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THE INDUCTION OF FUNCTIONAL VITAMIN B-12 DEFICIENCY IN RATS BY EXPOSURE TO NITROUS OXIDE

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Summary

One of the main problems in studying mammalian vitamin B-12 metabolism has been the lack of an experimental model, due to the difficulty of creating a truly vitamin B-12 deficient state in animals by dietary control. The effect of vitamin B-12 inactivation, induced by prior exposure of rats to nitrous oxide, on de novo pyrimidine biosynthesis as assessed by deoxyuridine suppression, was determined in short-term bone marrow cultures.

An abnormal suppression of [^{3}H]thymidine incorporation into DNA by the folate dependent de novo pathway was found at all four concentrations of deoxyuridine used, when compared to control marrows taken from rats not treated with nitrous oxide. This inhibition was completely reversible by addition to the culture medium of pteroylglutamate and 5-formyltetrahydropteroylglutamate and partly reversible by cyanocobalamin. It was unaffected by the addition of 5-methyltetrahydropteroylglutamate. This study indicates that nitrous oxide inactivation of vitamin B-12 can be used to produce animals who have functional deficiency of vitamin B-12.

The de novo biosynthesis of DNA thymine from deoxyuridylate (dUMP) involves methylation of dUMP to thymidylate (dTMP) and requires a normally functioning folate coenzyme, 5,10-methylenetetrahydropteroylglutamate (5,10-CH_2-H_4PteGlu), and, indirectly, vitamin B-12. In normal marrow in culture, added deoxyuridine (dUrd), by increasing de novo thymidylate biosynthesis, suppresses the incorporation of subsequently added tritiated thymidine

Abbreviations: dUrd, deoxyuridine; dThd, thymidine; PPO, 2,5-diphenyloxazole.

([^{3}H]dThd) by the salvage pathway into DNA. Reduction in the dUrd suppressive effect is abnormal, and signifies defective de novo pyrimidine biosynthesis. The lack of a dUrd suppressive effect has been used as a sensitive indicator of folate and vitamin B-12 deficiency in human bone marrow [1—3].

In animal studies using marrow from both rats and monkeys folate deficiency has been shown to cause an abnormal dUrd suppression test [4]. However, in contrast to humans, vitamin B-12 deficiency in the rat produced by control of diet, has been shown not to significantly reduce in vitro methylation of dUMP to dTMP in bone marrow cells as determined by the dUrd suppression test [5]. This was in spite of the fact that the vitamin B-12 deficiency was severe enough to produce a functional folate deficiency in liver as measured by a marked reduction in folate polyglutamate formation and increased formiminoglutamic acid excretion.

Amess et al. [6] have found transient megaloblastic changes in patients receiving N_2O. This gas has previously been used to inhibit bacterial vitamin B-12 dependent dioldehydrase and ethanolamine ammonia ligase in vitro [7,8] but some controversy exists as to the validity of these findings [9]. Inactivation of vitamin B-12 dependent enzymes by N_2O where it occurs, would almost certainly proceed by oxidation of the Co(I) state (B-12s), to the Co(II) state (B-12r) [10,11]. Since the Co(I) state is known to arise as an intermediate in the transfer of the methyl group of methylcobalamin to produce methionine [12], it appears possible that inactivation of the vitamin to produce B-12s could occur as this reaction proceeds. Thus functional vitamin B-12 deficiency may be induced in man by this mechanism at a rate which could be proportional to the turnover of the enzymes producing Co(I).

McGing et al. [13] have recently demonstrated in vivo the inhibition of vitamin B-12 dependent demethylation of 5-CH_3-H_4-PteGlu by N_2O in mice and shown this inhibition to cause decreased biosynthesis of folate polyglutamate forms. Similar observations have been made in rats (Scott, J.M., unpublished results). Thus the biochemical abnormality of decreased folate polyglutamate biosynthesis, which had been demonstrated previously in animals only by inducing combined vitamin B-12 and methionine deficiency [14] was brought about by N_2O inhibition alone. Decreased de novo pyrimidine biosynthesis, which is a feature of human vitamin B-12 deficient bone marrow [2], has not been demonstrated in rat bone marrow by dietary induced deficiency of vitamin B-12 or of methionine, either singly or combined [5]. The object of the present study was to determine if N_2O treatment could produce bone marrow in rats which showed dUrd suppression characteristics similar to those of vitamin B-12 deficient marrow from humans. This would then provide an animal experimental model to examine biochemical changes in de novo pyrimidine metabolism induced by a functional lack of vitamin B-12.

