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Synthesis of DNA as shown by the deoxyuridine suppression test is normal in the vitamin B_{12}-deficient fruit bat (*Rosettus Aegyptiacus*)

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1. DNA synthesis in the bone marrow and lymphocytes of the vitamin B_{12}-deficient fruit bat (*Rosettus Aegyptiacus*) has been studied via the deoxyuridine (dU) suppression test.

2. In contrast to vitamin B_{12} deficiency in man, the dU suppression test was not abnormal in the vitamin B_{12}-deficient bat, and this correlates with the reported finding that the latter does not develop megaloblastic anaemia.

3. The protection of haemopoiesis from vitamin B_{12} deficiency in the bat is probably related to the presence in the bat of separate pools of methylfolate and tetrahydrofolate, which enables the bat to overcome the trapping of methylfolate attendant on vitamin B_{12} deficiency.

4. These results confirm the central role of the vitamin B_{12} and folate-dependent pathway of *de novo* synthesis of DNA in the pathogenesis of vitamin B_{12}-deficient megaloblastic anaemia.

Severe vitamin B_{12} deficiency can be induced with relative ease in the fruit bat (*Rosettus Aegyptiacus*) (Green *et al.* 1975; van Tonder *et al.* 1975). Deficient bats show marked neurological changes, but megaloblastic anaemia does not occur. Thus in contrast to man, who may suffer both neurological and haematological effects of vitamin B_{12} deficiency, the deleterious effects in the bat are confined to the nervous system. Haemopoiesis in the bat is protected in some way from the effects of dietary depletion of vitamin B_{12}.

The exact mechanism whereby vitamin B_{12} deficiency in man produces megaloblastic anaemia is unknown, but it is thought to be related to the indirect role of vitamin B_{12} in the *de novo* synthesis of thymidine nucleotides for DNA synthesis (Noronha & Silverman, 1962; Herbert & Zalusky, 1962; Killman, 1964; Metz *et al.* 1968; Herbert *et al.* 1973). The vitamin B_{12} dependence of *de novo* synthesis of DNA in human haemopoietic cells can be shown readily via the deoxyuridine (dU) suppression test in cultures of human bone marrow (Killman, 1964; Metz *et al.* 1968; Herbert *et al.* 1973; van der Weyden *et al.* 1973; Wickramasinghe & Longland, 1974; Wickramasinghe & Saunders, 1976, 1977; Zittoun *et al.* 1978; Ganeshaguru & Hoffbrand, 1978) and of lymphocytes (Das & Hoffbrand, 1970; Das & Herbert, 1978; Das *et al.* 1978). In this test, DNA synthesis via the salvage pathway in cultures of marrow cells or lymphocytes, measured by [^{3}H]thymidine (^{3}H-TdR) incorporation, is suppressed by pre-incubation of the cells with excess of unlabelled dU. This action of dU is dependent on normal functioning of the *de novo* pathway of DNA synthesis, and is impaired in both vitamin B_{12} and folate deficiency in humans.

In the present investigation, DNA synthesis in haemopoietic cells was studied in the vitamin B_{12}-deficient bat in the hope that by elucidating the mechanism by which haemopoiesis is protected in the bat, information would be obtained on the significance of the biochemical lesion found in vitamin B_{12} deficiency in man.

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MATERIALS AND METHODS

Experimental animals

Fruit bats were captured in the wild and were maintained on a pest-free, all-fruit diet (Green *et al.* 1975; van Tonder *et al.* 1975). On this diet, liver vitamin B_{12} stores decrease exponentially with a half-life of 109 d. Control animals were maintained on an identical diet, but received 500 ng cyanocobalamin by injection every 2 weeks. At the time of study, the mean serum vitamin B_{12} concentration in the control bats, measured by radioisotope dilution assay using chicken serum as the vitamin B_{12}-binding protein (Green *et al.* 1974), ranged from 743 to 2000 pg/ml (mean 1418 pg/ml) and in the deficient bats, <10 to 267 pg/ml (mean 114 pg/ml). All the deficient bats showed neurological impairment as manifested by difficulty in climbing and changes in the normal flying cycle. These changes did not occur in the bats receiving vitamin B_{12} supplements.

