Special Article*

Studies on Anemia with Special Reference to Megaloblastic Anemia

Gyoichi WAKISAKA

The First Division, Department of Internal Medicine, Faculty of Medicine, Kyoto University

This paper deals with the studies on the pathologic physiology and pathogenesis of megaloblastic anemias caused by deficiency of vitamin \( B_12 \) and folic acid, especially of the former.

I. Intestinal absorption of vitamin \( B_{12} \) in patients with pernicious anemia

It was confirmed by fecal excretion test and Schilling's urinary excretion test using vitamin \( B_{12} \) labelled with \( {}^{60}Co \) (\( {}^{60}Co-B_{12} \)) that the intestinal absorption of vitamin \( B_{12} \) was markedly disturbed in patients with pernicious anemia. When \( {}^{60}Co-B_{12} \) was given by mouth together with hog intrinsic factor in patients with pernicious anemia, the intestinal absorption of \( {}^{60}Co-B_{12} \) was restored to normal. It was also shown that this vitamin \( B_{12} \) absorption test using \( {}^{60}Co-B_{12} \) was very useful for establishing the diagnosis of pernicious anemia in cases with subacute combined degeneration of the spinal cord even when there was no definite hematological manifestation of megaloblastic anemia.

II. Hematological investigation and intestinal absorption of vitamin \( B_{12} \) in patients with gastrectomy

Hematological observations of 126 patients with gastrectomy (5 cases with total gastrectomy, 13 cases with subtotal gastrectomy and 108 cases with partial gastrectomy) showed that there was a tendency to develop hyperchromic macrocytic anemia with the lapse of time following the operation. In some cases the bone marrow picture showed an increase of macroblasts, disturbed maturation of granulocytes and impairment of platelet formation. In patients with partial gastrectomy, the levels of vitamin \( B_{12} \), folic acid and citrovorum factor in the blood showed a tendency to decrease with the lapse of time following the operation, and by vitamin \( B_{12} \) load-

* Presented at the 61st Annual Meeting of the Japanese Society of Internal Medicine, May 8, 1964, in Kyoto.
ing test and folic acid loading test it was found that in these patients there was a tendency of vitamin $B_{12}$ deficiency and the conversion of folic acid to citrovorum factor was impaired (Fig. 2).

In patients with total gastrectomy the intestinal absorption of vitamin $B_{12}$ as determined by fecal excretion test or Schilling's urinary excretion test using $^{60}$Co-$B_{12}$ was markedly disturbed, and it was restored to normal by simultaneous administration of hog intrinsic factor as in patients with pernicious anemia (Fig. 1). It was found by Schilling's urinary excretion test using $^{60}$Co-$B_{12}$ that, even in patients with partial gastrectomy, the intestinal absorption of vitamin $B_{12}$ may be impaired in some cases, and that vitamin $B_{12}$ deficiency may develop with the lapse of time following gastrectomy. In some patients with subtotal gastrectomy, in whom the intestinal absorption of $^{60}$Co-$B_{12}$ was impaired, the absorption of $^{60}$Co-$B_{12}$ was not restored to normal by simultaneous administration of hog intrinsic factor, but it was restored to normal after the treatment with antibiotics (tetracycline) given by mouth. The impairment of intestinal absorption of vitamin $B_{12}$ in patients with partial gastrectomy might be attributed to the decrease of intrinsic factor secretion caused by atrophic gastritis of the residual stomach or to destruction or uptake of vitamin $B_{12}$ by intesti-
nal bacteria as a result of abnormal growth of intestinal flora.

III. Intestinal absorption of folic acid in patients with pernicious anemia, gastrectomy and intestinal diseases

By urinary excretion test using folic acid labelled with $^3$H it was found that the intestinal absorption of folic acid was slightly disturbed in some patients with pernicious anemia and partial gastrectomy (Fig. 3). In patients with partial resection of the small intestine and malabsorption syndrome the intestinal absorption of folic acid was found to be decreased as compared with normal controls.

IV. Experimental studies on the pathogenesis of megaloblastic anemia

In an attempt to produce experimental megaloblastic anemia in animals, 7-10 days young guinea pigs of AD-strain were fed with diet deficient in vitamin $\mathrm{B}_{12}$, folic acid or vitamin C or with diet deficient in two or three of these vitamins. In guinea pigs with single deficiency of vitamin $\mathrm{B}_{12}$ or folic acid or with combined deficiency of vitamin $\mathrm{B}_{12}$, folic acid and vitamin C there were observed a tendency of macrocytic anemia, decrease of leukocyte count, shift to the right of the mean nuclear count of neutrophils and decrease of platelet count. In the bone marrow, megaloblasts or intermediate megaloblasts appeared, and there was a tendency of impairment of leukopoiesis and maturation arrest of granulocytes. These changes were more marked in animals with combined deficiency of vitamin $\mathrm{B}_{12}$, folic acid and vitamin C than in those with single deficiency of vitamin $\mathrm{B}_{12}$ or folic acid.