Materials and Methods

Chemicals

Radioactive [*Me*-^{3}H]thymidine ([^{3}H]dThd) at a specific activity of 2.0 Ci/mmol was obtained from The Radiochemical Centre, Amersham, U.K. Deoxyuridine (dUrd), pteroylglutamate (PteGlu) and 5-methyltetrahydropteroyl-

glutamate (5-CH_3-H_4PteGlu) were supplied by Sigma, Poole, U.K. The latter compound was freshly prepared before use and made up in 1% sodium ascorbate and kept at 0°C. Vitamin B-12 in the form of cyanocobalamin was supplied by Glaxo, London, U.K. 5-Formyltetrahydropteroylglutamate (5-CHO-H_4PteGlu) was a gift of Lederle, Pearl River, NY.

Animal maintenance

Adult female Wistar rats (130—250 g) were placed in sealed chambers which were flushed with several times their volume using a gas mixture of 80% O_2 and 20% N_2O. The animals were maintained in the O_2/N_2O atmosphere for 48 h.

Marrow preparation

After killing by cranial fracture, bone marrow samples were prepared using a previous method with some modifications [2]. Bone marrow samples from the femora and tibiae were removed into 10 ml cold Hanks Balanced Salt solution containing 1000 units of heparin, both supplied by Gibco, Bio-cult, Glasgow, U.K. The sample was immediately chilled on ice and then centrifuged at 4°C for 10 min at $600 \times g$. As much of the supernatant as possible was decanted and the cell button was resuspended in the remaining supernatant using a Vortex mixer. This suspension was passed through needles of decreasing pore sizes 18, 21, 23, 26 gauge to break up any cell clumps present. The red blood cells present were lysed by the addition of an excess of Tris/NH_4Cl buffer, pH 7.2, followed by incubation for 20 min at 37°C. After lysis the cells were washed twice with Hanks Balanced Salt buffer and counted.

Suppression by dUrd

De novo synthesis of dTMP from dUMP was measured by the ability of dUrd to suppress the incorporation of added [^{3}H]dThd. Assays were carried out in microtitration plates supplied by Cooke, Billingshurst, U.K. Microtiter plates are sterile plastic plates (7.9×13.1 cm) containing an array of 96 wells, each well having a capacity of 300 μl. Assay mixtures were set up by adding to such plates 200 μl Hanks Balanced Salt buffer containing between $5 \cdot 10^6$ and $10 \cdot 10^6$ cells depending on the marrow preparation. Marrow cells from both normal and N_2O-treated animals were preincubated for 1 h at room temperature in the wells containing four different concentrations of dUrd (0.0004, 0.004, 0.04, 0.4 mM) with wells having no added dUrd acting as 100% controls. The amount of suppression effected at each concentration of dUrd was expressed as a percentage of that which was incorporated in the absence of dUrd. In addition the effect of the preincubation with addition of 50 μg PteGlu, 50 μg 5-CHO-H_4PteGlu, 50 μg 5-CH_3-H_4PteGlu and 1 μg cyanocobalamin was also determined. In each instance the relevant compound was added in 20 μl of Hanks Balanced Salts buffer and 20 μl of the same buffer was added to all other wells to adjust the volume. Control experiments showed that the amount of ascorbate added with the 5-CH_3-H_4PteGlu caused no alteration in [^{3}H]dThd incorporation. After 1 h of preincubation [^{3}H]dThd (0.6 μCi/20 μl) was added to all wells and incubation continued for a further 3 h at 37°C. After incubation, the well contents were transferred to type-faced GFC 2.5 cm diameter glass fibre discs (Whatman). The filters were communally washed

