Quantitative Effects of Nutritional Protein and Calorie Deficiency upon Immune Responses to Tumors in Mice

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SUMMARY

Protein calorie deficiency has been reported to increase resistance of animals to viral infection and malignant tumors while decreasing resistance to bacterial infection. Measurement of specific cellular and humoral immune responses to tumors in allogenic, syngenic, and autochthonous mouse systems showed both responses to be depressed by nutritional protein or calorie restriction, but humoral responses were affected at a higher nutritional level than are cellular responses. A range of moderate protein deficiency was found where cellular immune responses were enhanced due to lack of blocking serum antibody, and animals on this dietary protein level showed marked inhibition of tumor growth. Changes in lymphoid cell numbers and kinetics of the cellular response were found that may reflect mechanisms to conserve nutritional reserves.

INTRODUCTION

Rous (31) discovered in 1911 that undernourished chickens were almost totally resistant to infection with Rous sarcoma virus. Nutritional deprivation has since been reported to increase the resistance of rabbits to vaccinia virus (37), of mice to pseudorabies virus (7), and of man and mouse to poliomyelitis virus (14). Moreschi (28) and Rous (32) were the first to describe striking decreases in the incidence of spontaneous tumors in undernourished rodents. The incidence of spontaneous lung and breast tumors in calorie-restricted DBA, ABC, and Swiss mice reached only 16 to 55% of the incidence in normally fed controls at the same age; the mean time to the appearance of tumors was up to 1.9 times as long; and the mean total life-span of the malnourished animals was increased up to 5.7 times (41). Mice on low-calorie diets treated with carcinogenic hydrocarbons developed between 30 and 70% of the number of tumors and showed 1.2 to 1.9 times the latent period before appearance of the tumor in comparison with normally nourished animals. Caloric deficiency in animals has been consistently associated with marked reductions in incidence and delay in appearance of many tumors in rodents, including spontaneous mammary adenocarcinoma (42, 45, 48), spontaneous hepatoma (44), primary lung adenocarcinoma (41, 27), spontaneous leukemia (34), carcinogen-induced skin neoplasms (42, 44), UV-induced skin tumors (33), and carcinogen-induced sarcomata (41, 48). Decreases in incidence, growth, and metastatic spread of tumors could also be produced in animals with pure protein deficiency or with deficiency of essential amino acids. Particular examples are spontaneous mammary adenocarcinoma (15, 29, 44, 47), Walker rat tumor 256 (15), rat hepatoma 31 (46), rat lymphoma (30), and others (12, 13). A 20-year study of beef cattle showed the incidence of spontaneous ocular carcinoma to be 14% in heavier, larger animals fed on prime pastures and supplemented during the winter, compared to an incidence of 1.5% in animals on low-level feeding on poor pastures (1). In man, positive associations have been reported between cancer frequency and food intake (19), increasing body weight (43), and obesity (10, 36). The paradox of decreased host resistance to bacteria and increased resistance to virus infection and cancer in association with malnutrition, noted in 1945 (11), has never been adequately explained.

A possible immunological mechanism to explain these observations on the basis of increased host resistance to tumors was suggested by the demonstration that protein-deficient rats inoculated with tumor cells failed to develop serum-blocking antibody (21), which in normally nourished animals partly or completely inhibits cell-mediated immune destruction of the tumor. This paper reports further studies on the effect of nutritional deprivation upon host immune responses to allogenic, syngenic, and autochthonous tumors in mice.

MATERIALS AND METHODS

Animals. Male DBA2/J and C57BL/6J inbred mice were obtained from The Jackson Laboratories (Bar Harbor, Maine), and C3H/Bi/Um and C3Hf/Um male mice were obtained from the University of Minnesota colony.

Tumors. DBA/2 mastocytoma P-815-X2 ascites tumor (kindly provided by Dr. T. Brunner, Lausanne, Switzerland) was maintained by weekly serial passage and by in vitro culture in Dulbecco's modified Eagle's medium with 10% calf serum. A spontaneous C3Hf/Um mammary adenocarcinoma...
was maintained by s.c. passage in C3H/Bi/Umc mice and by in vitro culture RPMI\(^3\) Medium 1640 with 10% calf serum.