2) Incorporation of $^{32}$P into the nucleic acid fraction of hematopoietic organs

The incorporation of $^{32}$P into DNA and RNA of bone marrow cells was markedly
decreased in animals with single deficiency of vitamin B₁₂ or folic acid or with combined deficiency of these vitamins, especially in those with combined deficiency. These results suggest that vitamin B₁₂ and folic acid play an important role in nucleic acid synthesis, and that the maturation arrest of erythroblasts and the disturbance of leukopoiesis and thrombopoiesis in animals with deficiency of vitamin B₁₂ and folic acid are closely related to the disturbance of nucleic acid metabolism.

2) Estimation of DNA content of megaloblasts by microspectrophotometry

The average value of DNA content of basophilic and polychromatic megaloblasts of patients with pernicious anemia before treatment as determined by microspectrophotometry was slightly higher than that of basophilic and polychromatic normoblasts of normal controls, and it approached to that of normal controls after the treatment with vitamin B₁₂. In patients with pernicious anemia the distribution of DNA content of basophilic and polychromatic megaloblasts was shifted to the right as compared with that of basophilic and polychromatic normoblasts of normal controls, showing its peak between 2n and 4n (Fig. 4). After treatment of pernicious anemia with vitamin B₁₂, the number of erythroblasts with DNA content more than 4n was decreased, and the number of erythroblasts with DNA content between 2n and 4n was increased. The ratio of DNA content to nuclear area in megaloblasts did not differ from that in normoblasts. Therefore the increase of DNA content of megaloblasts might be attributed to the increase of their nuclear area. With the maturation and decrease of nuclear area the DNA content of normoblasts and megaloblasts decreased both in normal controls and in patients with pernicious anemia before and after treatment. The DNA content of megaloblasts in guinea pigs with combined deficiency of vitamin B₁₂, folic acid and vitamin C showed the same pattern as that in patients with pernicious anemia.

3) Mitotic activity, maturation and DNA synthesis of megaloblasts

The mitotic index of erythroblasts in patients with pernicious anemia before treatment was slightly lower than that in normal controls, and it became normal with the recovery of anemia following the treatment with vitamin B₁₂. The maturation index of erythroblasts in patients with pernicious anemia was increased when the bone marrow of these patients was cultured with the serum of normal controls.
as compared with that when the bone marrow was cultured with the serum of the patient. The maturation index of erythroblasts of these patients became normal after the treatment of the anemia.

Studies on the DNA synthesis of erythroblasts by autoradiography using \(^{3}H\)-thymidine showed that, in patients with pernicious anemia, the percentage of labelled cells and grain count of megaloblasts were decreased as compared with those of normal controls, suggesting impaired DNA synthesis in megaloblasts (Fig. 5). The incorporation of \(^{3}H\)-thymidine into erythroblasts was restored to normal after the treatment with vitamin B\(_{12}\). When the bone marrow of patients with pernicious anemia was cultured with the serum of normal controls, the percentage of \(^{3}H\)-thymidine-labelled basophilic and polychromatic megaloblasts was increased as compared with that when the bone marrow was cultured with the serum of the patient. In the bone marrow of guinea pigs with combined deficiency of vitamin B\(_{12}\), folic acid and vitamin C the percentage of \(^{3}H\)-thymidine labelled cells and grain count were decreased as compared with those in normal controls, suggesting impaired DNA synthesis of erythroblasts in these animals.

From the results mentioned above it might be concluded that the megaloblasts show decrease of DNA synthesis, decrease of mitotic activity and impairment of maturation. The reason for the increase of DNA content in megaloblasts in spite of impaired DNA synthesis might be explained, as suggested by Reisner et al., by impairment of DNA synthesis due to the deficiency of vitamin B\(_{12}\) or folic acid, which leads to disturbance of induction of mitosis, and consequently to an increase of erythroblasts with DNA content of approximately 4n.

