British Journal of Nutrition (2003), **89**, 441–444
DOI: 10.1079/BJN2002811

Short communication

Hypoxia-induced megaloblastosis in vitamin B_{12}-deficient rats

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(Received 23 May 2002 – Revised 25 October 2002 – Accepted 8 November 2002)

In rats, in contrast with human subjects who develop megaloblastic anaemia due to vitamin B_{12} deficiency, haematological abnormalities with anaemia were not observed under normoxic conditions even though plasma vitamin B_{12} concentration was reduced to $<15\%$ of a normal concentration by depleting dietary vitamin B_{12}. To elucidate whether erythropoiesis was affected by vitamin B_{12} deficiency in rats, these vitamin B_{12}-deficient rats were exposed to hypoxia (10·5 % O_2) to stimulate erythropoiesis. In the vitamin B_{12}-sufficient control rats, erythrocyte count was significantly ($P<0·05$) increased 1 week after starting the hypoxic exposure. However, the hypoxia-induced erythropoiesis was affected by vitamin B_{12} deficiency, and no significant increase in the erythrocyte count was observed even after 6-week exposure to hypoxia in the vitamin B_{12}-deficient rats. In the vitamin B_{12}-deficient rats in hypoxia, erythrocytes became abnormally enlarged, and haemoglobin concentration in peripheral blood was increased in proportion to the increase of mean corpuscular volume. However, the level of the increase in the haemoglobin concentration was significantly ($P<0·05$) lower in the vitamin B_{12}-deficient rats compared with that in the -sufficient controls. In addition, in the vitamin B_{12}-deficient rats, in contrast to the -sufficient rats, serum erythropoietin concentration was not normalized even after 6-week exposure to hypoxia. These results indicate that a megaloblastic anaemia-like symptom is induced when the vitamin B_{12}-deficient rats are exposed to hypoxia.

Hypoxia-induced erythropoiesis: Megaloblastic anaemia: Erythropoietin: Vitamin B_{12}-deficient rats

In experimental animals, in contrast with human subjects, it is very difficult to induce megaloblastic anaemia by depleting dietary vitamin B_{12} (England & Linnell, 1979). For example, in fruit bats (*Rousettus aegyptiacus*), Green *et al.* (1975) have reported that haematological abnormalities, except for a reduction of leucocyte count, are not induced even if dietary vitamin B_{12} is depleted for 47 weeks, although clear neurological abnormalities are observed. Thus, megaloblastic anaemia due to vitamin B_{12} deficiency seems to be unique to man. It may be that man is more reliant on the *de novo* synthesis of thymidylate, and less able to salvage it from DNA breakdown, than other species (Bender, 1992). In addition, since folate is sufficiently provided in the diet of most animals used as a model of vitamin B_{12} deficiency, anaemia might be masked in these animals (Stabler, 2000); details, however, remain obscure.

In mammals, hypoxia causes erythropoiesis to maintain an adequate level of O_2 in tissues. This is controlled by erythropoietin (Epo) produced in the kidneys (Jelkman, 1992; Bunn & Poyton, 1996). Epo operates on erythroid progenitor cells and enables them to proliferate into functioning erythrocytes (ERC).

To elucidate whether erythropoiesis is affected by vitamin B_{12} deficiency in mammals other than man, we have exposed rats with dietary vitamin B_{12} deficiency to hypoxia

Abbreviations: Epo, erythropoietin; ERC, erythrocyte; Hb, haemoglobin; MCV, mean corpuscular volume; MMA, methylmalonic acid.

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to stimulate erythropoiesis. In the present paper, we report that the hypoxia-induced erythropoiesis is affected in rats when plasma vitamin B_{12} concentration is reduced to $<15\%$ of a normal level, even though apparent haematological abnormalities are not induced in normoxia. In addition, it is also reported that a megaloblastic anaemia-like symptom appears in the vitamin B_{12}-deficient rats under hypoxic conditions.

