Influence of Nutrition on Anti-tumor Activity

Seung Hoon Choi¹, Eui Ho Hwang¹, Ki Il Park¹, Kyung Sik Lee¹ and Moritz M. Ziegler²

Nutritionally supporting the malnourished tumor bearing host may not benefit the disease outcome, but, rather, may preferentially "feed the cancer". We hypothesized that repletion is beneficial only when it augments an anti-tumor immune response. To support this hypothesis, 240 A/J mice were assigned to isocaloric dietary groups (24%, 5%, or 2.5% protein). On day 14 the mice received either immunogenic C1300- neuroblastoma (NB) or non-immunizing TBI-NB. On day 21 half of the restricted animals were repleted with 24% protein chow. At day 35, chromium-release cell-mediated cytotoxicity was measured. In the group of mice that received 2.5% protein chow, nutritional repletion specifically augmented anti-tumor activity for C1300-NB which elicits a host immune response (33.78 L.U. (repleted) vs 3.47 L.U. (depleted) p<0.01). In contrast, nutritional repletion was detrimental for non-immunizing TBI-NB, where further depression of cytotoxicity was seen (1.37 L. U. (repleted) vs 2.06 L.U. (depleted) 0<0.01). This suggests that the influence of nutritional repletion in tumor bearing animals is dependent on the integrity of host's anti-tumor immunity.

Key Words: Nutrition, tumor immunology

Nutritional support to the malnourished patients with malignant neoplasm can increase their tolerance of surgery, chemotherapy, or radiation therapy. Even though nutritional support led to improve early survival, there was no significant difference in long term survival (Daly and Copeland 1985). It remains as a supportive treatment modality. An adverse consequence of nutritional support is the risk of providing exogenous nutrient substrates for more rapid tumor growth. The influence of nutritional support on the host-tumor relationship is still controversial. Therefore, a more precise definition of the mechanisms involved in this adjuvant cancer therapy is needed. Controlled prospective clinical studies on the effect of nutritional support on tumor growth and the host immune systems are difficult in accurately measuring tumor growth (Stieger et al. 1975; Copeland 1982). For these reasons, the use of an animal model should allow a more precise measurement of the effect of protein-calorie malnutrition on immunological function. The animal tumor model differs from the human cancer by its inadequate blood supply due to subcutaneous location, the massive size of the tumor in relation to the host, and the extremely rapid growth of the tumors. The critical difference is the minimal immunogenicity of human tumors and the variable antigenicity of rodent tumors(McCarrick et al. 1986; Buzby et al. 1980). It was our concern that the disparities of reported results may have been secondary to a lack of careful examination of the host-tumor relationship. We speculated that the influence of malnutrition on tumor growth would depend almost exclusively on whether or not the tumor incited a host antitumor response. With use of our murine neuronlastoma model, in which two tumors of common progenitor cell origin differ in their immunogenicity as determined by in vivo and in vitro testing, many authors(McCarrick et al. 1986; Karpeh et al. 1987; Ziegler et al. 1986) previously reported a study of the impact of malnutrition on tumor growth. In this study, we have tested the hypothesis that nutritional repletion of the malnour-
ished tumor host will be of bebenefit and will not preferentially “feed the cancer”, if such therapy enhances anti-tumor immune activity.

MATERIAL AND METHODS

Animals

A/J mice were purchased from Jackson Laboratories, Bar Harbor, Maine. Male animals, six to eight weeks of age, weighing 20-25 grams were used, and a total of 240 animals were studied. They were maintained on commercial food pellets and tap water.

Tumors

C1300 neuroblastoma arose spontaneously in A/J mice showing behavioral characteristics not unlike the human tumor. This tumor obtained from Jackson Laboratories was maintained by serial transplantation in A/J mice or by serial passage in tissue culture in RPMI 1640 medium (Mediatech, Washington, DC) containing 10% heat-inactivated fetal calf serum (Gibco, Grand Island, NY) and 0.03% fresh glutamine (Mediatech). The variant termed TBJ neuroblastoma (TBJ-NB) was cloned from C1300-NB and was obtained from Dr. Arthur Bogden (E.G. and G. Mason Research Corp., Worcester, Mass.). During the past six years, we have maintained this tumor cell line both in vivo passage in A/J mice and by serial passage in tissue culture.

