DIRECT ACTION OF VITAMIN B_{12} UPON HUMAN BONE MARROW

THE EFFECT OF INSTILLATIONS OF VITAMIN B_{12} AND FOLIC ACID INTO THE BONE MARROW AS STUDIED BY NUCLEIC ACID STAINING TECHNIQUES [1, 2]

By DANIEL HORRIGAN, THOMAS JARROLD, and RICHARD W. VILTER

(From the Department of Internal Medicine, College of Medicine, University of Cincinnati, Cincinnati, Ohio)

(Submitted for publication July 5, 1950; accepted, October 10, 1950)

Folic acid and vitamin B_{12} are involved in chemical reactions which lead to the formation of nucleic acids in living cells. These relationships are amply demonstrated by studies in bacterial (1–5), animal (6), and human (7) metabolism. It appears likely that folic acid is essential to the formation of various purines and pyrimidines such as thymine from endogenous sources of carbon and amino nitrogen as well as to the interconversion of these substances or of their ribosides (8). Vitamin B_{12}, on the other hand, appears to be active in the formation of ribosides, such as thymidine, from these purines and pyrimidines. Folic acid and vitamin B_{12}, therefore, appear to be active at different stages of a chemical chain reaction which leads to the formation of nucleic acids.

In this paper, we wish to report a method of direct instillation of test substances into human bone marrow cavities. By such a method, observations may be made on the effect of various substances to be tested on marrow cells in their natural environment. By the use of this method, evidence has been obtained that vitamin B_{12} can be utilized unchanged by the megaloblasts and early erythroblasts of patients with pernicious anemia in relapse. On the other hand, folic acid has no such direct effect. Methyl green-pyronin stain applied to the erythroblasts of patients with pernicious anemia in relapse before and after the instillation of vitamin B_{12} into the bone marrow indicates that vitamin B_{12} corrects a qualitative abnormality of the cytoplasmic ribonucleic acid of these cells. Folic acid has a similar effect only after oral or parenteral administration and is presumably activated by enzymatic activity elsewhere in the body. These data offer additional evidence relating vitamin B_{12} and folic acid to nucleic acid metabolism.

[1] This work was aided by a grant from the Robert Gould Research Foundation.

[2] Read in part before the Forty-second Annual Meeting of the American Society for Clinical Investigation, Atlantic City, N.J., May 1, 1950.

MATERIAL AND METHODS

Injections into the marrow cavities of the iliac crests of six persons with pernicious anemia in relapse were performed as follows: three received 1 microgram of crystalline vitamin B_{12} [3] and three received 1 or 2 mgs. of folic acid.[4] In each instance, the material was injected slowly in 1 c.c. of solution after aspiration of 2–3 c.c. of marrow and blood. Moderate pain accompanied the injection and was diminished by slowing the rate of instillation. Forty-eight hours later, bone marrow was again aspirated from the exact site of previous instillation and also from the opposite iliac crest. The site of instillation was easily located by using the trauma produced by the earlier puncture as a landmark. In one case, marrow was aspirated also from sites 4 and 8 centimeters distant from the site of instillation of the vitamin B_{12}. Coverslip preparations were made of each marrow specimen obtained and stained *1)* with Wright-Giemsa stain for differential cell counts, *2)* with methyl green and pyronin B (9) for demonstration of cytoplasmic ribonucleic acid, and *3)* by a Stowell's modification of the Feulgen method (10) for demonstration of desoxyribonucleic acid. The methyl green-pyronin stain was prepared by mixing three parts of a 1 per cent solution of methyl green [5] containing 0.25 per cent liquefied phenol

[3] The material used was "Cobione," supplied by Merck and Co., Inc., Rahway, N.J., and provided in 1 c.c. ampules containing 10 micrograms of crystalline vitamine B_{12} per c.c. Appropriate dilutions were made for this study with physiologic saline solution.

[4] The material used was "Folvite," supplied as a powder by the Lederle division of the American Cyanamid Corp., Pearl River, N.Y. Solutions containing 1 or 2 mgs. of folic acid per c.c. were made in distilled water containing sodium bicarbonate sufficient for a pH of approximately 7.5.