twice with ice-cold 10% trichloroacetic acid for 5 min, and further twice washed in methanol. The volume of washing fluid to filter discs was in the order of 10 ml to one disc. After washing, the filters were dried at 60°C and placed in scintillation vials containing 10 mls toluene/0.5% PPO scintillation fluid and counted on a Packard Tri-Carb Model 3385 liquid scintillation counter. The microsystem, utilizing microtiter plates, was based on an original method developed by Penhale et al. [15], adapted for application to the dUrd test.

Results

The inhibition of [^{3}H]dThd incorporation for a range of dUrd concentrations is shown in Fig. 1. The dUrd suppression results are expressed as a percentage of [^{3}H]dThd incorporation into DNA in the presence of dUrd, when unsuppressed [^{3}H]dThd endogenous incorporation (i.e. in the absence of added dUrd) is taken as representing 100% incorporation.

Bone marrow cells from N_2O-treated rats show decreased ability to suppress [^{3}H]dThd incorporation when the de novo pathway is stimulated by the addition of dUrd, suggesting a decrease in the methylation of dUMP to dTMP. Addition of PteGlu but not of 5-CH_3-H_4PteGlu, to the incubation mixture decreased the [^{3}H]dThd incorporation in the N_2O-treated animals and almost completely corrected the defective dUrd suppression test observed in its absence.

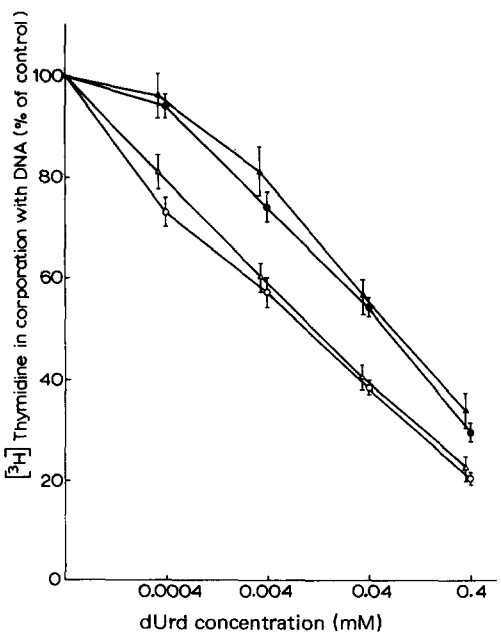

Fig. 1. Suppression of [^{3}H]dThd (0.6 Ci/20 l) incorporation into rat bone marrow cells in a 3 h incubation at 37°C after 1 h preincubation with various concentrations of dUrd, expressed as a percentage of control containing no dUrd (±S.E. n = 10) for normal animals (○——○) and N_2O-treated animals (●——●). The effect of in vitro addition of 5-CH_3-H_4PteGlu (50 μg) (▲——▲) and PteGlu (50 μg) (△——△) was found to be significantly different ($P < 0.001$) from (●——●) and (▲——▲), but not from each other.

TABLE I

EFFECT OF N_2O ON THE dUrd SUPPRESSIVE EFFECT

Incorporation of [^{3}H]dThd (0.6 Ci/20 l) into rat bone marrow cells in a 3 h incubation at 37°C after a 1 h preincubation with a 0.4 mM dUrd and various additions, expressed as a percentage of incorporation after preincubation without dUrd. Results are expressed as a mean ± S.E. (n = 10)

	[^{3}H]dThd incorporation (% control)
Normal, no N_2O	20.1 ± 0.8
N_2O-treated	29.4 ± 1.9
+ PteGlu (50 μg)	22.3 ± 2.3
+ 5-CHO-H_4PteGlu (50 μg)	15.7 ± 1.5
+ 5-CH_3-H_4PteGlu (50 μg)	33.0 ± 3.4
+ Cyanobalamin (1 μg)	25.1 ± 0.6

