

EFFECT OF VITAMIN B₁₂ *IN VITRO* ON INCORPORATION OF NUCLEIC ACID PRECURSORS BY PERNICIOUS ANEMIA BONE MARROW *

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Glaser and associates (1) reported that the ratios RNA to DNA and uracil to thymine were about 2.5 times higher in bone marrow from pernicious anemia (PA) patients before treatment than in bone marrow from normal subjects. Clinical cures caused these ratios to return to normal. Beck (2) has reviewed the evidence that the major consequence of vitamin B₁₂ deficiency is impaired deoxyribonucleotide synthesis, resulting in retardation of DNA synthesis and cell division. He suggests that the megaloblast of vitamin B₁₂ deficiency is in a state of "unbalanced growth" that is analogous to the filamentous forms of *Lactobacillus leichmannii* produced by vitamin B₁₂ or deoxyribonucleoside starvation. However, observations of the effect of vitamin B₁₂ on morphology of megaloblasts in suspension cultures have been contradictory. At least five studies reported no effect, whereas two investigators found some replacement by normoblastic cells (3).

Two investigations have tested the effect of vitamin B₁₂ *in vitro* on DNA synthesis by PA bone marrow. Thomas and Lochte (4) measured the incorporation of radioactive formate by PA marrows incubated for 5 and 10 hours in PA serum with and without added B₁₂. Specific activities of DNA thymine were 1.3 to 2.7 times higher with B₁₂. Lessner and Friedkin (5), on the contrary, were unable to demonstrate a reproducible effect of B₁₂ on incorporation of deoxyuridine-2-C¹⁴ by washed cell suspensions from B₁₂-deficient megaloblastic marrows incubated for 3 hours.

The availability of a number of patients with untreated pernicious anemia prompted us to extend these studies to the use of radioactive precursors of both DNA and RNA and of both py-

rimidines and purines. We thus measured the effect of vitamin B₁₂ on incorporation of cytidine-2-C¹⁴ and adenine-8-C¹⁴ by PA bone marrow. To evaluate the specificity of any stimulation or inhibition, we also measured the effect of B₁₂ on incorporation by nonmegaloblastic, hyperplastic erythroid marrows from hemolytic anemia.

METHODS

Seven to 10 ml heparinized bone marrow were removed by sternal aspiration from patients with untreated pernicious anemia or with hemolytic anemia (HA). Marrow from PA Patients 1 to 3 was passed through a wire screen attached to a syringe. This procedure was discontinued because of loss of marrow particles during manipulation. It was found that the marrow could be dispersed with a glass rod after delivery into a flask. Enough Robinson's glucose-salts medium (6) was added to give a total of 12 ml (or 18 ml with PA Patient 5). Samples of 3.0 ml were pipetted into 20-ml Warburg vessels containing 0.1 ml radioactive precursor and 0.05 ml vitamin B₁₂ (hydroxycobalamin)¹ or saline. Samples in the various experiments contained 1 to 3 × 10⁸ nucleated cells. The vessels were shaken for 2 hours at 38° C under an atmosphere of 95% air:5% carbon dioxide. They were then removed to an ice bath, and subsequent operations were carried out at 3° C. Contents of the vessels were centrifuged 10 minutes at 1,470 × g, and the supernatant fluid was discarded. The cells were washed with 10 vol of cold nonradioactive Robinson's medium, once in cytidine incorporations, and twice in adenine incorporations where cellular acid-soluble adenine was to be isolated. The washes were discarded and the centrifuged cells were extracted with three 3.0-ml portions of 0.2 M perchloric acid.

Purine and pyrimidine bases were isolated, purified, and measured for radioactivity as previously described (6). Differences in the present study were as follows. After separation of RNA and DNA fractions from cytidine incorporations, the RNA supernatant fluid was taken to dryness in a vacuum desiccator and hydrolyzed with 70% perchloric acid as described for the DNA precipitate. After hydrolysis, perchlorate was removed by

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¹ Merck & Co., Inc., Rahway, N. J.