[5] Methyl green and pyronin B were obtained from the National Aniline Division, Allied Chemical and Dye Corp., New York, N.Y. The stains used were certified by the Biological Stain Commission.

TABLE I

Nucleated erythroid cells in bone marrow before and after local instillation of vitamin B_{12}

	Case I			Case II			Case III		
	Before vit. B_{12}	After vit. B_{12}	Opposite ilium after vit. B_{12}	Before vit. B_{12}	After vit. B_{12}	Opposite ilium after vit. B_{12}	Before vit. B_{12}	After vit. B_{12}	Opposite ilium after vit. B_{12}
		1 gamma			*1 gamma*			*1 gamma*	
Megaloblasts } Per 100 WBC	16.0	3.5	11.0	13.5	1.5	17.5	15.5	3.0	13.0
Early erythroblasts } Per 100 WBC	32.0	15.5	32.5	26.5	26.5	28.5	35.5	26.5	37.5
Late erythroblasts } Per 100 WBC	18.0	25.5	14.5	15.0	35.0	18.5	12.0	28.0	20.5
Normoblasts } Per 100 WBC	43.0	100.5	43.5	40.5	90.5	48.0	24.5	87.5	21.5

to one part of a similarly prepared solution of pyronin B.[5] Coverslip marrow films were stained for ten minutes following fixation for one minute in 95 per cent alcohol.

Films of bone marrow from a patient with erythroid hyperplasia due to chronic blood loss and from a normal person were treated in the same manner and served as controls. The red color of the cytoplasm produced by the pyronin stain indicated the presence of ribonucleic acid since this color could not be developed following incubation of films for three hours at 60° C. in a 0.1 per cent solution of crystalline ribonuclease[6] buffered with sodium barbital to a pH of 6.75 (11).

RESULTS

Effect of local marrow instillation of vitamin B_{12} on erythrocyte maturation: Table I is a chart of differential counts of nucleated cells of the erythroid series in bone marrow from three patients with pernicious anemia in relapse before and after the instillation of vitamin B_{12} into the marrow cavity. Stimulation of erythroid maturation is shown in all three instances by a decrease in the number of megaloblasts and an increase in normoblasts in the specimens obtained 48 hours after the local marrow instillation of the Vitamin B_{12} (see Figures 1 and 2). The marrow obtained from the opposite iliac crest was unaffected, indicating that the stimulation induced by vitamin B_{12} was local when the amount instilled was 1 microgram. However, the local marrow instillations of 15 micrograms resulted in a systemic response. In the one instance that marrow was aspirated 4 and

[6] Crystalline ribonuclease was provided by the Worthington Biochemical Laboratory, Freehold, N. J.

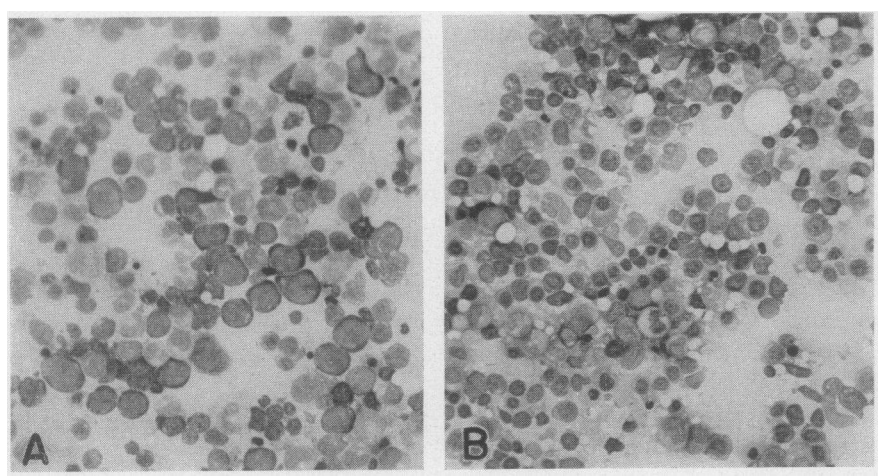

FIG. 1. THE EFFECT OF LOCAL MARROW INSTILLATION OF VITAMIN B_{12} ON WRIGHT-GIEMSA STAINED MARROW FILMS

A megaloblastic maturation arrest characteristic of pernicious anemia is seen in (A). A shift to a normoblastic maturation level at the injection site 48 hours after instillation of vitamin B_{12} is seen in (B). At this time, the marrow obtained from the opposite ilium was identical with (A). (× 320)