The effect of the additions of 5-CHO-H_4PteGlu and cyanocobalamin as well as PteGlu and 5-CH_3-H_4PteGlu were also studied and the [^{3}H]dThd incorporation at a single dUrd concentration is shown in Table I. As before, the N_2O-treated animals showed an abnormal dUrd suppression when compared to rats that had no N_2O exposure. Additions of 5-formyl-H_4PteGlu as well as PteGlu to the cultures of the N_2O-treated animals decreased the incorporation and returned it to the normal level while 5-CH_3-H_4PteGlu does not. Cyanocobalamin, when added to the N_2O cultures partially corrected the defective dUrd suppression.

Discussion

It was observed some years ago that while N_2O when used for short periods as an anaesthetic caused no marked clinical changes, longer exposure in the treatment of tetanus in man, resulted in acute marrow aplasia [16]. Subsequently a number of animal studies showed long-term exposure to N_2O to have depressive effects on various mammalian cell types [17—19].

Amess et al. [6] observed dUrd suppression results similar to those found in vitamin B-12 deficiency and concluded that inactivation of vitamin B-12 was responsible for the megaloblastic changes observed in patients exposed to N_2O.

In the chemical literature it has been accepted for some time that N_2O treatment in vitro can oxidise lower oxidation states of transition metal complexes and that such an oxidation could occur with the Co(I) form of vitamin B-12s [10,11]. Since it is generally accepted that 5-CH_3-H_4PteGlu:homocysteine methyltransferase proceeds via a Co(I) intermediate [12], it seems possible that this enzyme might be inactivated in vivo by N_2O oxidation [20].

It is generally agreed that it is extremely difficult to produce vitamin B-12 deficiency in experimental animals [21—23]. In the rat, apparently, the induction of biochemical changes due to vitamin B-12 deficiency also depends on the level of methionine ingestion [14]. Thus the need for a reproducible and simple method of inducing vitamin B-12 deprivation in an experimental animal exists. We have already shown that N_2O treatment inhibits folate polyglutamate formation in mouse liver [13]. The most likely explanation is that by inactivating vitamin B-12 and thus the conversion of 5-CH_3-H_4PteGlu to

H_4PteGlu which requires the vitamin B-12 dependent enzyme methionine synthetase, N_2O deprives the folate polyglutamate biosynthetic enzymes of their preferred substrate [24]. Subsequently, direct evidence for the inactivation of methionine synthetase has been obtained by Deacon et al. [20] who showed that exposure of rats to N_2O rapidly inactivated this enzyme. They also claimed that methyl malonyl CoA mutase seemed to be unaffected by N_2O exposure as evidenced by the absence of methylmalonic aciduria. This latter observation is in contrast to the results obtained by Scott et al. [25] who found abnormal propionate metabolism in rats exposed to N_2O. As propionate catabolism may be involved in the aetiology of the neurological lesions associated with vitamin B-12 deficiency [26,27], the findings of Dinn et al. [28] of neurological degeneration of the spinal column from a monkey exposed to nitrous oxide for 2 months tends to support the results of Scott et al. [25] that exposure to nitrous oxide inhibits the mutase enzyme.

The studies reported in this paper extend this work to rat bone marrow and to folate-dependent de novo pyrimidine biosynthesis.

Our results show that N_2O treatment inhibits the de novo pathway as evidenced by a decrease in dUrd suppression of [^{3}H]dThd incorporation (Fig. 1). In addition it was found that this abnormal dUrd test could be corrected by addition of PteGlu and 5-CHO-H_4PteGlu but not by 5-CH_3-H_4PteGlu (Table I). This is in agreement with previous studies on human bone marrow from vitamin B-12 deficient subjects [2,29,30]. This suggests that N_2O is specifically inactivating vitamin B-12, thus producing functional vitamin B-12 deficiency in the N_2O-treated marrow cells with concomitant functional folate deficiency. Addition of PteGlu and 5-CHO-H_4PteGlu have their corrective effect because, unlike 5-CH_3-H_4PteGlu, their utilization, initially at least, does not require functioning vitamin B-12. Addition of vitamin B-12 in the form of cyanocobalamin partially corrects the abnormal dUrd test possibly by flooding the system with excess vitamin B-12 since it is clear that cells normally have the capacity to convert cyanocobalamin to methylcobalamin.

