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Vitamin B_{12} and the Megaloblastic Development

Abstract. *In patients with vitamin B_{12} deficiency, phytohemagglutinin-stimulated cultured lymphocytes had little or no thymidylate synthetase activity. Ample activity was found in such lymphocytes from normal individuals and patients with folic acid deficiency or pernicious anemia in remission. It therefore appears that the megaloblastosis that is associated with vitamin B_{12} deficiency is related to low thymidylate synthetase activity.*

In a study of the folate coenzymes in an unusual case of megaloblastic anemia due to defective utilization or transport of vitamin B_{12}, it was found that during relapse although vitamin B_{12} in the serum was high (2000 to 3000 pg/ml) the thymidylate synthetase activity of cultured phytohemagglutinin-stimulated lymphocytes was low. Accordingly we studied this enzyme in patients with vitamin B_{12} deficiency both in relapse and in remission, in patients with folic acid deficiency, and in normal individuals. The enzyme activity was either low or absent only in lymphocytes of patients with vitamin B_{12} deficiency. We assumed that the various biochemical activities of the stimulated human lymphocyte are the same as those that occur in the human pronormoblast. Others have provided cytological and biochemical evidence that deficiency of vitamin B_{12} or folate cause derangement of DNA synthesis in the human lymphocyte (*1*).

The lymphocytes were collected and cultured as follows (*2*): blood (100 ml) was collected from each individual and placed in a heparinized flask. An equal volume of 3 percent high molecular weight dextran was added. In most of the experiments the lymphocytes (50×10^6 to about 100×10^6) were separated from the rest of the leukocytes by a Ficoll (Pharmacia) gradient. The cells were cultured in two portions (60 ml each) in medium 199 (Grand Island Biological Company) in the presence of 0.025 percent of phytohemagglutinin M (Difco) and newborn calf serum (20 percent). The final culture medium contained folic acid (8 ng/ml) and vitamin B_{12} (60 pg/ml). The amount of vitamin B_{12} in the medium is obviously extremely small and that of folic acid is still below the minimum amount needed for appreciable uptake by stimulated lymphocytes (*3*). The cells were incubated at 37°C in an atmosphere containing 5 percent CO_2 (General Electric Hotpack CO_2 incubator). The stimulated lymphocytes were harvested on day 3. A smear was stained and differential cell count made. The cells were counted. Some cells were used for the uptake of radioactive nucleosides, and the rest were lysed and used for protein determination (*4*) and the assay of thymidylate synthetase. The enzyme was assayed by the method of Kammen (*5*). The complete enzyme assay system (*5*) contained 0.1 ml of lysate or water and 0.1 ml of the following: formaldehyde (1 μmole); DL-tetrahydrofolate (0.1 μmole); tris·HCl buffer (*p*H 7.4) (10.0 μmole); magnesium chloride (4.0 μmole); 2-mercaptoethanol (20.0 μmole); and deoxyuridine monophosphate (dUMP) (0.02 μmole) (partly containing [5-^{3}H]-dUMP). Tetrahydrofolic acid was prepared by hydrogenation of folic acid (*6*). Tetrahydrofolic acid is spontaneously converted to the active form in the assay. Thymidylate synthetase activity results in the release of a tritium atom as a proton when thymidylic acid is formed. We made a dilution curve for the enzyme activity by using various dilutions of a strain of *Escherichia coli* (*7*). This established the minimum amount of cell lysate to be tested. The uptake of nucleosides by the stimulated lymphocytes was measured by incubating 10^6 cells (occasionally less) for 1 hour in Hanks buffered solution (*8*) at 37°C with one ^{14}C-labeled nucleoside, either thymidine, deoxyuridine, or uridine (no unlabeled nucleoside was added). At the end of the incubation, the tubes were chilled in ice. The cells were filtered on cellulose acetate membranes (pore size, 5 μm). The residue in the tubes was rinsed onto the membranes with 10 ml of ice-cold balanced saline. The membranes were washed with two 10-ml volumes of ice-cold 5 percent trichloroacetic acid and then placed in scintillation vials and digested overnight with 0.5 ml NCS solubilizer (Amersham/Searle). We measured radioactivity in a liquid scintillation counter (*9*). The nucleoside uptake was expressed as a percentage of the total amount of nucleoside in the incubation medium per 10^7 cells.

Many of the vitamin B_{12} deficient patients were suffering from leukopenia and so the recovery of lymphocytes from their peripheral blood was often reduced (but not by less than 50 percent). However, these lymphocytes did undergo blastogenesis following PHA stimulation. We detected blastogenesis by both morphological criteria and by assaying the uptake of [^{14}C]thymidine by the cells. The uptake of [^{14}C]thymidine in the vitamin B_{12} deficiency group ranged from 3.0 to 14.0 percent with an average of 6.0 ± 3.7 percent S.D.; in the normal group it ranged from 2.6 to 16.0 per-

Table 1. Thymidylate synthetase activity in phytohemagglutinin-stimulated lymphocytes. The enzyme activity is expressed as the uptake of dUMP by 10^7 cells. Two of the six patients with pernicious anemia were studied both in relapse and in remission; the others were studied only in remission.