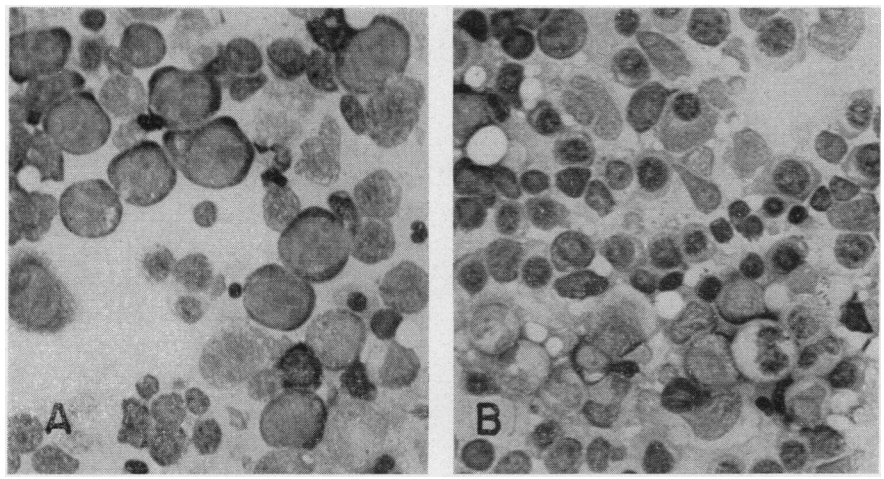

FIG. 2. MAGNIFICATION OF CELLS FROM FIGURE 1

(A) Megaloblasts of pernicious anemia. (B) Normoblasts. Wright-Giemsa stain. (× 650)

8 centimeters from the site of injection, the stimulating effect decreased as the distance increased.

Effect of local marrow instillation of folic acid on erythrocyte maturation: Table II is a chart of differential counts of nucleated erythroid cells in bone marrow from three patients with pernicious anemia in relapse before and after the local marrow instillation of 1 or 2 mgs. of folic acid. It is apparent that there was no stimulation to erythroid maturation at the site of injection of folic acid. The counts done on marrow before and after injection and on marrow from the opposite ilium are all similar. Furthermore, no change in the appearance of Wright-Giemsa stained marrow films was noted after instillation of folic acid into the marrow cavity.

Effect of local marrow instillation of vitamin B_{12} and folic acid on the nucleic acid content of erythroid cells: The character and distribution of ribonucleic acid in the cytoplasm of young erythroid cells were observed in methyl green-pyronin stained preparations of bone marrow from a patient with erythroid hyperplasia resulting from anemia of chronic blood loss. Study of such a preparation (Figure 3) reveals a diffuse and homogeneous distribution of the red granules throughout the cytoplasm of the early erythroblasts. In the later forms, they seem to become more condensed and clumped. Finally, during the normoblastic stage of the development of the erythrocyte, the red color, indicating cytoplasmic ribonucleic acid, disappears altogether.

Methyl green-pyronin stained marrow preparations from patients with pernicious anemia in relapse, one of which is presented in Figure 4-A, show that the distribution of the cytoplasmic ribonucleic acid is quite different in this disease. In these preparations, there is condensation and

TABLE II

Nucleated erythroid cells in bone marrow before and after local instillation of folic acid

	Case IV			Case V			Case VI		
	Before folic acid	After folic acid	Opposite ilium after folic acid	Before folic acid	After folic acid	Opposite ilium after folic acid	Before folic acid	After folic acid	Opposite ilium after folic acid
		1 mg.			*1 mg.*			*2 mgs.*	
Megaloblasts	6.0	7.0	3.5	7.5	8.5	5.5	13.5	15.0	11.0
Early erythroblasts (Per 100 WBC)	10.5	8.0	6.5	25.5	23.0	16.5	22.0	19.0	18.5
Late erythroblasts (Per 100 WBC)	7.0	3.0	4.5	17.5	21.5	16.0	15.0	12.0	10.5
Normoblasts (Per 100 WBC)	24.5	17.5	27.0	21.5	29.0	31.0	20.0	25.0	27.5