Nutritional regimens

Rat chow was constituted to vary the protein content with use of a casein base. The chow was obtained in pellet from Dyets (Bethlehem, PA) at a caloric density of 4.25 Kcal/gm. Diet composition for 24% protein (regular chow), 5% (moderately restricted chow) and 2.5% (severely restricted chow) is shown in Table 1. The total diet consumed by animals in each dietary group was approximately 4.5 gm of chow per mouse per day.

Tumor induction

Neuroblastoma cells were harvested from monolayer culture flask. Cell viability was determined by dye exclusion using 0.16% trypan blue solution (Eastman Kodak Co. Rochester, NY). Tumors could be induced 100 percent of the time using a dose 1 \times 10^6 tumor cells in both C1300-NB and TBJ-NB; this was chosen as standard for the experiments. A/J mice have been inoculated subcutaneously in our laboratory with either C1300-NB or TBJ-NB cells.

Experimental protocol

Mice were divided into three groups-48 mice for 24% chow, 96 mice for 5% chow and 96 mice for 2.5% chow. The mice were fed with three different chows for 14 days. Either C1300-NB or TBJ-NB cells were injected subcutaneously. Seven days after tumor inoculation, half of the restricted animals were replated with 24% protein chow. On the 21st day of tumor growth, all the mice were sacrificed and chromium release cell-mediated cytotoxicity was measured (Fig. 1).

Splenocyte suspensions

Spleens of tumor bearing animals were removed aseptically, placed in a complete medium (CM), gently washed with a rough surface of micro slides and aspirated in a cell suspension with a 10 ml syringe. They were allowed to sediment in an upright posit-

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>24% casein(gm/kg)</th>
<th>5% casein(gm/kg)</th>
<th>2.5% casein(gm/kg)</th>
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<tbody>
<tr>
<td>Casein</td>
<td>250</td>
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<td>25</td>
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<tr>
<td>Cornstarch</td>
<td>170</td>
<td>238</td>
<td>247</td>
</tr>
<tr>
<td>Sucrose</td>
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<td>240</td>
<td>248</td>
</tr>
<tr>
<td>Dextrose</td>
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</tr>
<tr>
<td>Cellulose</td>
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<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Soy bean oil</td>
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<td>80</td>
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<tr>
<td>Salt mix #200030</td>
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<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Vitamin mix #300050</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Caloric density</td>
<td>4.25 Kcal/gm</td>
<td>4.25 Kcal/gm</td>
<td>4.25 Kcal/gm</td>
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Number 1
**EXPERIMENTAL DESIGN**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Tumor</th>
<th>Repletion</th>
<th>Termination</th>
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<tbody>
<tr>
<td>24%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5%</td>
<td></td>
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<td></td>
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</table>

Day 0  Day 14  Day 21  Day 35

*Fig. 1. Experimental design. Tumor immunogenicity, nutritional repletion and cancer. Same experimental protocol was applied for C1300-NB and TBJ-NB.*

In Vitro generation of cytotoxic T lymphocytes

C1300-NB and TBJ-NB cells were harvested from monolayer culture flask as single cell suspension. These tumor cells were irradiated 5000 rad with Cesium irradiator. After counting living cells using 0.16% trypan blue dye exclusion, it was resuspended to a concentration of $1 \times 10^6$ cells/ml. One milliliter of CM containing $2.5 \times 10^7$ viable splenocytes of tumor bearing animals was added to each 25 cm² Corning Tissue culture flask (No. 25100). One milliliter of CM containing $1 \times 10^6$ viable irradiated neuroblastoma cells and 10 ml of CM were also added to each flask of the same tumor group. After six days' incubation at 37°C with 5% CO₂ the cells were harvested, counted using 0.16% trypan blue, and resuspended in CM.

Chromium release assay of cytotoxicity

A four-hour chromium release assay was performed. Four different effector-to-target ratio were examined to test the cytotoxicity of lymphocyte. The effector-to-target ratios 100:1, 20:1, 4:1, and 0:8:1 were examined. Various ratios of effector cells were plated in 96 well round-bottomed plates (Linbro Chemical Co., Hamden, CT) containing $10^4$ viable C1300-NB or TBJ-NB tumor target cells per well. Plates were then centrifuged at 80 × G for five minutes. They were incubated 37°C for four hours and then were resuspended at 500 × G for ten minutes. The supernatants were harvested by using SCS harvesting frames and SCS transfer tube strips(Skatron, Lier, Norway). The percentage of specific lysis was calculated by:

Sample counts – Background counts
Total counts – Background counts

Total counts were release by 0.1N Triton X and background counts by CM. In all experiments, the background counts were less than 30% of total counts. We arbitrarily defined the Lytic unit-30(L.U. -30) as the number of lymphocytes required to achieve 30% lysis of $10 \times 10^8$ ⁵¹Cr labeled target cell within four hours. We calculated cytotoxicity again and compared each group.

