Hypoxia-induced megaloblastosis in vitamin B12-deficient rats

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In rats, in contrast with human subjects who develop megaloblastic anaemia due to vitamin B12 deficiency, haematological abnormalities with anaemia were not observed under normoxic conditions even though plasma vitamin B12 concentration was reduced to 15% of a normal concentration by depleting dietary vitamin B12. To elucidate whether erythropoiesis was affected by vitamin B12 deficiency in rats, these vitamin B12-deficient rats were exposed to hypoxia (10.5% O2) to stimulate erythropoiesis. In the vitamin B12-sufficient control rats, erythrocyte count was significantly increased 1 week after starting the hypoxic exposure. However, the hypoxia-induced erythropoiesis was affected by vitamin B12 deficiency, and no significant increase in the erythrocyte count was observed even after 6-week exposure to hypoxia in the vitamin B12-deficient rats. In the vitamin B12-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 lower in the vitamin B12-deficient rats compared with that in the -sufficient controls. In addition, in the vitamin B12-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 B12-deficient rats are exposed to hypoxia.

Hypoxia-induced erythropoiesis: Megaloblastic anaemia: Erythropoietin: Vitamin B12-deficient rats

In experimental animals, in contrast with human subjects, it is very difficult to induce megaloblastic anaemia due to vitamin B12 deficiency, haematological abnormalities with anaemia were not observed under normoxic conditions even though plasma vitamin B12 concentration was reduced to <15% of a normal concentration by depleting dietary vitamin B12. To elucidate whether erythropoiesis was affected by vitamin B12 deficiency in rats, these vitamin B12-deficient rats were exposed to hypoxia (10.5% O2) to stimulate erythropoiesis. In the vitamin B12-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 B12 deficiency, and no significant increase in the erythrocyte count was observed even after 6-week exposure to hypoxia in the vitamin B12-deficient rats. In the vitamin B12-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 B12-deficient rats compared with that in the -sufficient controls. In addition, in the vitamin B12-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 B12-deficient rats are exposed to hypoxia.

In experimental animals, in contrast with human subjects, it is very difficult to induce megaloblastic anaemia by depleting dietary vitamin B12 (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 B12 is depleted for 47 weeks, although clear neurological abnormalities are observed. Thus, megaloblastic anaemia due to vitamin B12 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 B12 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 O2 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 B12 deficiency in mammals other than man, we have exposed rats with dietary vitamin B12 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 B12 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 B12-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 B12-deficient diet for 8 weeks, were used. The parent rats were obtained from Kiwa Laboratory Animals (Wakayama, Japan). The vitamin B12-deficient diet contained (g/kg): defatted soybean (about 500 g crude protein (N × 6·25) and 500 g carbohydrate; donated by Fuji Oil, Osaka, Japan) 400, glucose 438, soybean oil 100, mineral mixture 50, vitamin B12-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 ± 5 %) and lighting (lights on 08.00–20.00 hours), and fed the vitamin B12-deficient or -sufficient diet. The vitamin B12-sufficient diet was identical to the -deficient one, except that 25 μg cyanocobalamin/kg was included. After being maintained for 17 weeks from weaning on the experimental diet, these rats (20 weeks old) were exposed to hypoxia (10·5 % O2) in an ambient chamber for an additional 6 weeks. Hypoxic conditions were introduced by flowing a mixed gas (O2–N2 (10·5:89·5, v/v)), produced by Pana O2 apparatus (donated by Matsushita Electric, Osaka, Japan), into the chamber, and the O2 concentration in the chamber was maintained by monitoring with an O2 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 B12 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 B12-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 B12-sufficient and -deficient groups by two-way ANOVA followed by Newman–Keuls test. Data on vitamin B12 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<0·05.

Results
When weanling rats (3 weeks old) were fed a vitamin B12-deficient diet for 17 weeks, plasma vitamin B12 concentration was reduced to 0·15 (SE 0·011) nm (n 5), which was 13·5 % of the level of the vitamin B12-sufficient control rats (1·11 (SE 0·015) nm (n 5)). In addition, plasma MMA level was abnormally elevated in the vitamin B12-deficient rats (0·96 (SE 0·126) nm (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 B12-deficient rats at 20 weeks of age (Fig. 1). These rats were exposed to hypoxia (10·5 % O2) for 6 weeks, and changes in these haematological variables were examined. In the vitamin B12-deficient control rats, about 25 % of increase (P<0·05) 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 B12-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 B12-sufficient and -deficient groups, but the extent was significantly (P<0·05) greater in the -deficient rats. A significant elevation in the Hb concentration was observed in the vitamin B12-deficient rats as well as the -sufficient controls. However, the Hb concentration in the vitamin B12-deficient rats was significantly (P<0·05) lower than that in the -sufficient controls at any time points examined during the hypoxic exposure.