TABLE I
Effect of vitamin B₁₂ on 2-hour incorporations of adenine and cytidine by
megaloblastic bone marrow from untreated pernicious anemia patients

Adenine-8-C ¹⁴ , 1.0 μ mole 1,350,000 cpm/ μ mole	DNA adenine	DNA guanine	Cell acid-soluble adenine	RNA adenine	RNA guanine
Patient 1					
SA without B ₁₂ *	3,172 \pm 115†	511 \pm 40	226,700 \pm 1,600	34,660 \pm 1,340	2,891 \pm 54
SA with B ₁₂ , 5 $m\mu$ g	5,581 \pm 136	853 \pm 28	235,200 \pm 4,900	38,160 \pm 980	3,083 \pm 18
$\frac{B_{12}}$					
No B ₁₂	1.76	1.67	1.04	1.10	1.07
Patient 2					
SA without B ₁₂	4,296 \pm 124	528 \pm 32	213,700 \pm 7,000	37,330 \pm 290	3,224 \pm 84
SA with B ₁₂ , 5 $m\mu$ g	5,298 \pm 10	564 \pm 46	217,700 \pm 8,700	37,960 \pm 380	3,111 \pm 95
$\frac{B_{12}}$					
No B ₁₂	1.23	1.07	1.02	1.02	.96
Patient 3					
SA without B ₁₂	1,222 \pm 120		102,400 \pm 2,500	18,290 \pm 490	
SA with B ₁₂ , 5 $m\mu$ g	1,565 \pm 87		113,100 \pm 5,100	18,250 \pm 1,170	
$\frac{B_{12}}$					
No B ₁₂	1.28		1.10	1.00	
Cytidine-2-C ¹⁴ , 1.0 μ mole 662,000 cpm/ μ mole					
	DNA thymine	DNA cytosine		RNA uracil	RNA cytosine
Patient 4					
SA without B ₁₂	1,007 \pm 29	2,169 \pm 36		19,180 \pm 140	9,378 \pm 187
SA with B ₁₂ , 5 $m\mu$ g	1,358 \pm 27	2,516 \pm 82		19,440 \pm 160	8,867 \pm 170
$\frac{B_{12}}$					
No B ₁₂	1.35	1.16		1.01	.95
Patient 5					
SA without B ₁₂	1,558 \pm 33	2,860 \pm 10		21,940 \pm 120	9,137 \pm 113
SA with B ₁₂ , 5 $m\mu$ g	1,946 \pm 59	3,184 \pm 125		21,140 \pm 690	9,626 \pm 57
$\frac{B_{12}}$					
No B ₁₂	1.25	1.11		.96	1.05
SA with B ₁₂ , 50 $m\mu$ g	2,256 \pm 10	3,488 \pm 19		21,540 \pm 450	8,640 \pm 99
$\frac{B_{12}}$					
No B ₁₂	1.45	1.22		.98	.95

* Specific activities are given in counts per minute per micromole.

† Variation from the mean of duplicate flasks.

precipitation with KOH as described. The supernatant fluid was then chromatographed on paper without the extra step of column chromatography.

In experiments employing washed marrow cells, the procedure differed as follows. Aspirated marrow was centrifuged 5 minutes at $1,470 \times g$, and the plasma was discarded. The cells were stirred gently in 5 vol of Robinson's medium, recentrifuged, and suspended in a fresh portion of the medium. Three-ml portions of this suspension were used in the incubation.

RESULTS

The data of Table I show that 5 $m\mu$ g B₁₂ caused a small but consistent increase in the incorporation of both purine and pyrimidine precursors into DNA of PA marrows. Incorporation of cytidine

into DNA of marrow 5 was higher with 50 $m\mu$ g B₁₂ than with 5 $m\mu$ g. Only with marrow 1, where B₁₂ caused the greatest stimulation of incorporation into DNA (1.7 to 1.8 times), was there any increased labeling of RNA (1.1 times). Table II presents data for incorporation of cytidine by three hemolytic anemia marrows. Addition of 5 $m\mu$ g B₁₂ to these marrows caused no stimulation of incorporation into either RNA or DNA; the largest increase, with DNA thymine of HA marrow 1, was only 1.07 times. With no added B₁₂ (control vessels), the two PA marrows showed a higher incorporation of cytidine into RNA than did the three HA marrows, whereas incorporation into DNA by the two marrow types (incu-

TABLE II
Effect of vitamin B₁₂ on 2-hour incorporation of cytidine by hemolytic anemia bone marrow

