Resistance to L1210 Mouse Leukemia Cells in Moderately Protein-malnourished BALB/c Mice Treated in Vivo with Thymosin Fraction V

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ABSTRACT

Moderate protein malnutrition retarded the i.p. proliferation of L1210 mouse leukemia cells in BALB/c mice. The increased resistance against leukemia cell growth in protein-malnourished mice was correlated with increased in vitro mitogenic responsiveness of spleen lymphocytes to phytohemagglutinin and increased levels of serum corticosterone but could not be correlated with altered development of splenic lymphocyte-mediated cytotoxicity. The increased resistance against leukemia cells in well-fed mice treated with thymosin alone could not be correlated with an increase in any of these parameters.

Treatment with Thymosin Fraction V further increased the resistance of protein-malnourished mice to i.p. leukemia cell growth. The increased resistance of these mice to tumor cell growth was correlated with increased splenic lymphocyte mitogenic responsiveness to phytohemagglutinin, elevated serum corticosterone levels, and a slight increase in lymphocyte-mediated cytotoxicity 14 days after tumor challenge. For 7 days after the last treatment, protein-malnourished mice had reduced serum corticosterone levels. Nevertheless, the serum corticosterone levels were still higher than normal in these mice.

INTRODUCTION

In 1914 (25), Rous was one of the first investigators to note decreased incidence of spontaneous tumors in moderately malnourished mice. Since then, moderate malnutrition of various types has been associated with decreased incidence of spontaneous, transplanted, or induced tumors (1, 9, 13, 23–27, 33). Several mechanisms have been suggested to explain decreased tumor growth during nutritional stress including altered immune function.

Malnutrition, even moderate, has been associated with decreased resistance to a variety of bacterial infections in humans and animals (8, 16, 22, 28). Recent attempts by our laboratory have been successful in the restoration of the resistance of protein-malnourished mice against Listeria monocytogenes by treatment with Bovine Thymosin Fraction V.³ Therefore, in this paper, we have reported further studies on the effects of thymosin treatment upon the resistance of well-nourished and moderately protein-malnourished mice against a transplantable murine leukemia.

MATERIALS AND METHODS

Animals, Diet, and Experimental Design. We obtained approximately 240 female BALB/c mice from Harlan-Sprague-Dawley (Indianapolis, Ind.). The mice, 3 weeks of age, were divided equally and were fed throughout the experiment either a control 20% casein diet (Table 1) or a MPD* 4% casein diet (United States Biochemical Corp., Cleveland, Ohio) which were equal in calories. In the first experiment, after 8 weeks on their respective diets, 40 of these mice were given injections i.p. with 2.5 x 10⁶ L1210 mouse leukemia cells. In the second experiment, after 6 weeks on the respective diets, the remainder of the mice were given i.p. injections of either 100 µg of Bovine Thymosin Fraction V (Hoffman-LaRoche Inc., Nutley, N. J.) or PBS every other day for 2 weeks. Doses were on a per g of mouse basis, 6.25 versus 4.8 µg/g/day, for mice fed the MPD versus control diet, respectively. One, 7, or 21 days after the last thymosin or PBS treatment, 120 of the mice were given i.p. injections of 1 x 10⁷ L1210 mouse leukemia cells. Seven or 14 days after the injection of L1210 cells, mice from both experiments were sacrificed, and the number of PEC, spleen cell cytotoxicity against L1210 cells, and mitogenesis of spleen cells induced by PHA were assayed. Twenty mice from the first experiment and 60 mice from the second experiment that were not given injections of L1210 cells were sacrificed on Day 0 of the challenge with the murine leukemia cells. At that time, after sera were collected, the number of PEC, spleen cell cytotoxicity against L1210, spleen cell mitogenesis to PHA, and serum corticosteroid levels were assayed. Each datum consisted of the mean values from at least 5 mice.