Statistical analysis

The Student t test was used to assess differences of cytotoxicity between groups.
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RESULTS

During the first 14 days of nutritional manipulation, body weight was increased 12.8% in the 24% protein chow group. Body weight did not change during this period in the 5% protein chow group. There was 11.8% reduction of body weight in the severely malnourished 2.5% protein chow group (Table 2). Two weeks of nutritional repletion would restore dody weight and organ weight (spleen, liver and thymus), as well as serum albumin concentration, to near baseline values. This was determined in a previous analysis of organ assessments in a depletion-repletion experiment in our laboratory. Differences in diet composition did not effect the cellular cytotoxicity of host. To test the effect of nutritional repletion on severity of malnutrition, the 5% and 2.5% protein chows were included in this protocol. In the repleted group, the mice that received 5% protein chow did not show any significant change in cellular cytotoxicity (p = NS) either in C1300-NB injected mice of TBJ-NB injected mice. But the mice that received 2.5% protein chow and C1300-NB showed significantly increased cellular cytotoxicity after repletion. On the contrary, the mice that received 2.5% protein chow and TBJ-NB showed significantly decreased cellular cytotoxicity after repletion (Table 3). Figure 2 summarized data concerning the impact of diet on cellular immunity. There are no significant differences of cytotoxicity in C1300-NB between different diets. But nutritional depletion results in higher cellular cytotoxicity in TBJ-NB. Figure 3 summarizes the effects of depletion-repletion on the host cellular immunity in the 5% protein chow group. There was no difference in cellular cytotoxicity between the repleted and nonrepleted groups in C1300-NB. In TBJ-NB, cellular cytotoxicity is decreased in the repleted group;

Table 2. Body weight change during first 14 days of nutritional manipulation

<table>
<thead>
<tr>
<th>Diet</th>
<th>Body Weight</th>
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<tbody>
<tr>
<td>24%</td>
<td>12.8% increase</td>
</tr>
<tr>
<td>5%</td>
<td>No change</td>
</tr>
<tr>
<td>2.5%</td>
<td>11.8% decrease</td>
</tr>
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</table>

Table 3. Effects of depletion-repletion on the cellular cytotoxicity

<table>
<thead>
<tr>
<th>Diet</th>
<th>C1300-NB</th>
<th>TBJ-NB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonrepleted</td>
<td>Repleted</td>
</tr>
<tr>
<td>24%</td>
<td>2.23±0.14*</td>
<td>6.22±0.21</td>
</tr>
<tr>
<td>5%</td>
<td>7.39±0.94</td>
<td>33.78±2.38</td>
</tr>
<tr>
<td>2.5%</td>
<td>3.47±0.26</td>
<td>6.22±0.21</td>
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* L.U. = 30/10⁸ Effector cells

![Graph showing the effect of dietary regimens on cell mediated immunity](image.png)

Fig. 2. Import of dietary regimens on cell mediated immunity. Effector/Target ratio in various combinations of effector(lymphocyte) and tumor targets. C1300-NB; C1300-neuroblastoma TBJ-NB; TBJ-neuroblastoma

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**Fig. 3.** In the moderately malnourished host, nutritional repletion caused no significant change cell-mediated immunity, either in immunogenic C1300-NB or nonimmunogenic TBJ-NB.

**Fig. 4.** In the profoundly malnourished host, nutritional repletion augmented cellular cytotoxicity in the immunogenic C1300-NB bearing host. But nutritional repletion further deteriorated cellular cytotoxicity in the nonimmunogenic TBJ-NB bearing host.

however, there is no statistically significant difference between two groups. Figure 4 summarizes data of the severely protein restricted 2.5% protein chow group.

Nutritional repletion augmented cellular cytotoxicity in C1300-NB. These differences are statistically significant in all effector target ratio (p < 0.01). In contrast, nutritional repletion further deteriorated cytotoxicity in TBJ-NB. The differences of cytotoxicity are statistically significant in all effector target ratio (p < 0.01).