Materials and methods

Male weanling rats (3 weeks old), born to 14-week-old parent rats that had been fed a vitamin B_{12}-deficient diet for 8 weeks, were used. The parent rats were obtained from Kiwa Laboratory Animals (Wakayama, Japan). The vitamin B_{12}-deficient diet contained (g/kg): defatted soyabean (about 500 g crude protein ($N \times 6{\cdot}25$) and 500 g carbohydrate; donated by Fuji Oil, Osaka, Japan) 400, glucose 438, soyabean oil 100, mineral mixture 50, vitamin B_{12}-free vitamin mixture 5, choline chloride 2. The mineral and vitamin mixtures were prepared as described previously (Watanabe *et al.* 1991). Folic acid (5 mg/kg) was included in the diet. The animals used were maintained in accordance with the guidelines of the National Research Council (1985).

The weanling rats were housed individually in wire-bottomed cages at controlled temperature (22 ± 2°C), humidity ($55 \pm 5\%$) and lighting (lights on 08.00–20.00 hours), and fed the vitamin B_{12}-deficient or -sufficient diet. The vitamin B_{12}-sufficient diet was identical to the -deficient one, except that 25 μg cyanocobalamin/kg was included. After being maintained for 17 weeks from weanling on the experimental diet, these rats (20 weeks old) were exposed to hypoxia ($10{\cdot}5\%$ O_2) in an ambient chamber for an additional 6 weeks. Hypoxic conditions were introduced by flowing a mixed gas (O_2–N_2 (10·5:89·5, v/v)), produced by Pana O_2 apparatus (donated by Matsushita Electric, Osaka, Japan), into the chamber, and the O_2 concentration in the chamber was maintained by monitoring with an O_2 monitor during the exposure (Yamaji *et al.* 1996).

During the hypoxic exposure, blood samples were obtained from tail vein, and ERC count, mean corpuscular volume (MCV) and haemoglobin (Hb) concentration were measured with an automatic blood corpuscle count apparatus (Sysmex K-1000, Toua Iyou Electric, Kobe, Japan). At the end of the 6-week hypoxic exposure, blood was collected from inferior vena cava (under diethyl ether anaesthesia). Vitamin B_{12} and methylmalonic acid (MMA) were determined in plasma according to the methods of Ebara *et al.* (2001) and Toyoshima *et al.* (1996). Serum Epo concentration was determined by a radioimmunoassay method using a commercial kit (Recombigen Epo Kit, IatRon Laboratory, Ichikawa, Japan) according to the manufacturer's instruction.

Statistical analyses

Statistical analyses were performed with GB-Stat 5.4 (Dynamic Microsystems, Silver Spring, MD, USA). Haematological variables were compared between the vitamin B_{12}-sufficient and -deficient groups during the hypoxic exposure by two-way ANOVA for repeated measures, and *post-hoc* analyses were done by Newman–Keuls test. Change in Epo concentration during the hypoxic exposure was compared between the vitamin B_{12}-sufficient and -deficient groups by two-way ANOVA followed by Newman–Keuls test. Data on vitamin B_{12} and MMA were analysed by one-way ANOVA followed by Scheffé *post-hoc* test. All results are presented as mean values with their standard errors, and statistical significance is defined as $P<0{\cdot}05$.

Results

When weanling rats (3 weeks old) were fed a vitamin B_{12}-deficient diet for 17 weeks, plasma vitamin B_{12} concentration was reduced to 0·15 (SE 0·011) nM (n 5), which was $13{\cdot}5\%$ of the level of the vitamin B_{12}-sufficient control rats (1·11 (SE 0·015) nM (n 5)). In addition, plasma MMA level was abnormally elevated in the vitamin B_{12}-deficient rats (0·96 (SE 0·126) mM (n 5)), whereas it was undetectable in the -sufficient rats.