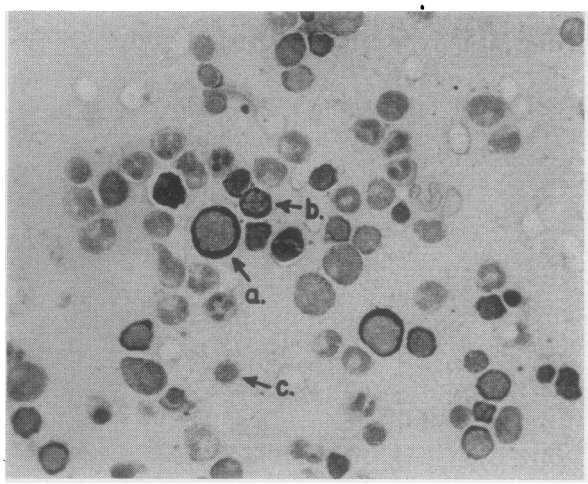

FIG. 3. A FILM OF MARROW FROM A PATIENT WITH CHRONIC BLOOD LOSS ANEMIA STAINED WITH METHYL GREEN AND PYRONIN B

Cytoplasmic ribonucleic acid stains red; nuclear structures stain green. a = early erythroblast; b = late erythroblast; c = normoblast. (× 650)

clumping of the red granules in the cytoplasm of the young cells of the erythroid series. In similarly stained marrow smears (Figure 4-B) after local marrow instillation of vitamin B_{12}, there is a change in the appearance of the cytoplasmic ribonucleic acid of these early erythroid cells. It becomes more diffuse and homogeneous; and, in general, it assumes the characteristics of that observed in early erythroblasts from a patient with anemia due to chronic blood loss.

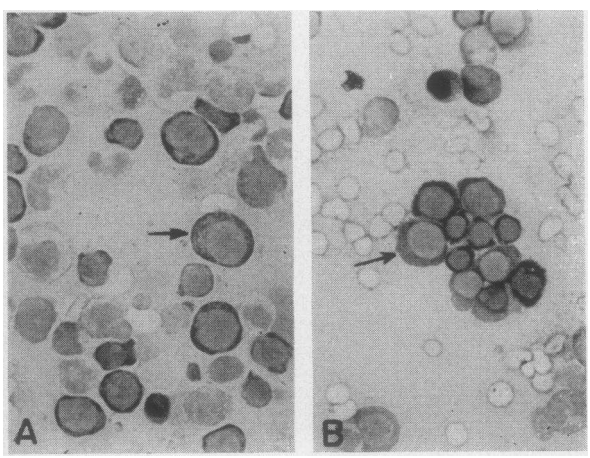

FIG. 4. THE EFFECT OF LOCAL MARROW INSTILLATION OF VITAMIN B_{12} ON METHYL GREEN-PYRONIN B STAINED MARROW FILMS

In (A) arrow points to early erythroblast in marrow from patient with pernicious anemia in relapse. In (B) arrow points to early erythroblast in marrow obtained 48 hours later from the injection site of vitamin B_{12}. (× 650)

No change in the clumped cytoplasmic ribonucleic acid was noted 48 hours after the local marrow instillation of folic acid. However, when folic acid was given orally or intramuscularly in therapeutic doses, the cytoplasmic ribonucleic acid became homogeneous and of more normal appearance.

The appearance of desoxyribonucleic acid stained by Stowell's modification of the Feulgen method was not altered in the preparations obtained after the instillation either of vitamin B_{12} or of folic acid. Furthermore, the desoxyribonucleic acid in erythroblasts from persons with pernicious anemia in relapse had the same appearance as that from a person with anemia of chronic blood loss.