Preparation of Lymphoid Cell Suspensions. The spleens were dispersed into a cellular suspension in sterile PBS by gently rubbing the spleens through a stainless steel screen. Erythrocytes were disrupted by washing the suspensions in Tris-buffered 0.15 M ammonium chloride (pH 7.2). Cells were then washed once in PBS and once in Roswell Park Memorial Institute Cell Culture Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.) containing 10% heat-inactivated fetal calf serum. The viability and concentration of spleen cells was assessed by microscopic hemocytometer examination of trypan blue exclusion. Cells from individual mice were adjusted to 5 x 10⁶/ml in culture media.

Measurement of Lymphocyte Mitogenesis. Quadruplicate samples of lymphocytes from an individual mouse were seeded into Costar Cluster 96-well plates at a concentration of 2.5 x 10⁵ cells/well. Mitogenesis was induced in 2 of the cultures after addition of 0.25 µg of purified PHA (Burroughs-Wellcome and Co., Research Triangle Park, N. C.) in media. After addition of only media, the remaining 2 cultures served as controls for spontaneous mitogenesis. The cell suspensions were incubated at 37°C in 5% CO₂ for 72 hr. Twenty-four hr prior to the end of incubation, 1 µCi of (methyl-³H)thymidine (Amer sham-Searle Corp., Arlington Heights, Ill.) was added to each culture. The cell suspensions were harvested onto glass fiber filters and washed with absolute methanol. The radioactivity present on each filter was measured using a liquid scintillation spectrometer.

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RESULTS

After 5 weeks on the diet, mice fed the control diet weighed 20.78 ± (S.D.) 1.01 g while mice fed the MPD diet weighed 15.96 ± 0.73 g. The mice fed the control diet consumed 2.91 ± 0.22 g food per mouse per day, while the mice fed the MPD diet consumed 3.77 ± 0.26 g food per mouse per day.

Response of Moderately Malnourished Mice to L1210 Cells. The levels of splenic LMC activity against L1210 cells, leukemia cell proliferation within the peritoneal cavity, spleen lymphocyte number, and mitogenic responsiveness to PHA were measured in mice from both dietary groups just prior to or 7 and 14 days after challenge with 2.5 × 10⁷ leukemia cells. The prechallenge PEC level was slightly higher in control mice than in mice fed the MPD diet (Table 2). This value is the normal level of lymphoid cells present in the peritoneal exudate. Seven days after i.p. injection of 2.5 × 10⁷ L1210 cells, there was a 70-fold increase in PEC levels due to the proliferation of L1210 cells. The 5-times-larger L1210 cells in the PEC are easily distinguishable from the low levels of smaller lymphoid cells. The i.p. proliferation of L1210 cells by Day 7 after challenge was significantly less in mice fed the MPD diet (p < 0.05). By 14 days after injection, there was a significant clearance of L1210 cells from the peritoneal cavity of mice fed either the control or MPD diet.

There was a similar level of splenic NK cell activity against L1210 cells in unchallenged mice from both dietary groups (Table 2). After injection of the L1210 cells, the total LMC, NK, and T-cell-mediated activity increased steadily in mice from both dietary groups, reaching a similar level by Day 14 after challenge. The mice fed the MPD diet had lower LMC activity on Day 7 compared to control mice. However, by Day 14, both dietary groups had similar levels of LMC activity.

The spleen cell number was significantly lower (p < 0.05) and spleen lymphocyte mitogenic responsiveness to PHA was significantly higher (p < 0.05) in mice fed the MPD diet than in mice fed the control diet (Table 3). In response to the leukemia cell challenge, the number of spleen lymphocytes increased in mice from both dietary groups during the 14 days after challenge. However, the spleen lymphocyte number remained lower in mice fed the MPD diet than in mice fed the control diet.