Cytidine-2-C ¹⁴ , 1.0 μmole 662,000 cpm/μmole	DNA thymine	DNA cytosine	RNA uracil	RNA cytosine
Patient 1				
SA without B ₁₂ *	1,093 ± 73†	2,013 ± 13	10,740 ± 401	6,410 ± 529
SA with B ₁₂ , 5 mμg	1,169 ± 51	1,935 ± 20	10,870 ± 200	6,491 ± 44
$\frac{B_{12}}$				
No B ₁₂	1.07	.96	1.01	1.01
Patient 2				
SA without B ₁₂	1,377 ± 67	2,490 ± 65	9,116 ± 647	6,990 ± 128
SA with B ₁₂ , 5 mμg	1,406 ± 43	2,500 ± 102	9,319 ± 328	6,187
$\frac{B_{12}}$				
No B ₁₂	1.02	1.00	1.02	.89
Patient 3				
SA without B ₁₂	1,321 ± 58	2,781 ± 30	9,249 ± 29	8,489 ± 354
SA with B ₁₂ , 5 mμg	1,279 ± 28	2,873 ± 28	9,421 ± 19	8,691 ± 243
$\frac{B_{12}}$				
No B ₁₂	.97	1.03	1.02	1.02

* Specific activities are given in counts per minute per micromole.

† Variation from the mean of duplicate flasks.

TABLE III
Effect of vitamin B₁₂ on 2-hour incorporations of cytidine by washed suspensions of hemolytic anemia (HA) and pernicious anemia (PA) bone marrow

Cytidine-2-C ¹⁴ , 1.0 μmole 662,000 cpm/μmole	DNA thymine	DNA cytosine	RNA uracil	RNA cytosine
Suspended in glucose-salts medium				
HA Patient 1				
SA without B ₁₂ *	454 ± 28†	851 ± 36		
SA with B ₁₂ , 5 mμg	452 ± 14	817 ± 46		
$\frac{B_{12}}$				
No B ₁₂	1.00	.96		
HA Patient 4				
SA without B ₁₂	726 ± 36	1,128 ± 16	7,835 ± 304	4,510 ± 65
SA with B ₁₂ , 5 mμg	714 ± 63	1,054 ± 21	8,020 ± 201	4,573 ± 101
$\frac{B_{12}}$				
No B ₁₂	.98	.93	1.02	1.01
PA Patient 6				
SA without B ₁₂	1,506 ± 84	3,094 ± 153	14,450 ± 580	7,607 ± 463
SA with B ₁₂ , ‡ 5 mμg	2,172	3,570	13,540	7,337
$\frac{B_{12}}$				
No B ₁₂	1.44	1.15	.94	.96
Suspended in PA plasma 6				
HA Patient 4				
SA without B ₁₂	628 ± 12	1,024 ± 20	10,060 ± 400	4,933 ± 84
SA with B ₁₂ , 5 mμg	645 ± 6	1,063 ± 30	10,170 ± 50	5,198 ± 396
$\frac{B_{12}}$				
No B ₁₂	1.03	1.04	1.01	1.05

* Specific activities are given in counts per minute per micromole.

† Variation from the mean of duplicate flasks.

‡ Only one flask containing B₁₂ with this marrow.

bated under the same conditions) was of similar magnitude.

The data of Table II demonstrated that added B₁₂ did not stimulate incorporation by HA marrow suspended in its own plasma diluted 1–1.2 to 1–1.7 with glucose-salts medium. The amount of B₁₂ added, 1.7 m μ g per ml, was well above the level of 0.15 to 0.9 m μ g per ml in normal and HA plasma (7). However, PA plasma would contain less than 0.1 m μ g per ml. Thus, the possibility remained that the extra amount of B₁₂ in the HA plasma was sufficient to give a maximal effect *in vitro*, and that the stimulatory effect was not specific for the B₁₂-deficient megaloblasts. Another sample of marrow from HA Patient 1 and a sample from a fourth HA patient were treated as follows. Cells were centrifuged, washed with 5 vol of glucose-salts medium, and resuspended in a fresh portion of the medium. This procedure would serve to remove intercellular B₁₂ but not protein-bound intracellular B₁₂. As shown in Table III, 5 m μ g B₁₂ did not stimulate incorporation by these washed HA marrows.