Under normoxic conditions, no statistically significant changes in ERC count, MCV and Hb concentration in peripheral blood were induced in the vitamin B_{12}-deficient rats at 20 weeks of age (Fig. 1). These rats were exposed to hypoxia ($10{\cdot}5\%$ O_2) for 6 weeks, and changes in these haematological variables were examined. In the vitamin B_{12}-sufficient control rats, about 25 % of increase ($P<0{\cdot}05$) in the ERC count was observed 1 week after starting the exposure, and the significantly higher level was maintained thereafter. In contrast, in the vitamin B_{12}-deficient rats, no significant increase was induced in the ERC count even after the 6-week exposure to hypoxia. The hypoxic exposure caused elevation in MCV in both the vitamin B_{12}-sufficient and -deficient groups, but the extent was significantly ($P<0{\cdot}05$) greater in the -deficient rats. A significant elevation in the Hb concentration was observed in the vitamin B_{12}-deficient rats as well as the -sufficient controls. However, the Hb concentration in the vitamin B_{12}-deficient rats was significantly ($P<0{\cdot}05$) lower than that in the -sufficient controls at any time points examined during the hypoxic exposure.

The plasma vitamin B_{12} level in the vitamin B_{12}-deficient rats was not significantly decreased by the hypoxic exposure for 6 weeks (0·15 (SE 0·011) (n 5) *v.* 0·14 (SE 0·031) (n 5) nM). In addition, no significant change in the plasma MMA concentration was observed after the 6-week exposure to hypoxia (0·96 (SE 0·127) (n 5) *v.* 0·89 (SE 0·090) (n 5) mM). These results suggest that vitamin B_{12} deficiency was not made more severe by the 6-week exposure to hypoxia.

Serum Epo concentration was followed during the hypoxic exposure for 6 weeks (Table 1). Before the exposure (in normoxia), the Epo concentration in the vitamin B_{12}-deficient rats was not significantly different from that in the -sufficient controls. One day after starting the hypoxic exposure, Epo in serum was significantly ($P<0{\cdot}05$) elevated in both the vitamin B_{12}-sufficient and -deficient groups. In the vitamin B_{12}-sufficient group, the elevated Epo concentration returned to a normal level (normoxic level) in 1 week after starting the exposure.