In response to the leukemia cell challenge, the mitogenic responsiveness of spleen lymphocytes to PHA increased by Day 7 after challenge in mice from both dietary groups. Also

Table 1

<table>
<thead>
<tr>
<th>Composition</th>
<th>Control</th>
<th>Low protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein high nitrogen</td>
<td>20.0</td>
<td>4.0</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Corn starch</td>
<td>15.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>50.0</td>
<td>66.0</td>
</tr>
<tr>
<td>Fiber-Cellulose</td>
<td>5.0</td>
<td>5.0</td>
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<tr>
<td>Corn oil</td>
<td>5.0</td>
<td>5.0</td>
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<tr>
<td>AIN mineral mix</td>
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<td>3.5</td>
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<tr>
<td>AIN vitamin mix</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* United States Biochemical Corp. Diet 10662.

** Percentage of lysis = (Test cpm - spontaneous release cpm) / (Total cpm - background cpm)

** Mean ± S.E.

** Effector cell:target cell ratio, 200:1.

** Mice sacrificed on day of injection but not given injections of L1210 cells.

** Mean ± S.E.

** Significantly different than mice fed the 20% casein diet; p < 0.05.

Table 2

<table>
<thead>
<tr>
<th>Time postinjection (days) of L1210 cells</th>
<th>PEC (× 10⁶)</th>
<th>LMC</th>
<th>20% dietary protein</th>
<th>4% dietary protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.1 ± 1.2</td>
<td>5.1 ± 1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>22.3 ± 8.1</td>
<td>9.8 ± 2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>69.4 ± 2.9</td>
<td>68.1 ± 8.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Weanling BALB/c mice fed either 20 or 4% casein diet for 5 weeks. Mice were given i.p. injections at that time of 2.5 × 10⁷ L1210 mouse leukemia cells.

** Effector cell:target cell ratio, 200:1.
Thymosin Fraction V in MPD Mice

Table 3

<table>
<thead>
<tr>
<th>Time post-injection (Day)</th>
<th>20% dietary protein</th>
<th>4% dietary protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>10.3 ± 2.3a</td>
<td>11.8 ± 1.2</td>
</tr>
</tbody>
</table>

Separate groups of mice were also challenged with 1 x 10^7 L1210 cells on Day 1, 7, and 21 post-thymosin treatment. They were assayed 7 and 14 days later with respect to LMC against L1210 cells and proliferation of L1210 cells i.p. Spleen lymphocyte numbers and mitogenic responsiveness to PHA on these days were similar to values obtained in the first experiment and were not affected by thymosin treatment (data not shown).

Spleen cell numbers were significantly lower in unchallenged

Table 5

<table>
<thead>
<tr>
<th>Level of dietary protein (%)</th>
<th>Treatment type</th>
<th>Time after last treatment (days)</th>
<th>cpm [³H]thymidine incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>PBS</td>
<td>1</td>
<td>18,836 ± 1,529 (16.91 ± 3.25)</td>
</tr>
<tr>
<td>20</td>
<td>Thymosin</td>
<td>1</td>
<td>18,099 ± 2,040 (17.19 ± 6.24)</td>
</tr>
<tr>
<td>4</td>
<td>PBS</td>
<td>1</td>
<td>53,036 ± 3,906 (25.30 ± 3.52)</td>
</tr>
<tr>
<td>4</td>
<td>Thymosin</td>
<td>1</td>
<td>45,710 ± 8,018 (47.27 ± 14.94)</td>
</tr>
<tr>
<td>20</td>
<td>PBS</td>
<td>1</td>
<td>22,012 ± 3,804 (9.63 ± 2.68)</td>
</tr>
<tr>
<td>20</td>
<td>Thymosin</td>
<td>7</td>
<td>33,261 ± 3,931 (17.82 ± 5.30)</td>
</tr>
<tr>
<td>4</td>
<td>PBS</td>
<td>7</td>
<td>87,457 ± 14,182 (54.07 ± 14.15)</td>
</tr>
<tr>
<td>4</td>
<td>Thymosin</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>PBS</td>
<td>21</td>
<td>21,892 ± 2,062 (6.28 ± 4.22)</td>
</tr>
<tr>
<td>20</td>
<td>Thymosin</td>
<td>21</td>
<td>29,439 ± 2,618 (8.52 ± 3.48)</td>
</tr>
<tr>
<td>4</td>
<td>PBS</td>
<td>21</td>
<td>35,997 ± 10,367 (32.93 ± 12.93)</td>
</tr>
<tr>
<td>4</td>
<td>Thymosin</td>
<td>21</td>
<td>81,247 ± 15,846 (60.85 ± 9.72)</td>
</tr>
</tbody>
</table>