Lear, Harris, Castle, and Fleming (8) have shown that both PA and normal sera have a factor or factors which bind some of the B₁₂ added *in vitro*. In their experiments, 1 ml of serum bound .45 to .66 m μ g B₁₂. To determine if these or other plasma factors were necessary to demonstrate a stimulatory effect of B₁₂ on PA marrows, the marrow from a sixth PA patient was washed and suspended in glucose-salts medium as described above. With 5 m μ g B₁₂, incorporation of cytidine into DNA thymine was increased 1.4 times (Table III). This experiment, however, was not entirely satisfactory, since one of the duplicate samples with B₁₂ was destroyed during a laboratory procedure. Results thus represent two control flasks and one flask with B₁₂.

To demonstrate in another way that the failure of B₁₂ to stimulate incorporation by HA cells suspended in glucose-salts medium was not owing to lack of plasma factors, another marrow sample from HA Patient 4 was washed in glucose-salts medium and then suspended in plasma from the marrow aspirate of PA Patient 6. However, 5 m μ g vitamin B₁₂ still did not stimulate incorporation by these HA marrow cells (Table III).

DISCUSSION

In seven tests with PA marrows incubated in PA serum for 5 and 10 hours, Thomas and Lochte reported that 10 and 100 m μ g B₁₂ per ml stimulated the incorporation of formate into DNA 1.3 to 2.7 times. However, in one experiment these workers obtained the same stimulation (1.4 times) with 1, 10, and 100 m μ g B₁₂ per ml. Moreover, they reported that the addition of B₁₂ to PA marrow incubated in normal serum produced no significant increase in DNA synthesis. These data indicated that the level of B₁₂ in normal sera might exert a maximal effect.

The amount of B₁₂ added in our experiments, 1.7 m μ g per ml, is about twice the highest level to be expected in plasma from normal and HA subjects. With six PA marrows (five suspended in their own plasma and one washed and suspended in glucose-salts medium), incorporation of adenine and cytidine into DNA adenine and thymine, respectively, was increased 1.2 to 1.8 times by this low level of added vitamin. Thus, we have confirmed the stimulation of incorporation into DNA with B₁₂ reported by the above workers and have shown that this effect occurs with both pyrimidine and purine precursors. Moreover, the present work has demonstrated that incorporation into RNA is not stimulated, at least when measured after the 2-hour incubation period. With the one marrow tested, 17 m μ g B₁₂ per ml caused a greater stimulation of incorporation into DNA than did 1.7 m μ g per ml, but yet no increased labeling of RNA. We have not attempted to test the hypothesis that vitamin B₁₂ participates in the conversion of ribonucleosides (or ribonucleotides) to deoxyribonucleosides (or deoxyribonucleotides). Adenine and cytidine were chosen as radioactive precursors because they were readily incorporated into both RNA and DNA of bone marrow incubated under conditions similar to those used in our studies with human white blood cells (6, 9).

Barker (10) has reviewed evidence that co-bamide coenzymes are the active forms of vitamin B₁₂ in three enzymatic reactions occurring in some microorganisms. One of these, the methylmalonyl isomerase reaction, also occurs in animals. Either a coenzyme form of B₁₂ is not required to stimulate *in vitro* DNA synthesis by megaloblasts.

blasts, or these cells can convert hydroxycobalamin to coenzyme B₁₂. Evidence of the local action of B₁₂ *in vivo* has been given by Horrigan, Jarrold, and Vilter (11), who demonstrated that direct instillation of cyanocobalamin into the marrow cavity of one iliac crest induced local transformation from megaloblastic to normoblastic proliferation without causing changes in the opposite iliac crest.

Added B₁₂ did not stimulate incorporation by washed HA cells suspended in a glucose-salts medium or in PA plasma. Evidently, the intracellular protein-bound B₁₂ in these nonmegaloblastic cells is sufficient for maximal incorporation under our *in vitro* conditions.