Condition	Number of individuals	Enzyme activity (100 pmole/hr)	Average activity
Vitamin B_{12} deficiency	8	0.0–2.9	1.1*
Pernicious anemia			
Relapse	2	0.0	
Remission	2	6.8, 6.1	
Relapse			
Remission	4	3.3–12.9	7.1†
Folic acid deficiency	4	7.4–10.5	8.9
Normal	15	4.7–13.8	8.2

* The enzyme activity is the average of the activities of the enzymes from patients with vitamin B_{12} deficiency including the activities of the two patients with pernicious anemia in relapse. † The enzyme activity is the average for the activities of the enzymes from all six patients with pernicious anemia in remission.

cent with an average of 6.7 ± 4.3 percent S.D. Similarly, there was no significant difference in the uptake of the other nucleosides by the various groups. Fewer cells from the vitamin B_{12} deficiency group survived the 3-day PHA cultures (70 percent), but this was taken into account when making the cell lysate for enzyme assay.

Vitamin B_{12} deficiency was established by determining the level of vitamin B_{12} in serum (*10*), by the characteristic appearance of the peripheral blood and by the megaloblastic appearance of the bone marrow. Pernicious anemia was confirmed by the Schilling test (*11*). We studied two patients with pernicious anemia during relapse and during vitamin B_{12}–induced remission. The other six patients had nutritional vitamin B_{12} deficiencies. As controls, we studied four patients with pernicious anemia (during remission) and four patients with nutritional folic acid deficiencies. We based our diagnosis of folic acid deficiency on low values of serum folic acid (*12*) but normal values of vitamin B_{12}, and the presence of macrocytes and neutrophils with hypersegmented nuclei in the peripheral blood smear. We performed bone marrow aspirations in two patients and found them to be megaloblastic. There were 15 normal individuals as additional controls.

The results of the thymidylate synthetase assay are shown in Table 1. The lowest value of enzyme activity from subjects in the pernicious anemia, in remission, folic acid deficiency, and normal groups is higher than even the highest value from subjects in the vitamin B_{12} deficiency in relapse group. The difference is significant at $P < .01$ [Wilcoxon rank sum test (*13*)].

There has been no prior evidence of vitamin B_{12} participation in the thymidylate synthetase reaction (*14*); the vitamin was not detected in highly purified synthetase preparations, and its addition to such preparations did not affect their activities. We now suggest that vitamin B_{12} is involved in the synthesis of thymidylate synthetase. The exact nature of this involvement is not known. This action of vitamin B_{12} in the megaloblastic process also clarifies the relationship between folic acid and vitamin B_{12} in megaloblastic anemia. Folic acid acts as a coenzyme for thymidylate synthetase in the de novo synthesis of DNA (*15*), whereas vitamin B_{12}, as indicated in this report, is involved in the synthesis of this enzyme. Therefore, vitamin B_{12} deficiency leads to a decreased synthesis and, consequently, activity of thymidylate synthetase. This results in an accumulation of unused 5-methyltetrahydrofolic acid. This "methyl trap" phenomenon has been observed and investigated in patients with vitamin B_{12} deficiency (*16*).

Inherited defects of vitamin B_{12} metabolism which cause abnormalities in methylmalonate catabolism or in the conversion of homocysteine to methionine have not been associated with megaloblastic development (*17*), and therefore these actions of the vitamin cannot be held responsible for megaloblastosis.

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Nerve Growth Factor: Relationship to the Cyclic AMP System of Sensory Ganglia

Abstract. *Nerve growth factor and* $N^6,O^{2'}$ *dibutyryl adenosine 3′,5′-monophosphate both stimulate neurite elongation by explanted ganglia. However, the addition of nerve growth factor does not lead to increased amounts of adenosine 3′,5′-monophosphate in intact ganglia, nor does it stimulate adenylate cyclase activity in broken ganglia cells.*

Both $N^6,O^{2'}$ dibutyryl adenosine 3′,5′-monophosphate (dibutyryl cyclic AMP) and nerve growth factor (NGF) stimulate the in vitro elongation of neurites from embryonic sensory and fetal trigeminal ganglia (*1–3*). When certain hormones bind to their target cells there is an increase in the intracellular concentration of adenosine 3′,5′-monophosphate (cyclic AMP) and cyclic AMP is said to be the "second messenger" for those hormones (*4*). It has been suggested that since both NGF and dibutyryl cyclic AMP stimulate neurite elongation, cyclic AMP might be the second messenger for NGF (*2, 3*).

Even though dibutyryl cyclic AMP and NGF both stimulate similar mor-

Table 1. Effect of NGF on the cyclic AMP content of embryonic sensory ganglia. Each value of cyclic AMP content per ganglion is the mean and standard deviation of the number of separate experiments shown in parentheses. Each determination included in the mean for that time interval was the result obtained from triplicate cyclic AMP assays.

Incubation time	Cyclic AMP content per ganglion	
	Control (pmole of cyclic AMP)	NGF (pmole of cyclic AMP)
15 minutes	0.77 ± 0.17 (2)	0.77 ± 0.07 (3)
30 minutes	0.77 ± 0.20 (5)	0.70 ± 0.21 (7)
1 hour	0.71 ± 0.18 (7)	0.70 ± 0.14 (7)
3 hours	0.95 (1)	0.75 (1)
6 hours	0.46 (1)	0.42 (1)
18 hours	0.66 ± 0.01 (2)	0.69 ± 0.10 (2)