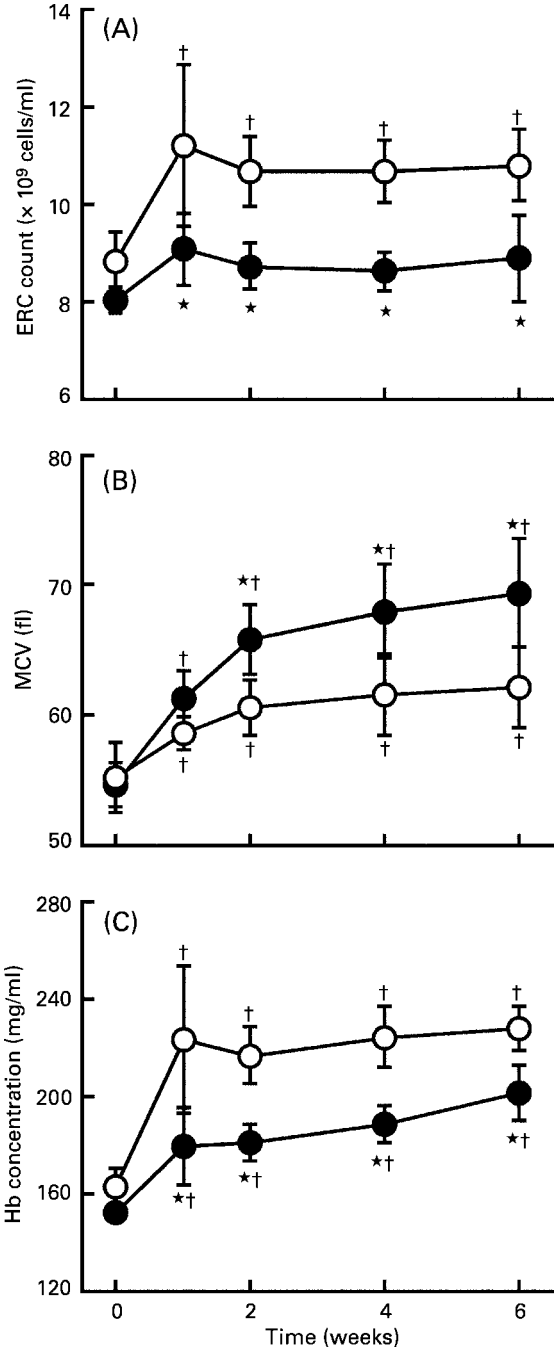

Fig. 1. Effect of 6-week exposure to hypoxia on erythrocyte (ERC) count (A), mean corpuscular volume (MCV, (B)) and haemoglobin (Hb) concentration (C) in peripheral blood of vitamin B_{12}-sufficient (○) and -deficient (●) rats. For details of diets and procedures, see p. 442. Values are means with their standard errors represented by vertical bars for five rats per group. Mean values were significantly different from those of the vitamin B_{12}-sufficient rats at the same time point: *$P<0{\cdot}05$. Mean values were significantly different from the values in normoxia (at week 0) in each group: †$P<0{\cdot}05$.

However, in the vitamin B_{12}-deficient rats, the Epo concentration remained at a significantly ($P<0{\cdot}05$) higher concentration compared with that in normoxia, throughout the 6-week exposure to hypoxia.

Table 1. Change in serum erythropoietin (Epo) concentration during exposure to hypoxia in vitamin B_{12}-deficient rats‡

(Mean values with their standard errors for five rats per group)

Time of hypoxic exposure	Serum Epo concentration (mIU/ml)			
	Vitamin B_{12}-sufficient		Vitamin B_{12}-deficient	
	Mean	SE	Mean	SE
0 d	24	7·9	28	6·2
1 d	104†	16·8	188*†	41·0
1 week	34	10·6	171*†	39·5
6 weeks	27	4·9	108*†	30·1

Mean values were significant different from those of the vitamin B_{12}-sufficient group at the same time point: *$P<0{\cdot}05$.

Mean values were significantly different from the values in normoxia (at 0 d) in each group: †$P<0{\cdot}05$.

‡ The vitamin B_{12}-sufficient or -deficient rats (20 weeks old) were exposed to hypoxia for 0 d, 1 d, 1 week or 6 weeks, and the serum Epo concentration was determined. For details of diets and procedures, see p. 442.

Discussion

Vitamin B_{12} deficiency results in the inactivation of methylmalonyl-CoA mutase and methionine synthase, which require 5′-deoxyadenosyl-cobalamin and methylcobalamin respectively, as a coenzyme, and consequently MMA and homocysteine are abnormally increased in plasma and excreted into urine (Toyoshima *et al.* 1996; Stabler, 2000). In human patients with severe vitamin B_{12} deficiency, megaloblastic anaemia, in addition to neurological abnormalities, is often observed. The megaloblastic anaemia observed in vitamin B_{12}-deficient patients is thought to result from the inactivation of methionine synthase, which leads to the impairment of folic acid metabolism closely related to thymidylate and purine biosynthesis (Shane, 1985, Koury *et al.* 2000). However, it has been reported that haematological abnormalities with anaemia do not appear in experimental animals, in contrast with human subjects, even under severe vitamin B_{12}-deficient conditions in which neurological abnormalities are induced (Kark *et al.* 1974; Green *et al.* 1975; Crampton *et al.* 1979).

Under our present experimental conditions, the plasma concentration of vitamin B_{12} was reduced to 0·15 nM (< 15 % of that in the vitamin B_{12}-sufficient controls) by depleting dietary vitamin B_{12} in rats. In addition, as observed in our previous paper (Toyoshima *et al.* 1996), the plasma MMA concentrations was abnormally raised in these rats. However, no statistically significant changes in the ERC count, MCV and Hb concentration in peripheral blood were observed in the vitamin B_{12}-deficient rats (Fig. 1). These results suggest that rats, as well as other experimental animals, do not develop megaloblastic anaemia due to vitamin B_{12} deficiency under normoxic conditions.

