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Influence of Cobalamin on the Survival of Mice Bearing Ascites Tumor

Key Words
Cobalamin
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Mice survival

Abstract
The effect of cobalamin (vitamin B_{12}) on the survival time of mice bearing P388 leukemia has been examined. Among the three cobalamins studied, the enzymatically active derivatives, methylcobalamin and 5′-deoxyadenosylcobalamin, were able to significantly increase the survival time of mice implanted intraperitoneally with the tumor cells. The pharmaceutical form, cyanocobalamin, was not active. The antitumor activity of these cobalamins may be associated with their functions in metabolism.

Evidence has accumulated in the recent past that vitamin B_{12} is associated with tumorigenesis. Patients with many types of malignant neoplasia and leukemia had elevated serum levels of vitamin B_{12} [1, 2] and vitamin B_{12}-specific binding proteins [3–5]. Certain cancer-bearing mice synthesized vitamin B_{12} and stored more vitamin B_{12} than normal controls did [1, 6]; but several spontaneous mammary tumors were shown to destroy this vitamin [6]. Studies with mouse spleen cells have shown that the addition of methylcobalamin to culture medium enhanced the production of antibody and suppressor T cells [7].

Vitamin B_{12} had been used with apparent benefit in the treatment of young children with neuroblastoma [8, 9], but the results of two survey studies using data from several hospitals failed to confirm that vitamin B_{12} therapy was effective either when it was administered alone or in conjunction with X-ray or chemotherapeutic agents [10, 11].

Experiments with laboratory animals also showed conflicting results. The administration of vitamin B_{12} inhibited the growth of certain tumors implanted in mice [12, 13]. Mice treated with vitamin B_{12} survived longer than did untreated controls. Vitamin B_{12} also inhibited the growth of liver tumors induced by *p*-dimethylaminoazobenzene in rats [14]. A mixture of vitamin B_{12} and vitamin C was able to inhibit the growth of certain mouse ascites or solid tumors and to increase the survival rate of tumor bearing mice [15–17]. However, in another study with Fischer CDF rats, a mixture of vitamins B_{12} and C had no effects on the growth of L_9 gliosarcoma, and no differences in survival time between treatment and control group were observed [18]. Furthermore, in some cases, vitamin B_{12} enhanced the growth of fibrosarcoma in rats and of Rous sarcoma in chickens [19]. Vitamin B_{12} was also reported to be procarcinogenic in rats and hamsters [20, 21]. In addition, a deficiency of vitamin B_{12} decreased the potency of certain carcinogens in rats [22, 23].

A recent in vitro study with cultured cells indicated that cobalamins were able to inhibit the growth of several malignant cell lines [24]. In these experiments, the meta-

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bolically active forms, methylcobalamin and 5′-deoxyadenosylcobalamin, were found to be effective but the metabolically inactive cyanocobalamin had practically no effect [24]. In the present study, in vivo experiments were carried out to examine the effects of three different forms of cobalamin on the survival of mice bearing ascites tumors.

Methods and Materials

Survival Time

Drug test was performed using a murine model, under the auspices of the NCI Division of Cancer Treatment, for screening chemical agents and natural products against animal tumors, model 3PS31, In Vivo Cancer Models, US Department of Health and Human Services, National Institutes of Health Publication No. 84-2635 (1984).

Female DBA and CDF_1 mice (18–20 g) were purchased from Simonsen Laboratories, Inc. (Gilroy, Calif.). They were fed a nonpurified diet (Purina Certified Rodent Diet, No. 5001, Ralston Purina Co., St. Louis, Mo.) throughout the experiment. Feed and water were offered ad libitum. Mouse lymphoid neoplasm cells P388 were propagated in DBA mice. The ascites cells were harvested on the 7th day after tumor implantation. The CDF_1 mice were injected intraperitoneally with 10^6 washed cells, suspended in 0.1 ml phosphate-buffered saline. Mice were then randomized into various test groups of 10 animals each. Starting 24 h after implantation, test mice were injected intraperitoneally with various doses of vitamin B_{12} in 0.2 ml of saline solution daily for 10 days. The control animals received an equal volume of saline. The mice were weighed twice a week and were killed 30 days after tumor implantation and the result evaluated. When 50% or more of the animals in a test group survived to the 30-day time period after tumor implantation, the experiment was extended to a total of 60 days. The number of days that each mouse lived after the transplantation of tumor cells was recorded as the survival time.

Statistical Calculations

The Student's two tailed t test was used to determine statistical differences between the control and experimental groups. The median survival time of each group was also used as an index of comparison of the test animals to their corresponding controls. The median survival time of the untreated control groups of these experiments was 16 ± 4 days. A test-to-control ratio of survival time of 1.3 for a test agent was considered to demonstrate activity, whereas a ratio of 1.75 or greater was considered significant activity.