It would thus appear that N_2O specifically inactivates vitamin B-12 in rats leading to deranged folate dependent de novo pyrimidine biosynthesis. This is in marked contrast to a previous study [5] on rat bone marrow where it had not been possible to demonstrate an abnormal deoxyuridine suppression of [^{3}H]thymidine incorporation into DNA by dietary induced vitamin B-12 deficiency.

In the light of the obvious presence of these biochemical changes it is of interest that, while megaloblastic changes occurred in humans very quickly [6], no such change was apparent in these rats even after upto 6 months exposure to 50% N_2O/50% O_2. In addition, while 2 months exposure to similar concentrations of N_2O can induce neurological changes in the monkey [28], no megaloblastic changes were found. Clearly, during the maturation of red cell precursors man has an unique susceptibility to vitamin B-12 deficiency. Perhaps these haematological differences may be more fruitfully examined by extending the folate trap hypothesis [31,32] to include consideration of the control of folates entering the trap in the first place. It seems likely that under normal conditions the rates of folates entering into the 5-CH_3-H_4PteGlu loop are controlled by feedback inhibition by *S*-adenosylmethionine on 5,10-CH_2-

H_4PteGlu reductase [33]. Krebs et al. [34] have argued that the primary consequence of vitamin B-12 deficiency is the interruption of methionine biosynthesis, which leads to a lowering of methionine levels and thus of *S*-adenosylmethionine levels. They claim that, as well as the decreased ability to form H_4PteGlu from 5-CH_3-H_4PteGlu during vitamin B-12 deficiency, the methyl trap comes about by the low activity of 10-CHO-H_4PteGlu dehydrogenase at low methionine concentrations, due to a lack of inhibition of 5,10-CH_2-H_4PteGlu reductase by *S*-adenosylmethionine. They would claim that the regulatory outlet for C_1 units are thus decreased and the C_1 derivatives of H_4PteGlu accumulated. Excess C_1 units would be reduced to 5-CH_3-H_4PteGlu because the thermodynamic equilibrium favours this reduction. In addition, because of lack of cobalamin, 5-CH_3-H_4PteGlu cannot form methionine from homocysteine, nor can the reaction leading to formation of 5-CH_3-H_4PteGlu be reversed. Thus most of the cell folate would be trapped as 5-CH_3-H_4PteGlu. We would agree with Krebs et al. [34] that the original methyl trap hypothesis [28,29], while essentially correct, must be extended to a consideration of folates entering the trap. However, in our view the position is much simpler than depicted [34]. If one agrees with the findings of Kutzbach and Stokstad [33] that the level of *S*-adenosylmethionine regulates the activity of 5-10-CH_2-H_4PteGlu reductase then decreased ability to make *S*-adenosylmethionine, which must be a secondary result of vitamin B-12 deficiency, would result in the cells' inability to prevent any 5-10-CH_2-H_4PteGlu formed, from being converted to 5-CH_3-H_4PteGlu. Since 5-10-CH_2-H_4PteGlu is unstable it probably has to be provided on a continuous basis for thymidylate synthetase and the unfettered activity of 5-10-CH_2-H_4PteGlu reductase may deprive this vital pyrimidine biosynthetic enzyme of its cosubstrate 5-10-CH_2-H_4PteGlu by its rapid conversion to 5-CH_3-H_4PteGlu. It seems reasonable that if susceptibility to vitamin B-12 differs from species to species, which from our studies appears to be the case, then a difference either in the trapping of 5-CH_3-H_4PteGlu or in the control of 5,10-CH_2-H_4PteGlu entering the trap must also occur in different species. It is to be hoped that the availability of a method to induce functional vitamin B-12 deficiency in animals will be of assistance in resolving some of these outstanding problems.

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