V. Nature of intrinsic factor

1) Purification of intrinsic factor from human gastric juice

An attempt was made to purify intrinsic factor from human gastric juice by sephadex-G-100 and DEAE-cellulose column chromatography using \(^{60}Ca-B_{12}\) as an indicator. The vitamin B\(_{12}\) binding capacity of gastric juice after the treatment with DEAE-cellulose column chromatography in patients with pernicious anemia was only 1/5-1/15 as compared with that in normal controls. Purified intrinsic factor obtained by this method from 100 mg. of lyophilized normal human gastric juice was found to be effective at the dose of 2.83 mg. by Schilling's urinary excretion.
Purification of intrinsic factor from hog intrinsic factor concentrate

To 10 gm. of hog intrinsic factor concentrate ("Organon") was added 0.3 μg. of \(^{60}\text{Co-}B_{12}\), and the intrinsic factor was further concentrated according to Holdsworth's method using DEAE-SF cellulose column chromatography. The second peak of protein corresponding to bound \(^{60}\text{Co-}B_{12}\) was collected, dialyzed, lyophilized, and purified further by CM-cellulose column chromatography. Thus about 13 mg. of purified intrinsic factor was obtained from 10 gm. of hog intrinsic factor concentrate. This purified intrinsic factor was found to be effective at the dose of 1.0 mg. by Schilling's urinary excretion method in patients with total gastrectomy. The vitamin \(B_{12}\) binding capacity of this purified intrinsic factor was increased eightyfold, namely from the original value of 0.22 μg./mg. to the final value of 17.4 μg./mg. By the same method as mentioned above purified intrinsic factor was obtained from hog intrinsic factor concentrate ("Nordmark"). On ultracentrifugal analysis of this purified intrinsic factor, one major peak of 3.4 S and one minor peak of 12.7 S were obtained. The major peak was a symmetrical peak containing 94% of the total protein. Purified intrinsic factor obtained from hog intrinsic factor concentrate ("Organon") showed one peak by paper electrophoresis, which corresponded to the third peak of semi-purified intrinsic factor (Wes 818) supplied by the courtesy of Dr. Ellenbogen. The peak observed by PAS stain corresponded to that of protein.

Analysis of the amino acid composition of the purified intrinsic factor obtained from hog intrinsic factor concentrate ("Nordmark") gave the result almost similar to that reported by Holdsworth, while the composition of polysaccharides was slightly different from that reported by Holdsworth. From the result of the ultracentrifugal analysis the molecular weight of the purified intrinsic factor was presumed to be 50,000–55,000. When the molecular weight was assumed to be 55,000, it was calculated that one molecule of purified intrinsic factor combines with 0.72 molecule of vitamin \(B_{12}\), in other words approximately one molecule of purified intrinsic factor combines with one molecule of vitamin \(B_{12}\).

VI. Metabolism of coenzyme \(B_{12}\)

In patients with pernicious anemia the urinary excretion of \(^{64}\text{Co-coenzyme } B_{12}\) following its oral administration was low, and it was restored to normal by simultaneous administration of hog intrinsic factor, suggesting that the intestinal absorption of coenzyme \(B_{12}\) is also dependent on intrinsic factor. Tissue distribution studies in rats using \(^{64}\text{Co-coenzyme } B_{12}\) and \(^{64}\text{Co-}B_{12}\) showed that the hepatic uptake of \(^{64}\text{Co-coenzyme } B_{12}\) following intravenous administration was higher and long-lasting than that of \(^{64}\text{Co-}B_{12}\), and that the simultaneous intravenous administration of hog intrinsic factor enhanced the hepatic uptake of coenzyme \(B_{12}\) and vitamin \(B_{12}\), especially the former.

The content of coenzyme \(B_{12}\) as determined by the method of Abeles and Lee
employing the intramolecular oxidation reduction reaction which converts 1, 2-
propandiol to propion aldehyde was found to be markedly decreased in the liver and
kidney of rats with vitamin $\text{B}_12$ deficiency as compared with that of normal controls,
suggesting that coenzyme $\text{B}_12$ is formed from vitamin $\text{B}_12$. In rats with liver injury
induced by $\text{CCl}_4$ the content of coenzyme $\text{B}_12$ of the liver and kidney was markedly
decreased, and it was gradually restored to normal with the lapse of time following
$\text{CCl}_4$ administration, suggesting that the conversion of vitamin $\text{B}_12$ to coenzyme $\text{B}_12$
is impaired in liver injury.

![Fig. 6. Hepatic uptake of CN-$\text{B}_12$
OH-$\text{B}_12$ and DBCC in rat following admini-
stration with or without H. I. F.](image)

![Fig. 7. Conversion rate of CN- and OH-
$\text{B}_12$ to DBCC in rat and human livers.](image)

Furthermore the tissue distribution of cyanocobalamin, hydroxocobalamin and
coenzyme $\text{B}_12$ was compared in rats using these compounds labelled with $^{60}\text{Co}$ or $^{57}\text{Co}$. The hepatic uptake of coenzyme $\text{B}_12$ was highest, followed by that of hydroxocobalamin, while that of cyanocobalamin was lower than that of hydroxocobalamin (Fig. 6).