**DISCUSSION**

The present data supports our previous report that nutritional supplementation of the malnourished tumor-bearing host will either benefit the host of “feed the tumor”. This difference relates to the differing immunogenicity or antigenicity of C1300-NB and TBJ-NB tumors. C1300-NB was originally reported to be similar to human neuroblastoma, but our anatomic search has failed to confirm metastatic potential. TBJ-NB variant is an aggressive and systemically disseminating tumor which frequently metastasizes to lung, liver, kidney and spleen (McCarrick et al. 1986; McAlack et al. 1977; Pons et al. 1982). C1300-NB elicits a host anti-tumor response in immunologically-competent strain A mice when assessed by in vivo immunization-excision challenge assay, by in vitro MLTC technique, and in vitro chromium release cell mediated cytotoxicity assay. Utilizing the same in vivo and in vitro techniques, TBJ-NB recipients do not produce a significant host anti-tumor response, a response shown to influence tumor growth characteristics.
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(McCarrick et al. 1986; Karpeh et al. 1987; Ziegler et al. 1986; Ziegler et al. 1979; Ziegler et al. 1980)

Nutritional depletion plays an insignificant role in the host cell-mediated immune response in immunogenic C1300-NB; in other words, cell-mediated immunity is not reduced by dietary restriction. In contrast, cell-mediated immunity is augmented by dietary restriction in nonimmunogenic TBJ-NB.

That cell-mediated immunity is not depressed during moderate or severe malnutrition has been demonstrated by many investigators. The enhanced T-cell function in malnutrition as demonstrated by others was thought to be due to a depressed production of a blocking antibody(Jose and Good 1973; Bill 1971) or due to inhibition of suppressor cell proliferation(Good et al. 1976; Broder and Waldmann 1978).

Nutritional repletion of immunogenic C1300-NB hosts results in augmentation of the cell-mediated immune response. This immunologic enhancement is definite in severely malnourished(2.5% protein diet) mice. This data supports our previous work and agrees with findings from recent extensive studies which focused on animal survival and tumor burden(McCarrick et al. 1986; Karpeh et al. 1987). Nutritional repletion produces further deterioration of cell-mediated immunity in nonimmunogenic TBJ-NB. The phenomenon is statistically significant in severely malnourished mice. Although not statistically significant, a decrease of cell-mediated immunity following repletion in moderately malnourished (5% protein diet) mice was noticed in TBJ-NB. Under this circumstance, nutritional support might preferentially feed the tumor, and an increased tumor weight/carcass weight ratio will occur.

Mandatory protein-calorie deprivation did not significantly impede tumor growth relative to chow controls, despite the more severe host depletion. All cancer cells are less sensitive to host nutritional regulation; and, so, have a growth advantage over the host tissues. This would enable the tumor in the malnourished state to compete successfully for nutrients(Lawson et al. 1982).

The assessment of the effects of nutritional repletion on tumor growth and survival in experimental animal tumor systems have been controversial. These reports have mainly included the Morris Hepatoma(Cameron et al. 1977; Daly et al. 1978; Cameron and Pavlat 1976; Weber et al. 1983), the Walker 256 carcinosarcoma(Mills et al. 1981; Daly et al. 1980; Jensen and Muntzing 1970), the Lewis AC-33 mammary adenocarcinoma(Kishi et al. 1982; Ota et al. 1977; Goodgame et al. 1979) and the methylcholanthrene induced sarcoma (Cameron 1981; Popp et al. 1983). Numerous reports demonstrated significant acceleration of tumor growth during periods of nutritional repletion. Cameron et al. (1979) and Weber et al. (1983) reported tumor growth acceleration after nutritional repletion in the Morris Hepatoma.

Daly et al.(1980) and Mills et al.(1981) demonstrated growth acceleration of the Walker 256 carcinosarcoma with nutritional repletion. Studies utilizing the chemically induced Lewis AC-33 mammary adenocarcinoma showed nutritional repletion is associated with an increase of tumor burden(Stieger et al. 1975; Buzby et al. 1980; Torosian et al. 1983).