**Immunization.** For the allogenic system, C57BL/6J male mice received 30 X 10\(^6\) viable DBA/2J mastocytoma cells by i.p. injection, and spleen cells and serum were harvested 11 days later. For the syngenic system, solid C3H/Bi/Umc mammary adenocarcinoma was cut into small pieces with scissors, further dispersed with a Teflon tissue homogenizer, washed with calcium-magnesium-free Hanks' solution (Grand Island Biological Corp., Grand Island, N. Y.), and treated for 30 min at 37\(^\circ\)C with 0.05% trypsin-EDTA to obtain a single-cell suspension. After washing, 30 X 10\(^6\) viable mammary tumor cells in single-cell suspension were given by i.p. injection to C3Hf/Umc male mice, and spleen cells and serum were harvested 11 days later. Each tumor was established by in vitro culture at the time of immunization. For determination of secondary immune responses, a 2nd i.p. injection of 20 X 10\(^6\) viable cells was given 21 days after the primary immunization, and spleens and serum harvested 7 days later; for determination of late secondary response, a 3rd injection of tumor cells was given 14 days after the 2nd immunization, and spleens and serum were harvested 5 days later. Variations on this protocol are described for the results of individual experiments.

**In Vitro Assay.** Cytotoxic cellular immunity was measured in the allogenic system by the mastocytoma \(^{31}\)Cr release method of Brunner (3) as modified by Canty and Wunderlich (6). The assay measures radioactivity released into the supernatant from damaged radioactive isotope-labeled tumor target cells in the presence of sensitized or nonsensitized lymphocytes. Specific target cell lysis is considered to measure thymus-dependent cell-mediated immunity (6). Percentage specific lysis of target cells was calculated from the formula

\[
\% \text{ specific lysis } = \left( \frac{\text{cpm of specimen} - \text{cpm with nonimmune lymphocytes} \times 100\%}{\text{total releasable activity} - \text{cpm with nonimmune lymphocytes}} \right)
\]

Radioactivity was measured with a well-type \(\gamma\) counter (Nuclear Chicago Corp., Des Plaines, Ill.).

Cytotoxic cellular immunity was measured in syngenic and autochthonous systems by a modification of the in vitro assay described by Jagarlamoody et al. (20). The assay depends upon the loss of adherence of radioactivity-labeled target cells when damaged or killed by cytotoxic lymphoid cells, thus allowing these nonadhering cells to be washed out of the microcultures. Results are expressed as the percentage of decrease in radioactivity remaining in cultures of target cells with lymphocytes, compared to target cells alone, and is considered to measure thymus-dependent cytotoxic lymphocytic activity (23). Target cells consisting of C3H/Bi/Umc mammary tumor in monolayer cultures were labeled by the addition of tritiated thymidine (Amesham-Searle Corp., Arlington Heights, Ill.) (specific activity, 26 Ci/m mole; 10 \(\mu\)Ci/ml final concentration) for 7 days. Before testing, target cells were suspended with 0.05% trypsin-EDTA in calcium-magnesium-free Hanks' solution, washed 3 times, and adjusted to 5 X 10\(^4\) viable cells/ml in RPMI Medium 1640 with 20% fetal calf serum containing nonradioactive thymidine (2 mg/ml). Aliquots of 0.05 ml of labeled target cells (approximately 2500 cells) were placed in flat-bottomed wells of a Falcon Microtest 11 tissue culture plate. Aliquots of 0.05 ml spleen lymphoid cells, adjusted to 10\(^7\) viable cells/ml of RPMI media, were added to the appropriate target cells on the microculture plate. Lids were sealed and microculture plates were incubated for 72 hr at 37\(^\circ\)C in 5% CO\(_2\) and air. Cultures were terminated by gentle washing, with phosphate-buffered saline (pH 7.2), of each microculture well to remove nonadherent cells. Remaining cells adherent to the well base were air dried, lysed in 0.05 ml Hyamine hydroxide, and incorporated into dioxane-based liquid scintillation fluid; radioactivity was measured by a Beckman Model LS-233 liquid scintillation counter.

