) and while inhaling N_2O/O_2 (1/1) for five days (●). Three rats were returned to a normal atmosphere for three days after exposure to N_2O for five days. There is a rise in thymidylate activity following exposure to N_2O with a return to normal values on its withdrawal.

therefore, indirect and presumably through the provision of single carbon units, initially at the formate state of reduction [10,11]. The single carbon unit for thymidine synthesis is required in the methylene form. There is no impairment in serine transhydroxymethylase activity, which supplies methylene groups, in the B_{12}-inactivated rat [12] and presumably this pathway is unable to expand to meet the requirement for methylene units. Indeed much of the single carbon for thymidine synthesis is derived from formate [13] and the supply of formate is B_{12}-dependent [10,11].

It is interesting that in the B_{12}-inactivated rat some 80% of thymidine requirements continue to be met by synthesis whereas in B_{12} depleted man this value is probably less than 40%. Thymidylate synthetase activity doubles in the N_2O-inactivated rat as compared to nine-fold increase in B_{12}-deficient man. The much greater dependance of man on the B_{12}-dependent formate contribution for thymidine synthesis may explain why DNA synthesis is so much more severely affected in man, so that man alone develops a megaloblastic anemia in B_{12}-deficiency.

References

1. Banks, R.G.S., Henderson, R.J. and Pratt, J.M. (1968) J. Chem. Soc. (A) 2886-2889.
2. Deacon, R., Lumb, M., Perry, J., Chanarin, I., Minty, B., Halsey, M.J. and Nunn, J.F. (1978) Lancet, ii, 1023-1024.
3. Killmann, S-A. (1964) Acta Med. Scand. 175, 483-488.
4. Metz, J., Kelly, A., Swett, V.C., Waxman, S. and Herbert, V. (1968) Br. J. Haematol. 14, 575-592.
5. Sakamoto, S., Niina, M. and Takaku, F. (1975) Blood 46, 699-704.
6. Haurani, F.I. (1973) Science 182, 78-79.
7. Kammen, H.O. (1966) Anal. Biochem. 17, 553-556.
8. Lowry, O.H., Rosenbrough, N.J., Lewis-Farr, A. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
9. De la Chapelle, A. and Gräsbeck, R. (1964) Acta Med. Scand. Suppl. 412, 59-64.
10. Chanarin, I., Deacon, R., Lumb, M. and Perry, J. (1980) Lancet ii, 505-508.
11. Chanarin, I., Deacon, R., Lumb, M. and Perry, J. (1981) Br. J. Haematol. 47, 487-491.
12. Deacon, R., Perry, J., Lumb, M. and Chanarin, I. (1980) Biochem. Biophys. Res. Commun. 97, 1324-1328.
13. Lajtha, L.G. (1954) Nature 174, 1013-1014.