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Research Article

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EFFECT OF VITAMIN B_{12} *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 B_{12} deficiency is impaired deoxyribonucleotide synthesis, resulting in retardation of DNA synthesis and cell division. He suggests that the megaloblast of vitamin B_{12} deficiency is in a state of "unbalanced growth" that is analogous to the filamentous forms of *Lactobacillus leichmannii* produced by vitamin B_{12} or deoxyribonucleoside starvation. However, observations of the effect of vitamin B_{12} 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 B_{12} *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 B_{12}. Specific activities of DNA thymine were 1.3 to 2.7 times higher with B_{12}. Lessner and Friedkin (5), on the contrary, were unable to demonstrate a reproducible effect of B_{12} on incorporation of deoxyuridine-2-C^{14} by washed cell suspensions from B_{12}-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 pyrimidines and purines. We thus measured the effect of vitamin B_{12} on incorporation of cytidine-2-C^{14} and adenine-8-C^{14} by PA bone marrow. To evaluate the specificity of any stimulation or inhibition, we also measured the effect of B_{12} 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 B_{12} (hydroxycobalamin)[1] or saline. Samples in the various experiments contained 1 to 3×10^8 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 $1,470 \times g$, 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 B_{12} on 2-hour incorporations of adenine and cytidine by megaloblastic bone marrow from untreated pernicious anemia patients

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

bated under the same conditions) was of similar magnitude.

The data of Table II demonstrated that added B_{12} 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 B_{12} added, 1.7 mμg per ml, was well above the level of 0.15 to 0.9 mμg per ml in normal and HA plasma (7). However, PA plasma would contain less than 0.1 mμg per ml. Thus, the possibility remained that the extra amount of B_{12} in the HA plasma was sufficient to give a maximal effect *in vitro,* and that the stimulatory effect was not specific for the B_{12}-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 B_{12} but not protein-bound intracellular B_{12}. As shown in Table III, 5 mμg B_{12} 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 B_{12} added *in vitro.* In their experiments, 1 ml of serum bound .45 to .66 mμg B_{12}. To determine if these or other plasma factors were necessary to demonstrate a stimulatory effect of B_{12} on PA marrows, the marrow from a sixth PA patient was washed and suspended in glucose-salts medium as described above. With 5 mμg B_{12}, 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 B_{12} was destroyed during a laboratory procedure. Results thus represent two control flasks and one flask with B_{12}.

To demonstrate in another way that the failure of B_{12} 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 mμg vitamin B_{12} 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 mμg B_{12} 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 mμg B_{12} per ml. Moreover, they reported that the addition of B_{12} to PA marrow incubated in normal serum produced no significant increase in DNA synthesis. These data indicated that the level of B_{12} in normal sera might exert a maximal effect.

The amount of B_{12} added in our experiments, 1.7 mμg 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 B_{12} 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 mμg B_{12} per ml caused a greater stimulation of incorporation into DNA than did 1.7 mμg per ml, but yet no increased labeling of RNA. We have not attempted to test the hypothesis that vitamin B_{12} 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 cobamide coenzymes are the active forms of vitamin B_{12} in three enzymatic reactions occurring in some microorganisms. One of these, the methylmalonyl isomerase reaction, also occurs in animals. Either a coenzyme form of B_{12} is not required to stimulate *in vitro* DNA synthesis by megalo-

blasts, or these cells can convert hydroxycobalamin to coenzyme B_{12}. Evidence of the local action of B_{12} *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 B_{12} did not stimulate incorporation by washed HA cells suspended in a glucose-salts medium or in PA plasma. Evidently, the intracellular protein-bound B_{12} in these nonmegaloblastic cells is sufficient for maximal incorporation under our *in vitro* conditions.

The stimulation by B_{12} 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 B_{12}-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 B_{12} 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 B_{12} (hydroxycobalamin), 1.7 mμg per ml, stimulated the incorporation of adenine-8-C^{14} and cytidine-2-C^{14} into DNA bases but not into RNA bases. Addition of this level of B_{12} to hemolytic anemia marrows, incubated in their own plasma, pernicious anemia plasma, or glucose-salts medium did not stimulate incorporation of cytidine-2-C^{14} into either RNA or DNA.

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