Daly et al. (1978) and Ota et al.(1977), using the Morris Hepatoma, and Kishi et al. (1982) using the Walker 256 carcinosarcoma demonstrated no significant stimulation of tumor growth following nutritional support. In the model of the methylcholanthrene-induced sarcoma with intravenous nutritional support, tumor growth is not consistent after repletion. Goodgame et al. (1979) reported no stimulation of the tumor growth after nutritional repletion; whereas, Popp et al. (1983) demonstrated that the tumor growth is responsive to the level of nutritional support. The explanations for these diverse results include dissimilar experimental techniques(Popp et al. 1981) and varying antigenicity of different rodent tumor systems(McCarrick et al. 1986; Karpeh et al. 1987).

Malnutrition in the cancer patient correlated with significantly shorter survival and a poor response to chemotherapy(Dewys et al. 1981). Nutritional support in patients with cancer caused reversal from malnutrition to adequate nutrition(Shamberger et al. 1984; Rickard et al. 1985; Eys et al. 1982). Clinical application of nutritional support in the cancer patient proved to be effective in reduction of postoperative morbidity and mortality(Muller et al. 1982). However, a group of other investigators reported no survival of therapeutic advantage for the adjuvant parenteral nutrition(Shamberger et al. 1984; Fischer 1984).

They postulated that nutritional support not only restores the patient's deteriorated metabolic condition, but also stimulates tumor growth. Consequently, the ultimate outcome is the unchanged duration of remission-free interval(Fischer 1984). Proper nutritional support in previously malnourished patients benefits the host by either improving tolerance to chemotherapy or by acceleration of normal bone marrow function(Eys et al. 1980; Hays...
et al. 1983).

Utilizing intravenous hyperalimentation in small cell bronchogenic carcinoma, Valdiveso et al. (1981) demonstrated an increased complete remission rate over conventional treatment (85% vs 59%). In reports by Serrou et al. (1981), Nixon et al. (1981), Popp et al. (1981), no such advantage was found for patients with small cell anaplastic lung carcinoma, metastatic colon carcinoma, or diffuse lymphoma. As a treatment modality for the cancer patient, intravenous hyperalimentation remains in the supportive category. Uniform implementation of either nutritional supplementation or minimal feeding of the malnourished cancer patient cannot be justified.

Many studies in the past have demonstrated impaired antibody production and cell-mediated immune response in malnourished patients and protein depleted experimental animals (Dionigi et al. 1977; Selvaraj and Bhat 1972; Ricci and Ziegler 1984; Seth and Chandra 1972; Smythe et al. 1971; Woodruff 1970; Neumann et al. 1975). A study in children with protein calorie malnutrition showed that synthesis of IgG, IgA, and IgM was not quantitatively impaired (Watson and Freesemann 1970). In malnutrition, the thymus is the first organ to show atrophy and the last to recover (Kahan 1981). Consequently, depression of T-cell cytotoxicity is prolonged, while increased antibody production has been seen after nutritional repletion of previously depleted animals (Good et al. 1976; Law et al. 1973; Law et al. 1974). Blocking antibody or suppressor T-cells are markedly sensitive to malnutrition. So, specifically committed lymphoid effector cells operate more efficiently due to lack of inhibition, even though the total lymphocyte number is reduced. With further restriction of protein intake, both cell-mediated and humoral immune responses are suppressed. A previous report showed that profound depression of cellular response occurred at a dietary level of 3% protein (Jose and Good 1973).

The present study demonstrates that host anti-tumor immune response is influenced not only by the host's nutritional status but also by immunizing properties of the tumor. These findings are compatible with a clinical course of in vivo murine neuroblastoma. When malnourished tumor bearers were repleted. There was a statistically insignificant reversal of growth of the immunogenic C1300-NB with a diminution in tumor size. However, after repletion, the nonimmunizing tumor increased in size almost nine fold (Karpeh et al. 1987).

In summary, anti-tumor immunity is not reduced by short term dietary restriction. Anti-tumor immunity is augmented in TBJ-NB, a mechanism made possible by elimination of suppressor cell activity. Nutritional repletion specifically benefits the host by augmenting tumor-specific immune activity in immunogenic C1300-NB. Nutritional repletion further impaired tumor specific immune activity in nonimmunogenic TBJ-NB.

Many factors should be taken into consideration in determining whether nutritional supplementation should be undertaken for cancer patients: patient's nutritional state, immunizing characteristics of the tumor, and further treatment plan. Most of the human tumors are thought to be minimally immunogenic; so, inadvertent application of nutritional supplementation in cancer patient will be harmful to the patient.

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