Both bone marrow and lymphocytes were studied, the latter because blood could be obtained without killing the bats.

Radioactive and non-radioactive compounds

[^{3}H]thymidine (^{3}H-TdR) (specific activity 15–20 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks. Phytohaemagglutinin (PHA) was purchased from Wellcome Laboratories, methotrexate (MTX) from Lederle Laboratories, dU from Sigma Laboratories, cyanocobalamin from Glaxo Laboratories, and folic acid (pteroylglutamic acid) from Sigma Laboratories.

Bone marrow culture

Bats were killed by exsanguination via cardiac puncture. The humerus bones were removed immediately after death. Marrow was flushed from these bones by injecting 2 ml culture medium TC199 (Wellcome Laboratories) via a 26 gauge needle through the marrow cavity.

All subsequent preparative procedures were carried out in the cold. The suspension of marrow cells was washed twice with 4 ml culture medium, centrifuging each time at 400 *g* for 15 min at 4°. A final suspension of concentration 2×10^6 cells/ml was prepared in culture medium, comprising TC199 and foetal calf serum (FCS) (2:1, v/v).

For the individual experiments, 0·2 ml cell suspension containing 4×10^5 cells was pipetted into each well of a microtitre plate. All cultures were set up in triplicate. The plates were incubated in sealed boxes in which the atmosphere was kept moist.

After incubation with various additives, according to the individual experiments, the reactions were terminated by harvesting the cells on paper (Skatron AS) in a Titetek harvester. The discs were cut out of the paper, and placed in plastic disposable vials containing 4 ml toluene. Scintillation fluid was added, and the radioactivity assayed in a Packard Liquid-Scintillation counter.

Lymphocyte culture

Blood was drawn by cardiac puncture, and 5 ml was layered carefully onto a Hypaque-Ficoll gradient. The gradient was centrifuged at 600 *g* for 30 min at 18°. The lymphocyte layer was removed with a pipette and washed twice in 15 ml TC199, centrifuging each time at 600 *g* for 10 min at 18°. The cells were resuspended at a concentration of 1×10^6/ml medium containing FCS (250 mg/ml).

Preliminary experiments were carried out to determine the concentration of PHA which would produce optimum stimulation of bat lymphocytes. Concentrations of PHA ranging from 5 to 50 μg/10^5 cells were added to the lymphocyte suspensions, which were then incubated for 48 h at 37°. The uptake of ^{3}H-TdR after a further 24 h incubation was determined. The uptake of ^{3}H-TdR was maximal at a concentration of 10 μg PHA/10^5 cells in the

lymphocytes from both control and deficient bats. All subsequent experiments were carried out using this concentration of PHA.

Portions of 0·2 ml containing 2×10^5 cells were pipetted into each well of a microtitre plate, and 20 μg PHA added to each well. Controls comprised cells only, without added PHA. All cultures were set up in triplicate. The plates were incubated for 48 h, under the same conditions as described for the bone marrow cultures. After 48 h incubation, various compounds were added, as detailed in the individual experiments, and the cultures re-incubated. At the end of the experiments, the lymphocytes were harvested and the radioactivity assayed as described for the bone marrow cultures.

As the number of cells/well in the marrow cultures (4×10^5) was double that for the lymphocyte cultures (2×10^5), the amount of additives used was always halved for the latter.

The dU suppression test

The test was carried out in bone marrow and lymphocyte cultures by standard techniques (Metz *et al.* 1968; Das & Hoffbrand, 1970). Three different concentrations of dU were used, 0·1, 0·01, and 0·001 μM. Each concentration was added in a volume of 10 μl. In the instance of lymphocyte cultures, the dU was added in concentrations of 0·1, 0·01, and 0·001 μM/well after the cultures had been incubated for 48 h with PHA, and the cultures then incubated for 1 h at 37°. ^{3}H-TdR (0·2 μCi in 10 μl) was then added, and the cells incubated for a further 24 h before harvesting.