* Mean ± S.E.

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on Day 7 after the challenge, background mitogenesis, as measured by the incorporation of tritiated thymidine, was higher by 17-fold in mice from both dietary groups compared to unchallenged mice. The responsiveness of spleen lymphocytes to PHA remained higher in mice fed the MPD diet compared to controls throughout the 14-day period following challenge.

Response of Thymosin-treated Mice to L1210 Cells. Mice from both dietary groups were treated every other day for 2 weeks with a total of 700 μg of thymosin or PBS. Doses were therefore 6.25 μg per g mouse per day for mice fed the MPD diet and 4.8 μg per g mouse per day for mice fed the control diet. On 1, 7, and 21 days after the last treatment, the levels of splenic LMC against L1210 cells, PEC levels, spleen lymphocyte numbers, and spleen lymphocyte mitogenic responsiveness to PHA were determined in mice from both dietary groups.

Table 4

<table>
<thead>
<tr>
<th>Dietary protein level (%)</th>
<th>Treatment type</th>
<th>Time after last treatment (days)</th>
<th>Spleen lymphocytes/mouse x 10^7</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>PBS</td>
<td>1</td>
<td>9.7 ± 0.3b</td>
</tr>
<tr>
<td>20</td>
<td>Thymosin</td>
<td>1</td>
<td>13.2 ± 1.0b</td>
</tr>
<tr>
<td>4</td>
<td>PBS</td>
<td>7</td>
<td>2.0 ± 0.6b</td>
</tr>
<tr>
<td>4</td>
<td>Thymosin</td>
<td>7</td>
<td>1.7 ± 0.7b</td>
</tr>
<tr>
<td>20</td>
<td>PBS</td>
<td>7</td>
<td>13.7 ± 0.7</td>
</tr>
<tr>
<td>20</td>
<td>Thymosin</td>
<td>7</td>
<td>12.1 ± 1.9</td>
</tr>
<tr>
<td>4</td>
<td>PBS</td>
<td>7</td>
<td>5.1 ± 0.5b</td>
</tr>
<tr>
<td>4</td>
<td>Thymosin</td>
<td>7</td>
<td>4.7 ± 1.4b</td>
</tr>
<tr>
<td>20</td>
<td>PBS</td>
<td>21</td>
<td>10.4 ± 1.2</td>
</tr>
<tr>
<td>20</td>
<td>Thymosin</td>
<td>21</td>
<td>6.3 ± 0.6b</td>
</tr>
<tr>
<td>4</td>
<td>PBS</td>
<td>21</td>
<td>5.4 ± 1.9b</td>
</tr>
<tr>
<td>4</td>
<td>Thymosin</td>
<td>21</td>
<td>3.3 ± 0.8b</td>
</tr>
</tbody>
</table>

* Mean ± S.E.

b Significantly different than PBS-treated mice fed the control diet; p ≤ 0.05.