DISCUSSION

The normal maturation of erythroblasts in the bone marrow of persons with pernicious anemia in relapse observed at the site of instillation of vitamin B_{12} but not in marrow from the opposite ilium demonstrates that vitamin B_{12} can be utilized directly by bone marrow cells without alteration by any other organ or tissue. Therefore, if vitamin B_{12} and extrinsic factor of food are the same substance, as seems probable, this experiment provides direct evidence that extrinsic factor need not be chemically changed by intrinsic factor or by the liver before becoming active for erythrocyte maturation. This, of course, is not a new concept. Studies of Berk and his associates (12) on hematologic responses in persons with pernicious anemia, who received vitamin B_{12} and refined liver extract orally along with normal human gastric juice, suggest that vitamin B_{12} (presumably extrinsic factor of food) and the antipernicious anemia principle of liver are identical or closely related compounds. After observing the magnitude of fecal elimination of vitamin B_{12} in patients with untreated pernicious anemia and the hematologic responses in similar patients obtained by the administration of 10 per cent alcoholic extracts of beef muscle, they concluded that ". . . the function of the intrinsic factor of normal human gastric juice is to facilitate the absorption

by the intestine of vitamin B_{12} . . . rather than to react with the extrinsic factor as hitherto assumed."

On the other hand, in our experiment, folic acid was not utilized unchanged within 48 hours after instillation of the substance into the marrow cavity. Observations by others provide further data that folic acid, acting as a substrate, in some way must be altered before becoming active in hematopoiesis. Norris and Majnarich have reported that folic acid has no effect on hematopoiesis as observed by an *in vitro* rabbit bone marrow culture technique (13). However, after this substance was incubated with milk xanthine oxidase, liver homogenate, or extract of rat gastric mucosa, it became hematopoietically active as demonstrated by this method (14). Tove and Elvejhem (15) have reported the presence of a substance in methanol extract of liver which has a sparing effect on folic acid in the metabolism of mink. Furthermore, this substance protected these animals from folic acid deficiency produced by feeding them 7-methyl folic acid.

Further stimulation to the study of the mode of action of folic acid in hematopoiesis was provided by the concentration by Sauberlich (16) of a substance in rat urine which is a growth factor for *Leuconostoc citrovorum* 8081. Studies on this factor, termed the "citrovorum factor," have demonstrated that *1*) the urinary excretion of the substance is increased by oral administration of folic acid both in rats (17) and in human beings (17,18), and *2*) the substance counteracts the toxicity of aminopterin as measured by growth of *L. citrovorum* (17). Data are not yet available on the hematopoietic activity of this substance in erythrocyte maturation factor deficiency anemia. Preliminary studies in this laboratory indicate that the local marrow instillation of four million units of "citrovorum factor" does not result in stimulation of erythrocyte maturation in persons with pernicious anemia in relapse.

The changes observed in the cytoplasmic ribonucleic acid of erythroid precursor cells in marrow from subjects with pernicious anemia in relapse as compared with those from persons with anemia of chronic blood loss provide data linking erythrocyte maturation factor deficiency anemia to abnormalities in nucleoprotein metabolism. Thorell, in 1947 (19), correlated, by cytochemical and ultraviolet microphotographic studies, endocellular growth process in hematopoiesis with the presence of ribonucleic acid in the granulocytic and erythroid precursor cells in bone marrow. Briefly, he found that growth and new cellular protein formation during hematopoiesis are associated with high concentrations of ribonucleic acid in the cytoplasm and the nucleolus of these cells; as growth activity declines, the concentrations of ribonucleic acid decrease proportionately. In normal erythropoiesis, hemoglobin synthesis does not begin until the ribonucleic acid metabolism is completed; after this, the synthesis of hemoglobin proceeds rapidly. In pernicious anemia, there is an abnormality in these relationships of growth and differentiation; in this disease, hemoglobin synthesis begins while the ribose polynucleotide content of the cell is still high. The apparent correction of this abnormality observed in our study after the local marrow instillation of vitamin B_{12} provides additional data linking vitamin B_{12} and nucleoprotein metabolism.

SUMMARY AND CONCLUSIONS

1. A method of direct instillation of test substances into human bone marrow cavities is described.

2. Vitamin B_{12} can be utilized locally by the bone marrow cells and corrects a qualitative abnormality in cellular ribonucleic acid in persons with pernicious anemia in relapse. It need not be altered by stomach or liver to exert this effect.