When mammals are exposed to low O_2 conditions, Epo is produced in kidney and erythropoiesis is induced by the action of Epo to provide O_2 into tissues at an adequate level (Hill *et al.* 1987; Jelkman, 1992; Bunn & Poyton, 1996). To examine the effect of vitamin B_{12} deficiency on the hypoxia-induced erythropoiesis, these vitamin B_{12}-deficient rats were exposed to 10·5 % O_2 conditions for 6

weeks. In the vitamin B_{12}-sufficient control rats, a significant ($P<0{\cdot}05$) increase (about 25 %) in the ERC count was observed in 1 week after starting the exposure (Fig. 1). However, the hypoxia-induced erythropoiesis was inhibited by vitamin B_{12} deficiency, and the ERC count was not significantly increased even after the 6-week exposure to hypoxia in the vitamin B_{12}-deficient group. It is thus reasonable to postulate that thymidylate and purine biosynthesis are affected under vitamin B_{12}-deficient conditions in rats as well as human subjects, and the increase in the ERC count is inhibited due to the impairment of DNA synthesis in the deficient rats when exposed to hypoxia. MCV was increased, with concomitant increase in Hb content per ERC, in hypoxia (Hill *et al.* 1987). The extent of the increase was significantly ($P<0{\cdot}05$) greater in the vitamin B_{12}-deficient rats than the -sufficient rats. In addition, the Hb concentration in peripheral blood was increased in proportion to the increase in MCV in the deficient rats in hypoxia. Thus, in these rats, ERC become abnormally enlarged to increase Hb in peripheral blood as much as possible, since the hypoxia-induced erythropoiesis is inhibited under the vitamin B_{12}-deficient conditions. However, the Hb concentration was significantly ($P<0{\cdot}05$) lower in the vitamin B_{12}-deficient rats than the -sufficient controls at any time point examined during the 6-week exposure to hypoxia. These observations suggest that a megaloblastic anaemia-like symptom is induced in the vitamin B_{12}-deficient rats when exposed to hypoxia.

Carmel & MacPhee (1992) have observed that Epo in serum is abnormally increased in response to the reduction of the Hb concentration in peripheral blood in vitamin B_{12}-deficient patients with anaemia. No significant difference was observed in the serum Epo concentration between the vitamin B_{12}-sufficient and -deficient rats in normoxia (Table 1), confirming that the -deficient rats do not have anaemia under the present conditions. It has been reported that a great increase in the serum Epo concentration is transiently induced in rats after exposure to low O_2 conditions (Tan *et al.* 1992). Indeed, when the vitamin B_{12}-sufficient rats were exposed to hypoxia for 6 weeks, the serum Epo concentration was significantly ($P<0{\cdot}05$) greater, compared with a normoxic level, in early phase (on day 1), but it was normalized in 1 week after starting the exposure. These results indicate that the vitamin B_{12}-sufficient rats are able to adapt to hypoxia in 1 week by increasing the ERC count. However, in the deficient rats, the Epo concentration did not return to a normal level (normoxic level) even after the 6-week exposure to hypoxia. It is thus suggested that O_2 is not provided into tissues at an adequate level in the vitamin B_{12}-deficient rats, in contrast to the -sufficient controls, throughout the exposure to hypoxia for 6 weeks.

Results obtained in the present paper show that the hypoxia-induced erythropoiesis is affected in rats when the plasma vitamin B_{12} concentration is lowered to $<15\,\%$ of a normal level, although haematological abnormalities with anaemia are not observed under normoxic conditions. Consequently, megaloblastic anaemia-like symptoms appear when these vitamin B_{12}-deficient rats are exposed to low O_2 conditions. This is the first report showing a way in which a rat model can be used to study megaloblastic changes associated with vitamin B_{12} deficiency.

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