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Female BALB/c mice were fed either 20 or 4% casein diet for 5 weeks from the time of weaning. While continuing on the respective diets, the mice were given injections of 0.5 ml of PBS or Thymosin Fraction V (100 μg) i.p. every other day for 2 weeks. Groups of mice were then either sacrificed or challenged with 1 x 10^7 L1210 cells 1, 7, or 21 days after the last thymosin injection. The spleen cell responsiveness to PHA was elevated at this time in unchallenged mice.
mice fed the MPD diet \( (F = 136.10; p < 0.001) \) (Table 4). In unchallenged mice fed the MPD diet, the thymosin treatment had a slight but not significant suppressive effect upon spleen lymphocyte numbers. The effect of thymosin treatment was different in unchallenged mice fed the control diet. On Day 1 after the last treatment, the spleen lymphocyte number was enhanced compared to PBS-treated control mice. By 21 days after the last treatment, the spleen lymphocyte number was depressed in thymosin-treated unchallenged mice.

As in the first experiment, spleen lymphocyte mitogenic responsiveness to PHA was significantly higher in unchallenged mice fed the MPD diet \( (F = 61.05; p < 0.001) \) than in well-nourished controls (Table 5). The thymosin treatment affected PHA-induced splenic lymphocyte mitogenesis of mice fed the MPD diet differently than it affected mice fed the control diet \( (F = 13.14; p < 0.001) \). On Day 1 post-thymosin treatment, both dietary groups had slightly suppressed spleen lymphocyte mitogenic responsiveness to PHA compared to PBS-treated controls. By 7 and 21 days post-thymosin treatment, the spleen lymphocyte mitogenic responsiveness to PHA was not affected in mice fed the control diet but was enhanced further in mice fed the MPD diet.

The level of PEC in mice given injections of L1210 cells was determined on Days 7 and 14 after challenge (Table 6). As shown above, consumption of the low-protein diet independently lowered levels of PEC on Day 7 after challenge \( (F = 22.37; p < 0.001) \) compared to mice fed the control diet. Thymosin treatment also independently lowered levels of PEC on Day 7 after challenge \( (F = 22.37; p < 0.001) \) compared to mice fed the control diet. Thymosin treatment also independently lowered the Day 7 level of PEC irrespective of dietary protein level \( (F = 9.36; p < 0.006) \). By Day 14 after challenge, all groups had a significant clearance of leukemia cells from the peritoneal cavity.

As the leukemia cells were cleared from the peritoneal cavity by Day 14 after challenge, splenic lymphocytes from all dietary-treatment groups of mice developed an enhanced ability to kill L1210 cells (Table 7). When taken together, both treatment groups of mice fed the MPD diet tended to have better LMC activity by Day 14 after challenge, which proved to be significant \( (F = 8.68; p < 0.005) \). However, there was no consistent correlation between degree of tumor cell growth inhibition and presence of native LMC or development of LMC activity in mice from each dietary-treatment group.

**Corticosterone Levels.** Corticosteroids have a suppressive effect upon leukemia cell or other tumor cell proliferation (11, 17) including L1210 cells (14), and malnutrition raises serum corticosteroid levels (5). Mice treated with 0.9% NaCl solution and fed the MPD diet had significantly higher levels of serum corticosterone than did 0.9% NaCl solution-treated controls \( (p < 0.05) \) (Table 8). The thymosin treatment significantly lowered the serum corticosterone levels of mice fed the MPD diet up to 7 days after the last treatment. However, this effect waned by 21 days after the last treatment. The thymosin treatment had no effect upon the serum corticosterone levels of mice fed the control diet.

**DISCUSSION**

The data from this experiment indicate that consumption of a diet low in protein rendered mice better able to retard leukemia cell proliferation. Many previous investigations have shown that moderate protein malnutrition renders animals more resistant to spontaneous and transplanted cancer cells than are well-nourished controls (1, 2, 9, 13, 23-27). A partial explanation may be, as we showed in this report, that protein-

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### Table 6

**Effect of thymosin treatment upon resistance of control and protein-malnourished mice against L1210 leukemia cell growth**