Calculation of Therapeutic Index

The therapeutic index, referring to the dose ratio between toxic and therapeutic effect, is expressed as the ratio LD_{50}/ED_{90}, where LD_{50} is the dosage lethal to 50% of the untreated animals and ED_{90} is the dosage that give a 90% cell kill in the experimental mice [25]. Values of ED_{90} were measured using CDF_1 mice. The mice were injected intraperitoneally with 10^6 cells, suspended in 0.1 ml phosphate-buffered saline. The mice were then randomized into various groups of 10 animals each. Starting 24 h after implantation, test mice were injected intraperitoneally with various doses of the test agents in 0.2 ml of saline solution. On the tenth day after tumor implantation, the ascites cells were harvested and washed. Then, a homogeneous representation of the cells was stained with erythrosin B. The numbers of viable cells and dead cells were counted with a hemocytometer. The value of ED_{90} was calculated by comparing viable cell numbers in treated and control animals. The survival inocculum curves from the studies of survival time described above were also used for the calculation of ED_{90}. A good agreement was obtained between the two methods of calculation.

Chemicals

Cyano-, methyl- and 5′-deoxyadenosyl cobalamin were purchased from Sigma Chemical Co., St. Louis, Mo. The cobalamin derivatives were prepared by introducing the dry crystals into small sterile tubes. A sufficient number of tubes were prepared and stored at –20 °C. Immediately before injection, sterile saline solution was added to the tubes to dissolve the compounds.

Results and Discussion

We have tested the antitumor activity of three cobalamin derivatives that were available to us, at three different dosages: 25,50 and 100 mg/kg body weight/injection. The mice were able to tolerate the injection of cobalamin at a daily dose of 100 mg/kg body weight without any apparent adverse effect. Table 1 shows the survival times of test and control mice bearing P388 leukemia after treatment with cobalamins. The data indicate a statistically significant increase in the survival of mice treated with methylcobalamin or adenosylcobalamin when compared with the controls. Adenosylcobalamin was apparently more effective than methylcobalamin. However, cyanocobalamin has practically no effect on tumor growth. These observations were in good agreement with results in previous experiments in which in vitro cell culture technique was used [24]. The ratios of median survival time of a test group to median survival time of control for these mice are shown in parentheses in table 1. These ratios are in accordance with the corresponding p values.

The therapeutic index is the ratio between toxic and therapeutic effect, or the ratio of LD_{50} and ED_{90} [25]. The values of ED_{90} for methyl- and 5′-deoxyadenosylcobalamin were 120 and 100 mg/kg body weight, respectively, whereas cyanocobalamin did not affect cell growth at a daily dose as high as 1,000 mg/kg body weight. It has been shown that the addition of vitamin B_{12} to food in amounts far in excess of need or absorbability appears to be without hazard. Cyanocobalamin has caused no toxicity in animals at levels several thousand times their nutritional requirements [29]. Toxicity tests in our laboratory indicated that these three cobalimin derivatives were

Table 1. Effect of cobalamin derivatives on the survival of mice bearing ascites tumor

Test agent	Dosage, mg/kg body weight/day				Therapeutic Index
	0	25	50	100	
Cyanocobalamin	17.8 ± 2.9	12.6 ± 7.5 (0.97)	19.3 ± 4.2 (1.03)	18.7 ± 1.5 (1.06)	–
	19.6 ± 3.5	17.1 ± 3.5 (0.94)	20.5 ± 2.7 (1.17)	20.5 ± 1.4 (1.17)	
	18.1 ± 3.6	17.9 ± 3.4 (0.97)	18.3 ± 4.5 (1.00)	18.5 ± 4.2 (1.03)	
Methylcobalamin	18.0 ± 6.0	17.5 ± 2.8 (1.00)	17.3 ± 0.5 (1.00)	38.0 ± 18.4[a] (2.12)	15
	17.0 ± 3.2	–	18.9 ± 0.3 (1.06)	22.7 ± 9.7 (1.61)	
	16.7 ± 0.9	–	17.3 ± 6.1 (0.91)	32.7 ± 10.6[c] (2.18)	
Adenosylcobalamin	9.4 ± 2.4	9.1 ± 1.4 (1.18)	8.9 ± 2.3 (1.18)	29.1 ± 1.9[c] (3.53)	20
	15.5 ± 0.9	16.7 ± 4.5 (1.11)	21.2 ± 6.2[b] (1.43)	34.3 ± 10.3[c] (2.86)	
	15.0 ± 2.1	–	28.4 ± 13.7[b] (1.33)	40.7 ± 25.2[b] (3.83)	

Values are means ± SD of survival times (days) for n = 10. Significance of the difference between control and experimental values: [a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$. Values in parentheses are ratios of median survival time of test group to median survival time of control group. A value of 1.75 or greater for a test agent was considered to demonstrate significant activity.