In bone marrow suspensions, the dU in concentrations of 0·2, 0·02, and 0·002 μM/well was added and the cultures then stood for 30 min at room temperature. ^{3}H-TdR (0·4 μCi in 10 μl) was then added to each well, and the cells incubated for a further 2 h at 37°. Control cultures were kept at 4° for 2 h.

Effect of methotrexate, cyanocobalamin and folic acid on dU suppression

When the effect of these compounds on dU suppression was studied, they were added to the cultures in 10 μl volumes before adding the dU. In the bone marrow cultures, the cells were then incubated for 15 min at room temperature, the dU added, and the dU suppression test completed as described previously. The lymphocyte cultures were incubated at 37° for 30 min, the dU then added and the test completed as described previously.

RESULTS

Bone marrow

The dU suppression test was carried out on five control and five deficient bats. As all cultures were set up in triplicate, the number of cells obtained was not always adequate to perform the test at all three concentrations of dU. When limited numbers of cells were available, the higher concentrations of dU received priority.

The results are shown in Table 1. In control bats, pre-incubation of marrow cells with dU suppressed the uptake of subsequently added ^{3}H-TdR and the effect was dose dependent. Expressed as a percentage of the control sample without dU, uptake was suppressed to mean values of 5·0, 23·2, and 45·0 at concentrations of dU of 0·2, 0·02, and 0·002 μM respectively. In the vitamin B_{12}-deficient bats, the dU suppression was slightly more than in the control animals, but the differences are not statistically significant.

Lymphocytes

The results of dU suppression tests carried out at various concentrations of dU in six control and ten deficient bats are shown in Table 2. The results are similar to those found

Table 1. *The deoxyuridine (dU) suppression test in bone marrow from vitamin B_{12}-replete and vitamin B_{12}-deficient fruit bats* (Rosettus Aegyptiacus), *together with the effect of added cyanocobalamin and folic acid (PGA)*

(Results of ^{3}H-TdR uptake into control cultures (without added dU) are expressed as disintegrations/min per 4×10^5 cells. All other results are expressed as the percentage uptake of ^{3}H-TdR after pre-incubation with dU as compared with the uptake in cultures to which no dU was added)

	Vitamin B_{12}-replete*			Vitamin B_{12}-deficient†		
Treatment (dU/μM)	No. of bats	Mean	Range	No. of bats	Mean	Range
0	5	4619	2227–8080	5	4241	2033–6974
0·2	5	5·0	1–14	5	1·6	0–3
0·02	5	23·2	5–41	4	13·7	6–19
+ 1 μg cyanocobalamin	3	22·0	14–33	3	16·3	8–24
+ 50 μg pteroylglutamic acid	1	18	—	3	13·0	4–18
0·002	3	45·0	16–60	3	41·0	23–56
+ 1 μg cyanocobalamin	2	53·5	53–55	3	52·7	44–65
+ 50 μg pteroylglutamic acid	1	54	—	2	51·0	47–55

* Serum vitamin B_{12} concentration 1520–2000 pg/ml (mean 1673).
† Serum vitamin B_{12} concentration 167–267 pg/ml (mean 210).