<table>
<thead>
<tr>
<th>Dietary protein level (%)</th>
<th>Treatment type</th>
<th>Time after last treatment (days)</th>
<th>PEC ( \times 10^{-5} ) at following time after injection of L1210 cells</th>
<th>Source of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 0</td>
<td>Day 7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 14</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>PBS</td>
<td>1</td>
<td>7.2 ± 1.1b</td>
<td>398.3 ± 105.4</td>
</tr>
<tr>
<td>20</td>
<td>Thymosin</td>
<td>1</td>
<td>11.3 ± 1.6c</td>
<td>377.3 ± 68.7</td>
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<tr>
<td>4</td>
<td>PBS</td>
<td>1</td>
<td>1.2 ± 0.8c</td>
<td>166.2 ± 41.2c</td>
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<tr>
<td>4</td>
<td>Thymosin</td>
<td>1</td>
<td>0.2 ± 0.1c</td>
<td>42.7 ± 24.4c</td>
</tr>
<tr>
<td>20</td>
<td>PBS</td>
<td>7</td>
<td>562.0 ± 43.3</td>
<td>18.9 ± 3.4</td>
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<tr>
<td>20</td>
<td>Thymosin</td>
<td>7</td>
<td>353.4 ± 64.5c</td>
<td>19.2 ± 6.1</td>
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<td>4</td>
<td>PBS</td>
<td>7</td>
<td>450.7 ± 113.5</td>
<td>11.3 ± 1.6c</td>
</tr>
<tr>
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<td>Thymosin</td>
<td>7</td>
<td>300.9 ± 73.9c</td>
<td>11.7 ± 1.9c</td>
</tr>
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<td>PBS</td>
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<td>667.8 ± 44.1</td>
<td>30.8 ± 17.5</td>
</tr>
<tr>
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<td>Thymosin</td>
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<td>512.4 ± 91.7</td>
<td>52.4 ± 25.0</td>
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<td>PBS</td>
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<td>339.5 ± 127.6c</td>
<td>52.0 ± 46.1</td>
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<tr>
<td>4</td>
<td>Thymosin</td>
<td>21</td>
<td>231.7 ± 82.5c</td>
<td>19.0 ± 5.7</td>
</tr>
</tbody>
</table>

*Challenged with \( 1 \times 10^7 \) L1210 mouse leukemia cells.

b Mean ± S.E.

c Significantly different than PBS-treated mice fed the control diet; \( p < 0.05 \).
malnourished animals have increased T-cell activity even though lymphocyte numbers are lower than those of well-nourished controls (3, 22). B-cell activity may be reduced (32). Mastocytoma-challenged protein-malnourished mice did not develop significant levels of serum blocking antibodies allowing tumor cells to escape immunosurveillance (13). Furthermore, peritoneal macrophages from protein-malnourished animals may be slightly more active than those of well-nourished controls (3, 32). While moderate malnutrition increases resistance against cancer cells, it decreases the resistance of the host to a variety of pathogens (8, 16, 22, 28). We have shown that

Bovine Thymosin Fraction V can partially restore the cell-mediated resistance of protein-malnourished mice against L. monocytogenes, suggesting that thymic hormone production is decreased by nutritional stress. Treatment with thymic hormones also enhances resistance to growth of cancer cells (15, 18, 29). In this report, we confirm these observations by showing that thymosin increased the resistance of mice to cancer cell proliferation at certain times after treatment. It was also shown, for the first time, that the combination of thymosin treatment and moderate protein malnutrition enhanced resistance of mice to cancer cell proliferation above the level that either treatment had alone. This combined effect did not seem to be synergistic but rather to be additive.