The plasma vitamin B12 level in the vitamin B12-deficient rats was not significantly decreased by the hypoxic exposure for 6 weeks (0·15 (SE 0·011) v. 0·14 (SE 0·031) nm (n 5)). In addition, no significant change in the plasma MMA concentration was observed after the 6-week exposure to hypoxia (0·96 (SE 0·127) v. 0·89 (SE 0·090) nm (n 5)). These results suggest that vitamin B12 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 B12-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<0·05) elevated in both the vitamin B12-sufficient and -deficient groups. In the vitamin B12-sufficient group, the elevated Epo concentration returned to a normal level (normoxic level) in 1 week after starting the exposure.
However, in the vitamin B\textsubscript{12}-deficient rats, the Epo concentration remained at a significantly ($P$<0.05) higher concentration compared with that in normoxia, throughout the 6-week exposure to hypoxia.

### Discussion

Vitamin B\textsubscript{12} deficiency results in the inactivation of methylmalonyl-CoA mutase and methionine synthase, which require 5\textsuperscript{0}-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\textsubscript{12} deficiency, megaloblastic anaemia, in addition to neurological abnormalities, is often observed. The megaloblastic anaemia observed in vitamin B\textsubscript{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\textsubscript{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\textsubscript{12} was reduced to 0.15 nM (15% of that in the vitamin B\textsubscript{12}-sufficient controls) by depleting dietary vitamin B\textsubscript{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\textsubscript{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\textsubscript{12} deficiency under normoxic conditions.

When mammals are exposed to low O\textsubscript{2} conditions, Epo is produced in kidney and erythropoiesis is induced by the action of Epo to provide O\textsubscript{2} into tissues at an adequate level (Hill et al. 1987; Jelkman, 1992; Bunn & Poyton, 1996). To examine the effect of vitamin B\textsubscript{12} deficiency on the hypoxia-induced erythropoiesis, these vitamin B\textsubscript{12}-deficient rats were exposed to 10.5% O\textsubscript{2} conditions for 6 weeks of exposure to hypoxia. The serum Epo concentration during exposure to hypoxia in vitamin B\textsubscript{12}-deficient rats is given in Table 1.

### Table 1. Change in serum erythropoietin (Epo) concentration during exposure to hypoxia in vitamin B\textsubscript{12}-deficient rats†‡

<table>
<thead>
<tr>
<th>Time of hypoxic exposure</th>
<th>Vitamin B\textsubscript{12}-sufficient</th>
<th>Vitamin B\textsubscript{12}-deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean SE</td>
<td>Mean SE</td>
</tr>
<tr>
<td>0 d</td>
<td>24 7.9</td>
<td>28 16.8</td>
</tr>
<tr>
<td>1 d</td>
<td>104† 16.8</td>
<td>188* † 41.0</td>
</tr>
<tr>
<td>1 week</td>
<td>34 10.6†</td>
<td>171† † 39.5</td>
</tr>
<tr>
<td>6 weeks</td>
<td>27 4.9†</td>
<td>108† 30.1</td>
</tr>
</tbody>
</table>

Mean values were significant different from those of the vitamin B\textsubscript{12}-sufficient group at the same time point: *$P$<0.05, †$P$<0.05.

Mean values were significantly different from the values in normoxia (at 0 d) in each group: †$P$<0.05.

† The vitamin B\textsubscript{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.

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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\textsubscript{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\textsubscript{12}-sufficient rats at the same time point: *$P$<0.05. Mean values were significantly different from the values in normoxia (at week 0) in each group: †$P$<0.05.
weeks. In the vitamin B₁₂-sufficient control rats, a significant (P<0.05) 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 B₁₂ deficiency, and the ERC count was not significantly increased even after the 6-week exposure to hypoxia in the vitamin B₁₂-deficient group. It is thus reasonable to postulate that thymidylate and purine biosynthesis are affected under vitamin B₁₂-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<0.05) greater in the vitamin B₁₂-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 B₁₂-deficient conditions. However, the Hb concentration was significantly (P<0.05) lower in the vitamin B₁₂-deficient rats than the -sufficient controls at any time point examined during the 6-week exposure to hypoxia. These observations suggest that a megaloblastic anemia-like symptom is induced in the vitamin B₁₂-deficient rats when exposed to hypoxia.

Carmel & MacPhee (1992) have observed that Epo concentration in serum is abnormally increased in response to the reduction of the Hb concentration in peripheral blood in vitamin B₁₂-deficient patients with anaemia. No significant difference was observed in the serum Epo concentration between the vitamin B₁₂-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 O₂ conditions (Tan et al. 1992). Indeed, when the vitamin B₁₂-sufficient rats were exposed to hypoxia for 6 weeks, the serum Epo concentration was significantly (P<0.05) 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 B₁₂-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 O₂ is not provided into tissues at an adequate level in the vitamin B₁₂-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 inhibited in rats when the plasma vitamin B₁₂ 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 B₁₂-deficient rats are exposed to low O₂ conditions. This is the first report showing a way in which a rat model can be used to study megaloblastic changes associated with vitamin B₁₂ deficiency.

References

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