In order to demonstrate the conversion of cyanocobalamin and hydroxocobalamin
to coenzyme $\text{B}_12$, cyanocobalamin and hydroxocobalamin labelled with $^{57}\text{Co}$ or $^{60}\text{Co}$ were injected intravenously in rats or man and the radioactivity in the fractions of
cyanocobalamin, hydroxocobalamin and coenzyme $\text{B}_12$ in the liver tissue was deter-
mined at 3, 12, 24, 42 and 72 hours following the administration (Fig. 7). It was
found that both cyanocobalamin and hydroxocobalamin were converted to coenzyme
$\text{B}_12$ with the lapse of time, and the conversion rate of hydroxocobalamin to coenzyme
B$_{12}$ was higher than that of cyanocobalamin.

In rats with liver injury induced by CCl$_4$, the conversion rate of cyanocobalamin and hydroxocobalamin to coenzyme B$_{12}$ was lower than that in normal controls, but even in this case the conversion rate of hydroxocobalamin to coenzyme B$_{12}$ was higher than that of cyanocobalamin. In rats with vitamin B$_{12}$ deficiency the conversion of hydroxocobalamin to coenzyme B$_{12}$ was also higher than that of cyanocobalamin.

**VII. Increase of urinary excretion of methylmalonic acid in coenzyme B$_{12}$ deficiency**

It was confirmed in our experiments that the urinary excretion of methylmalonic acid was markedly increased in guinea pigs with combined deficiency of vitamin B$_{12}$, folic acid and vitamin C. In patients with pernicious anemia before treatment the urinary excretion of methylmalonic acid was markedly increased, and it was decreased to normal following the treatment with vitamin B$_{12}$ (Fig. 8). From these results the increase of urinary excretion of methylmalonic acid may be regarded as a useful indicator of vitamin B$_{12}$ deficiency or disturbance in the utilization of vitamin B$_{12}$.

In patients with pernicious anemia the intramuscular injection of coenzyme B$_{12}$ in the dose of 5 $\mu$g. or 10 $\mu$g. was found to be effective. The effect of coenzyme B$_{12}$ in pernicious anemia was almost similar to that of vitamin B$_{12}$, when their molecular weights were taken into consideration.

It has been demonstrated by our experiments that the liver plays an important role in the conversion of vitamin B$_{12}$ to coenzyme B$_{12}$, but further studies are needed to see whether megaloblastic anemia may develop as a result of impairment of the conversion of vitamin B$_{12}$ to coenzyme B$_{12}$ in liver injury.

Since the discovery of liver therapy for pernicious anemia by Minot and Murphy, the advocation of intrinsic factor and extrinsic factor by Castle and the isolation of vitamin B$_{12}$ by Rickes and Smith, there has been a great progress in the pathogenesis, diagnosis and treatment of pernicious anemia, but much remains to be clarified as to the nature and mechanism of action of intrinsic factor and the mechanism of development of megaloblastic anemia and gastric atrophy, which is responsible for the lack of intrinsic factor in pernicious anemia.

It is hoped that further studies will be made by new techniques in order to
solve these problems.

Co-Workers
Haruto UCHINO, Akima MIYOSHI, Shigeo KARIYONE, Kenichiro ITO, Hisao KUGE, Takeo MIYAKE, Yoshiaki OKUDA, Nobuyoshi IKEMOTO, Shigeo UKYO, Hidenori SOTOYASHI, Hirobumi ANDO, Nobuo YAMAGUCHI, Shigeru SHIRAKAWA, Susumu MITSUTANI, Masahiro FUJI, Yoshinobu YAMAMURA, Yoshiho YAGIRI, Masami INADA, Toshiaki YOSHINO, Shigetoshi OKAWA, Reiji MORISHITA, Akio TODO, Yataro YOSHIDA, Shojiro ISHIHARA, Kazuo WATANABE, Yasuo TANABE, Kyoichi INOUE, Tadashi KANO, Hiroyoshi SAWADA and Shojiro KONOBU

Acknowledgement

The author wishes to express his heartfelt thanks for his co-workers and Professors Hayaishi, Miyake, Kimura, Okamoto, Kyogoku, Fukui, Shimizu, Iwai, Tokuda and Kawaide, Kyoto University, Professor Witts, Oxford University, Professor Glass, New York University, Professor Glass, New York Medical College, Dr. Mezey, Merck Sharp & Dohme, Dr. Perlman, Squibb, Dr. Thompson, Organon, Dr. Ellenbogen, Lederle, and Winthrop and Deutsche Nordmark Co. for their cooperation and kind help in this work. This work was partly supported by the grant from the Ministry of Education, Fujiwara Foundation and Rockefeller Foundation, for which thanks are due.

References