Just after the last treatment, thymosin had a slightly suppressive effect upon spleen lymphocyte mitogenic responsiveness to PHA as shown previously. Prior to thymosin treatment, there was an increase in spleen lymphocyte mitogenic responsiveness to PHA per cell in untreated mice fed the MPD diet with a concomitant decrease in spleen lymphocyte number. By 7 and 21 days after the last thymosin treatment, there was an even more dramatic increase in spleen lymphocyte mitogenic responsiveness to PHA in protein-malnourished mice. The increase in spleen cell responsiveness to PHA in thymosin-treated moderately protein-malnourished mice may partially account for the concomitant increase in resistance to leukemia cell growth. The cytotoxic lymphocytes which occur before tumor transplantation have been termed NK cells, do not have \( \theta \) antigen, and are at a low level (10). The cytotoxic lymphocytes which develop after leukemia transplantation are derived from the \( \theta \) antigen-bearing population which are also responsive to PHA (12) and a slight increase in NK cell activity (10).

Therefore, the total \( \theta \) LMC is important in the resistance against cancer cell growth. There did not seem to be a difference in Day 1 NK cell activity among the dietary-treatment groups in this report. By Day 7 after leukemia cell transplantation, there was a small increase in splenic LMC activity which was present in all groups to an equal extent. The differences in Day 7 LMC activity could not be correlated with the differences in resistance to leukemia cells seen in the protein-malnourished and/or thymosin-treated mice. There was a slight discrepancy between the Day 7 LMC in the first and the second experiment. Variation at this time might be expected since this is an intermediate point in the response against L1210 cells. Peak LMC activity did not occur until 2 weeks after leukemia challenge, a result consistent with previous observations (13). The peak LMC activity was correlated with the clearance of leukemia cells from the peritoneal cavity of all groups of mice by Day 14 after leukemia transplantation.

Jose and Good (13) have suggested that the increased resistance to tumors in protein-malnourished mice was partially correlated with a decrease in serum blocking antibody, which normally develops about 9 days after tumor transplantation. However, we observed an apparent decrease in leukemia cell growth in protein-malnourished mice 7 days after transplantation. An event which must have occurred on or before that day rendered the protein-malnourished mice more resistant to cancer cell growth. Therefore, it seems unlikely that a decrease in synthesis of blocking antibody could have played a significant role in the increased resistance to cancer cells seen here.

Since the observed decreases in leukemia cell growth of protein-malnourished mice occurred prior to the time in which

### Table 7

<table>
<thead>
<tr>
<th>Dietary protein level (%)</th>
<th>Treatment type</th>
<th>Time after last treatment (days)</th>
<th>Cell-mediated cytotoxicity (%) at following time after injection of L1210 cells</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
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<td>Day 7</td>
</tr>
<tr>
<td>20</td>
<td>PBS</td>
<td>1</td>
<td>9.9 ± 1.8</td>
</tr>
<tr>
<td>20</td>
<td>Thymosin</td>
<td>1</td>
<td>9.0 ± 1.1</td>
</tr>
<tr>
<td>4</td>
<td>PBS</td>
<td>1</td>
<td>15.3 ± 1.8</td>
</tr>
<tr>
<td>4</td>
<td>Thymosin</td>
<td>1</td>
<td>8.7 ± 1.5</td>
</tr>
<tr>
<td>20</td>
<td>PBS</td>
<td>7</td>
<td>13.7 ± 0.8</td>
</tr>
<tr>
<td>20</td>
<td>Thymosin</td>
<td>7</td>
<td>18.3 ± 1.8</td>
</tr>
<tr>
<td>4</td>
<td>PBS</td>
<td>7</td>
<td>20.7 ± 2.4</td>
</tr>
<tr>
<td>4</td>
<td>Thymosin</td>
<td>7</td>
<td>13.4 ± 1.5</td>
</tr>
</tbody>
</table>

* Mice challenged with 1 x 10^7 L1210 mouse leukemia cells; effector cell-target ratio 200:1.

* Mean ± S.E. of spleen cells.

* Significantly different PBS-treated mice fed the control diet; P ≤ 0.05.