Table 2. *The deoxyuridine (dU) suppression test in PHA-stimulated lymphocytes from vitamin B_{12}-replete and vitamin B_{12}-deficient fruit bats* (Rosettus Aegyptiacus), *together with the effect of added cyanocobalamin and folic acid (PGA)*

(Results of ^{3}H-TdR uptake into control cultures (without added dU) are expressed as disintegrations/min per 2×10^5 cells. All other results are expressed as the percentage uptake of ^{3}H-TdR after pre-incubation with dU as compared with the uptake in cultures to which no dU was added)

	Vitamin B_{12}-replete*			Vitamin B_{12}-deficient†		
Treatment (dU/μM)	No. of bats	Mean	Range	No. of bats	Mean	Range
0	6	1230	611–2232	10	1404	483–2617
0·2	6	1·9	0–5	7	2·6	0–8
0·02	5	25·4	0–40	10	20·8	2–43
+ 1 μg cyanocobalamin	4	31·0	0–52	6	22·6	3–35
+ 50 μg pteroylglutamic acid	3	23·5	4–29	2	15·0	9–21
0·002	5	74·8	64–95	5	67·8	42–100

* Serum B_{12} concentration 499–1736 pg/ml (mean 1053).
† Serum B_{12} concentration < 10–169 pg/ml (mean 67).

with the bone marrow cultures, i.e. there was no impairment in dU suppression in the vitamin B_{12}-deficient animals compared with the controls.

Effect of added cyanocobalamin and folic acid

The possible effect of added cyanocobalamin and folic acid on the dU suppression test was studied in the bone marrow and lymphocytes of vitamin B_{12}-deficient and control bats. The results are shown in Tables 1 and 2. The addition of cyanocobalamin or folate failed to enhance significantly the ability of dU to suppress the uptake of ^{3}H-TdR.

Table 3. *The effect of* 10 *μg methotrexate* (*MTX*) *on the deoxyuridine* (*dU*) *suppression test in bone marrow from fruit bats* (Rosettus Aegyptiacus)

(Results are expressed as disintegration/min per 4×10^5 cells. Values in parentheses are the percentage uptakes of ^{3}H-TdR after pre-incubation with dU with and without added MTX, as compared with the uptakes in cultures to which no dU was added)

Bat No.	Serum vitamin B_{12} (pg/ml)	Treatment: No dU	0·2 μM dU	0·2 μM dU+MTX	0·02 μM dU	0·02 μM dU+MTX
1	167	4415	51 (1)	2274 (50)	796 (18)	3605 (79)
2	209	6974	203 (3)	3023 (43)	414 (6)	4904 (70)
3	237	2033	66 (3)	580 (29)	382 (19)	900 (44)
4	1579	2227	141 (6)	830 (37)	430 (19)	1291 (58)

Effect of methotrexate

The effect of MTX on dU suppression at two dose levels of dU (0·2 and 0·02 μM) was studied in bone marrow from four bats. The results are shown in Table 3. In all the experiments, MTX markedly inhibited the ability of dU to suppress the uptake of subsequently added ^{3}H-TdR.

DISCUSSION

In the present study, pre-incubation of cultures of bat bone marrow or lymphocytes with dU produced suppression of the uptake of subsequently added ^{3}H-TdR. This action of dU is similar to that found in human bone marrow and lymphocytes, indicating the presence of *de novo* pathways for DNA synthesis in the bat cells. As in man, the *de novo* pathway is dependent on a supply of reduced folate, for MTX inhibits markedly the ability of dU to suppress the uptake of ^{3}H-TdR.

In vitamin B_{12} deficiency in man, dU fails to suppress the uptake of subsequently added ^{3}H-TdR, and there is a clear difference between healthy subjects and vitamin B_{12}-deficient patients (Metz *et al.* 1968; Das & Hoffbrand, 1970; Herbert *et al.* 1973). Abnormal DNA synthesis in vitamin B_{12} deficiency in man is attributed to impairment of thymidylate synthesis via the *de novo* pathway. The defect is partially corrected by the addition of vitamin B_{12}, but corrected completely by the addition of non-methylated folate (Metz *et al.* 1968; van der Weyden *et al.* 1973; Zittoun *et al.* 1973). Thus vitamin B_{12} deficiency is thought to produce its effect on DNA synthesis via a secondary derangement in folate metabolism. The most widely held explanation for this is the methylfolate trap hypothesis, whereby decreased activity of the vitamin B_{12}-dependent enzyme methionine synthetase (*EC* 2.1.1.13) necessary for the conversion of methylfolate to tetrahydrofolate (THFA) results in deficient THFA for *de novo* synthesis of thymine DNA (Herbert & Zalusky, 1962; Noronha & Silverman, 1962). Further evidence in favour of trapping of methylfolate is the rise in serum folate (largely methylfolate) levels that occurs in vitamin B_{12} deficiency in man.