Therapeutic index is expressed as the ratio LD_{50}/ED_{90}, where LD_{50} is the dose lethal to 50% of a population and ED_{90} is the dose that gives a 90% cell kill.

remarkably nontoxic when administered intraperitoneally. The LD_{50} for methylcobalamin was 1,800 mg/kg body weight, which was 15 times the effective dose. The LD_{50} for 5′-deoxyadenosylcobalamin was higher than that for methylcobalamin. However, when a dose of 2,000 mg/kg body weight was injected into these mice, they started to lose weight. Thus, the value of 2,000 mg/kg body weight was used and the therapeutic index for 5′-deoxyadenosylcobalamin was 20. Although very large doses of cobalamins injected intraperitoneally were nontoxic, it became lethal when a much smaller dose was injected intravenously into the mouse tail vein. This may be the effect of the large volume of fluid entering the mouse bloodstream during a relatively short period of injection time.

Vitamin B_{12} and folate are involved in the process of one-carbon-unit metabolism and methylcobalamin is a source of one-carbon functional groups [26, 27]. Although the mechanism of the antitumor activity is not known, it is quite evident that the roles of vitamin B_{12} in carcinogenesis may be associated with its functions in normal metabolism, particularly in the one-carbon-unit metabolism and in the positive and negative control of DNA synthesis by normal and malignant cells [26, 27]. Vitamin B_{12} is expected to correct defective DNA-thymine synthesis in vitamin B_{12}-defective marrow [28], because thymidylate synthase requires N^5,N^{10}-methylenetetrahydrofolate as methyl donor. For the same reason, in patients with folate and vitamin B_{12} deficiency, the addition of these vitamins to marrow and lymphocyte cultures enhanced the incorporation of ^{3}H-deoxyuridine into DNA [29].

Previous in vitro findings have indicated that the metabolically active cobalamins were able to inhibit malignant cell growth, while the metabolically inactive forms had practically no effect [24]. Folic acid and vitamin B_{12} are intimately related to the synthesis of DNA and RNA; lack of either damages DNA synthesis. The primary damage is to de novo DNA synthesis, with the result that there may be a secondary increment in salvage DNA synthesis [27, 29]. In those tumors in which synthesis of DNA by the salvage pathway is relatively greater than in normal cells, as compared to the de novo pathway of DNA synthesis, it is theoretically possible that folate and vitamin B_{12}, by enhancing de novo DNA synthesis, could be relatively more helpful to normal than to tumor cells and relatively more harmful to certain tumor cells [27, 29]. It has been suggested that these vitamins and their antagonists could be involved in the control of normal gene expression and that deficiency of folate or vitamin B_{12} or any cause of failure to methylate DNA or RNA can activate malignancy by hypomethylating oncogenes, leading to such gene expression or gene amplication, and that methylating oncogenes can inhibit malignancy by making them dormant. Furthermore, these vitamins can be useful in controlling tumors that grow more rapidly as more of them are supplied, because the tumor cells can be stimulated into the DNA synthesis phase in which a number of cancer chemotherapy agents exert their deadly effects. These agents can be used in a sequence right after folate or vitamin B_{12} [27, 30]. Large doses of folic acid and vitamin B_{12} were able to potentiate cytotoxicity of fluoropyrimidine by stabilizing the ternary complex and between fluorodeoxyuridylate and thymidylate synthase [30]. The finding that adenosylcobalamin was more effective than methylcobalamin indicated that the mechanism of cobalamin in carcinogenesis involved not only methylation but also other metabolic pathways of vitamin B_{12} metabolism.

Most of the cobalamin in animals exists as the two coenzymatically active forms, methylcobalamin and adenosylcobalamin [26]. Methylcobalamin constitutes 60–80% of the total plasma cobalamin. Adenosylcobalamin is the major cobalamin in cellular tissues. The stable pharmaceutical form, cyanocobalamin, is not nutritionally active. Although animals have the biochemical machinery to convert cyanocobalamin and other cobalamins into the two metabolically active cobalamins, the difference in activity of the three cobalamins indicated that the rate of the conversion was probably very low and insufficient in view of the rather high required dosage of the active cobalamins.

In summary, these studies indicate that methyl- and 5′-deoxyadenosylcobalamin were able to significantly increase the survival time of mice implanted with the P388 tumor cells. Because there has been criticism of the use of P388 as a tumor system for drug discovery and development, other tumor systems are being studied in our laboratory for further evaluation.

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