### Table 8

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>F</th>
<th>p</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein level (P)</td>
<td>3.0</td>
<td>≤ 0.088</td>
<td>8.7</td>
<td>≤ 0.005</td>
</tr>
<tr>
<td>Treatment type (T)</td>
<td>3.1</td>
<td>≤ 0.085</td>
<td>2.0</td>
<td>≤ 0.165</td>
</tr>
<tr>
<td>Duration after treatment (D)</td>
<td>12.6</td>
<td>≤ 0.001</td>
<td>10.1</td>
<td>≤ 0.001</td>
</tr>
<tr>
<td>P x T</td>
<td>11.5</td>
<td>≤ 0.001</td>
<td>3.7</td>
<td>≤ 0.052</td>
</tr>
<tr>
<td>P x D</td>
<td>0.3</td>
<td>≤ 0.771</td>
<td>2.3</td>
<td>≤ 0.113</td>
</tr>
<tr>
<td>T x D</td>
<td>1.4</td>
<td>≤ 0.244</td>
<td>2.4</td>
<td>≤ 0.102</td>
</tr>
<tr>
<td>P x T x D</td>
<td>1.3</td>
<td>≤ 0.276</td>
<td>3.3</td>
<td>≤ 0.046</td>
</tr>
</tbody>
</table>

* Mean ± S.E.

* Significantly different from PBS-treated mice fed the control diet; P ≤ 0.05.
significant LMC activity developed, it was possible that the increased resistance was due, at least in part, to increased levels of corticosterone. Corticosteroids have been shown to have inhibitory effects upon lymphoid tissues (2, 6). They have also been widely used in the successful treatment of acute leukemia (17) as well as malignant melanoma (11) and breast cancer (19). The success of corticosteroid therapy has been correlated with a direct interaction between corticosteroids and tumor cell via glucocorticoid receptors (17). The murine leukemia L1210 has been shown to be sensitive to high levels of glucocorticoids in vivo (14).

We observed that thymosin treatment decreased serum corticosterone levels in protein-malnourished mice for a short time after the last treatment compared to PBS-treated malnourished mice. The serum corticosterone levels still remained somewhat higher than normal. The thymosin treatment did not alter significantly the serum corticosterone levels of mice fed the control diet. The different effects of thymosin treatment between the 2 dietary groups with respect to corticosterone levels may be due to a reduction in glucocorticoid receptors on cells of the malnourished mice. Cells from protein-deficient rats (32) and monkeys (21) have decreased corticosteroid receptors. Cells with lower levels of glucocorticoid receptors are less sensitive to corticosteroid suppression of macromolecular synthesis (20). It is possible that thymosin treatment increased glucocorticoid receptors in lymphocytes from protein-malnourished mice, thereby decreasing serum corticosterone levels in these mice. These data suggest that there is an interaction between nutritional stresses and the immune and adrenal systems.

It may seem paradoxical that the in vitro mitogenic responsiveness of lymphocytes to PHA were increased in protein-malnourished animals with high in vivo levels of corticosterone. However, several previous reports have indicated that lymphocytes from malnourished animals have higher mitogenic responsiveness to PHA (3, 22). Guinea pigs treated in vivo with corticosteroids exhibited lymphocyte proliferation in vivo but lymphocytes which were more responsive in vitro to PHA (6). Therefore, even though lymphocytes may be suppressed in vivo with high corticosterone levels, they may not be less responsive to PHA in an in vitro environment without exogenous corticosterone.

Clearly, there is a difference between the very immunosuppressive effects of severe protein malnutrition (32) and moderate protein malnutrition which has some suppressive and stimulatory features upon the immune system. As demonstrated in this report, there was an enhancement in certain cell-mediated immune responses and resistance to leukemia cell growth by the combination of moderate dietary stress and exogenous thymosin treatment. These combined treatments offer a superb model to help understand the mechanism of alteration of the immune system by moderate nutritional stress and thymosin treatment.

ACKNOWLEDGMENTS

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Resistance to L1210 Mouse Leukemia Cells in Moderately Protein-malnourished BALB/c Mice Treated *in Vivo* with Thymosin Fraction V

Thomas M. Petro and Ronald R. Watson


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