In the fruit bat, no significant difference could be demonstrated between vitamin B_{12}-deficient and control animals, in spite of very low levels of serum vitamin B_{12} and severe neurological changes in the deficient bats. The failure of vitamin B_{12} deficiency in the bat to lead to changes in the dU suppression test as seen in vitamin B_{12}-deficient man may be due to a number of factors. It is possible that in the bat, the conversion of methylfolate to

THFA is not vitamin B_{12}-dependent. However, the finding that serum folate levels are elevated in the vitamin B_{12}-deficient bat (van Tonder *et al.* 1975) suggests that the reaction is in fact vitamin B_{12}-dependent. Furthermore, methionine synthetase activity in the liver of vitamin B_{12}-deficient bats is significantly reduced compared with that of control bats (I. Chanarin & J. Perry, unpublished observations). Thus it is unlikely that failure to demonstrate a defect in DNA synthesis in the vitamin B_{12}-deficient bat is due to the fact that the enzyme methionine synthetase is not vitamin B_{12}-dependent.

The results of other studies in the bat suggest a more likely explanation for the present findings. Perry *et al.* (1979*a*,*b*) demonstrated significan differences in the way injected labelled methylfolate and THFA are used by the bat. The results suggest the presence of two distinct pools of folate activity, the methylfolate pool being separate from the THFA pool, and not a single pool as previously supposed. If, as indicated from this work, the THFA is distinct from the methylfolate pool, it would suggest a source of THFA for haemopoeisis for the bat, which is not dependent on constant regeneration of THFA from methylfolate. This would provide the bat with an escape mechanism whereby the effect of trapping of methylfolate in vitamin B_{12} deficiency could be overcome via the separate THFA pool.

The vitamin B_{12}-deficient all-fruit diet fed to the bats is high in folate, and the initial rise in red cell folate levels in the bats during captivity (van Tonder *et al.* 1975) may be a reflexion of this. The high dietary folate intake may be a factor in protecting haemopoeisis from megaloblastic change in vitamin B_{12} deficiency, and as such these bats would resemble human vegans with a low vitamin B_{12}–high folate diet, and in whom vitamin B_{12} deficiency leads to more severe neurological than haematological changes (Wokes & Smith, 1962). However, this is unlikely to be the only reason for the absence of megaloblastic change in the bat, for some human vegans do develop megaloblastic change due to vitamin B_{12} deficiency (Mollin & Ross, 1954).

Secondary changes in the concentration of serum transcobalamins which occur in the vitamin B_{12}-deficient bat, but not in vitamin B_{12}-deficient man, may be of relevance in protecting the vitamin B_{12}-deficient bat from developing megaloblastic anaemia. As vitamin B_{12} deficiency develops in the bat fed on the all-fruit diet, the levels of transcobalamin II (TCII), the transport binder which promotes cellular uptake of vitamin B_{12}, rises markedly (Green *et al.* 1979). Such a rise does not occur in vitamin B_{12}-deficient man (Retief *et al.* 1967). As suggested by Green *et al.* (1979), this change in TCII could represent a compensatory adjustment to the lowered state of vitamin B_{12} nutrition in the bat. However, this change is unlikely to afford protection for haemopoiesis at the very low levels of serum vitamin B_{12} that occurred in some of the vitamin B_{12}-deficient bats in this study.

The findings of the present study reinforce the concept that the defect in *de novo* synthesis of DNA, as demonstrated via the dU suppression test, is the central theme in considering the mechanism whereby vitamin B_{12} deficiency leads to megaloblastic anaemia.

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