The stimulation by B₁₂ of incorporation into DNA but not into RNA of PA marrows would be in agreement with the clinical findings that the abnormally high RNA to DNA ratio in megaloblasts returns to normal after successful treatment with this vitamin. When B₁₂-requiring *L. leichmannii* is grown in the absence of the vitamin, DNA synthesis and cell division are impaired without concomitant inhibition of RNA and protein synthesis (12, 13). The resulting cytoplasmic growth produces filamentous organisms with an elevated RNA to DNA ratio. It is likely that the increased incorporation into DNA of PA marrows when B₁₂ is added is related to a similar state of unbalanced growth in the megaloblast.

SUMMARY

Five pernicious anemia bone marrows were incubated for 2 hours in their own plasma diluted with a glucose-salts medium, and one in glucose-salts medium alone. Added vitamin B₁₂ (hydroxycobalamin), 1.7 mμg per ml, stimulated the incorporation of adenine-8-C¹⁴ and cytidine-2-C¹⁴ into DNA bases but not into RNA bases. Addition of this level of B₁₂ to hemolytic anemia marrows, incubated in their own plasma, pernicious anemia plasma, or glucose-salts medium did not stimulate incorporation of cytidine-2-C¹⁴ into either RNA or DNA.

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REFERENCES

1. Glaser, H. S., J. F. Mueller, T. Jarrold, K. Sakurai, J. J. Will, and R. W. Vilter. Effect of vitamin B₁₂ and folic acid on nucleic acid composition of the bone marrow of patients with megaloblastic anemia. *J. Lab. clin. Med.* 1954, 43, 905.
2. Beck, W. S. The metabolic functions of vitamin B₁₂. *New Engl. J. Med.* 1962, 266, 708, 765, 814.
3. Reisner, E. H., Jr. The nature and significance of megaloblastic blood formation. *Blood* 1958, 13, 313.
4. Thomas, E. D., and H. L. Lochte, Jr. Studies on the biochemical defect of pernicious anemia. I. *In vitro* observations on oxygen consumption, heme synthesis and deoxyribonucleic acid synthesis by pernicious anemia bone marrow. *J. clin. Invest.* 1958, 37, 166.
5. Lessner, H. E., and M. Friedkin. The *in vitro* incorporation of deoxyuridine and thymidine into deoxyribonucleic acid of human megaloblastic and normoblastic bone marrows. *Clin. Res.* 1959, 7, 207.
6. Williams, A. M., and R. F. Schilling. The *in vitro* incorporation of nucleic acid precursors into leukemic human leukocytes. I. Methodology and effects of therapy on incorporation *in vitro*. *J. Lab. clin. Med.* 1961, 58, 76.
7. Mollin, D. L. The megaloblastic anemias. *Ann. Rev. Med.* 1960, 11, 333.
8. Lear, A. A., J. W. Harris, W. B. Castle, and E. M. Fleming. The serum vitamin B₁₂ concentration in pernicious anemia. *J. Lab. clin. Med.* 1954, 44, 715.
9. Williams, A. M. Nucleic acid metabolism in leukemic human leukocytes. I. *In vitro* incorporation by leukocytes from chronic granulocytic leukemia. *Cancer Res.* 1962, 22, 314.
10. Barker, H. A. Chemistry and biology of the vitamin-B₁₂-coenzymes *in Vitamin B₁₂ und Intrinsic Faktor*, 2nd Europäisches Symposium, Hamburg, 1961, H. C. Heinrich, Ed. Stuttgart, Ferdinand Enke, 1962, p. 82.
11. Horrigan, D., T. Jarrold, and R. W. Vilter. Direct action of vitamin B₁₂ upon human bone marrow. The effect of instillations of vitamin B₁₂ and folic acid into the bone marrow as studied by nucleic acid staining techniques. *J. clin. Invest.* 1951, 30, 31.
12. Beck, W. S., S. Hook, and B. H. Barnett. The metabolic functions of vitamin B₁₂. I. Distinctive modes of unbalanced growth behavior in *Lactobacillus leichmannii*. *Biochim. biophys. Acta (Amst.)* 1962, 55, 455.
13. Beck, W. S., M. Goulian, and S. Hook. The metabolic functions of vitamin B₁₂. II. Participation of vitamin B₁₂ in the biosynthesis of deoxyribonucleic acid and its acid-soluble precursors. *Biochim. biophys. Acta (Amst.)* 1962, 55, 470.