3. Folic acid is not utilized locally by bone marrow cells within 48 hours of instillation into the marrow cavity; but when given orally or parenterally, it has the same cytologic and cytochemical effects as vitamin B_{12}. It is probable that folic acid must be converted to an active hematopoietic substance by enzymatic activity elsewhere in the body.

REFERENCES

1. Stokes, J. L., Substitution of thymine for "folic acid" in the nutrition of lactic acid bacteria. J. Bact., 1944, **48**, 201.
2. Snell, E. E., and Mitchell, H. K., Purine and pyrimidine bases as growth substances for lactic acid bacteria. Proc. Nat. Acad. Sc., 1941, **27**, 1.
3. Rogers, L. L., and Shive, W., Biochemical transformation as determined by competitive analogue-metabolite growth inhibitions. VII. Relationship

of purines and thymine to folic acid. J. Biol. Chem., 1948, **172**, 751.

4. Shive, W., Ravel, J. M., and Eakin, R. E., An interrelationship of thymidine and vitamin B_{12}. J. Am. Chem. Soc., 1948, **70**, 2614.
5. Shive, W., Ravel, J. M., and Harding, W. M., An interrelationship of purines and vitamin B_{12}. J. Biol. Chem., 1948, **176**, 991.
6. Koch-Weser, D., and Popper, H., Correlation between ribose nucleic acid depletion and other signs of liver damage as influenced by vitamin B_{12}. Proc. Central Soc. Clinical Res., 1949, **22**, 91.
7. Vilter, R. W., Horrigan, D., Mueller, J. F., Jarrold, T., Vilter, C. F., Hawkins, V., and Seaman, A., Studies on the relationships of vitamin B_{12}, folic acid, thymine, uracil and methyl group donors in persons with pernicious anemia and related megaloblastic anemias. Blood, 1950, **5**, 695.
8. Jukes, T. H., Broquist, H. P., and Stokstad, E. L. R., Vitamin B_{12} and "citrovorum factor" in the nutrition of *Lactobacillus leichmannii* and *Leuconostoc citrovorum*. Arch. Biochem., 1950, **26**, 157.
9. Brachet, J., and Shaver, J. R., The effect of nucleases on cytochemical reactions for amino acids and on staining with acid dyes. Stain Technol., 1948, **23**, 177.
10. Stowell, R. E., Feulgen reaction for thymonucleic acid. Stain Technol., 1945, **20**, 45.
11. Popper, H., Personal communication.
12. Berk, L., Castle, W. B., Welch, A. D., Heinle, R. W., Anker, R., and Epstein, M., Observations on the etiologic relationship of achylia gastrica to pernicious anemia. X. Activity of vitamin B_{12} as food (extrinsic) factor. New England J. Med., 1948, **239**, 911.
13. Norris, E. R., and Majnarich, J. J., Effect of xanthopterin on cell proliferation in bone marrow cultures. Am. J. Physiol., 1948, **152**, 175.
14. Norris, E. R., and Majnarich, J. J., Action of enzymes on vitamin B_{14} and pteridines. Science, 1949, **109**, 33.
15. Tove, S. B., and Elvejhem, C. A., Relation of a factor in a methanol extract of liver and pteroylglutamic acid in mink. Federation Proc., 1949, **8**, 399.
16. Sauberlich, H. E., The relationship of folic acid, vitamin B_{12}, and thymidine in the nutrition of *Leuconostoc citrovorum* 8081. Arch. Biochem., 1949, **24**, 224.
17. Sauberlich, H. E., The effect of folic acid on the urinary excretion of the growth factor required by *Leuconostoc citrovorum*. J. Biol. Chem., 1949, **181**, 467.
18. Anker, R. M., Boehne, J. W., and Welch, A. D., A folic acid-derived growth factor for *Leuconostoc citrovorum* in human urine. Federation Proc., 1950, **9**, 351.
19. Thorell, B., Studies on the formation of cellular substances during blood cell production. Acta med. Scandinav., 1947, Suppl. **200**, 1.