**Antibody Determinations.** Hemagglutinating antibody was determined by the polyvinylpyrrolidine method (38), with the use of allogenic erythrocytes from the immunizing mouse strain as indicator cells. Complement-dependent cytotoxic antibody titers were determined by release of radioactive label from target cells incubated with inactivated serum in serial dilution for 60 min at 4\(^\circ\)C, followed by incubation at 37\(^\circ\)C for 30 min with guinea pig complement at 1/20 dilution. Blocking antibody was measured by inhibition of target cell lysis by sensitized spleen lymphoid cells from immunized animals on normal diets in presence of serum tested.

Statistical analysis was performed with Student's \(t\) test.

**Mouse Diets.** Normal protein diets were prepared according to the formula (g dry weight/100 G diet): casein (N: 15%), 30; mineral mixture USP XIV, 4; vitamin mixture, 2; agar, 1; dextrose, 28; cornstarch, 28; corn oil, 7; distilled water, 100. Other ad libitum diets were prepared in similar proportions with substitution of dextrose-cornstarch for casein to achieve final casein concentrations of 12, 8.6, 6, and 3.5%. In these agar diets, protein contributed approximately 28, 11, 8, 5, and 3% of the available calories. Ad libitum diets were continuously available to the animals, but amounts actually consumed were measured regularly. Restricted calorie diets were given to the animals in quantities equivalent to one-half the dry weight of food consumed by mice fed the corresponding ad libitum diet. The concentrations of protein, minerals, vitamins, and corn oil were at twice the concentration of the corresponding ad libitum diet; thus each animal on calorie-restricted diets received the same total quantity of all constituents except dextrose and cornstarch. All animals were commenced on diets between 32 and 38 days of age and were tested between 10 and 14 weeks of age, except as specified in individual experiments.

**RESULTS**

**Kinetics of the Immune Response in Protein Deficiency.** Determination of cytotoxic cell-mediated immunity and serum-blocking activity were determined by in vitro assay at different time intervals after primary immunization of C3Hf/Umc mice, in different dietary groups, with syngenic mammary adenocarcinoma. Results (Chart 1) indicate that cellular cytotoxicity expressed as percentage of target cell lysis
Cancer Immunity in Dietary Protein-restricted Mice

Primary Immune Responses in Protein-Calorie-deficient Mice. Cellular cytotoxic immunity and serum-blocking activity, tested by \textit{in vitro} assay, were measured in animals fed different diets 11 days after i.p. inoculation of \(30 \times 10^6\) tumor cells in both allogenic (Table 1) and syngenic systems (Table 2). Mice fed protein-restricted or protein- and calorie-restricted diets sufficient to retard body weight development similar degrees of cellular cytotoxic immunity \textit{in vitro} down to a dietary level of 5\% protein-calories (Tables 1 and 2). Animals fed diets containing 3\% protein-calories failed to develop significant cellular cytotoxic immunity. Small but consistent increases in cellular cytotoxicity were found in mice on restricted diets \((p < 0.01)\). Serum cytotoxic antibody and hemagglutinating antibody titers developed in animals fed 28 or 11\% protein, but progressive reduction in these titers was found at dietary protein levels of 8 and 5\%, and no response could be detected in animals on 3\% protein-calories. Restriction of calories in mice on protein-deficient diets further reduced their body weight and antibody responses. Eleven-day sera from immunized animals on 28 and 11\% protein diets inhibited \textit{in vitro} cellular immunity (Tables 1 and 2). This blocking antibody activity was markedly reduced in animals on 8\% protein diets \((p < 0.01)\) and was undetectable at a 5\% protein level. Incubation of both cells and serum from immunized animals on 5\% protein diets produced 10-fold greater target cell lysis \textit{in vitro} than that for cells and serum from normally fed animals.

Secondary and Tertiary Immune Responses. Secondary and tertiary immunization by i.p. inoculation of tumor cells resulted in increases in cellular cytotoxicity, particularly at lower ratios of lymphocyte to target cell. An increasing proportion of protein-restricted mice began to show serum-blocking activity \textit{in vitro} on repeated inoculation, but the blocking activity in serum from these restricted mice was consistently less than in serum from normally fed mice. Animals restricted in both protein and calories showed smaller increases in cellular immunity and in blocking activity.

Table 1

\textit{Allogenic system: primary cellular and humoral immune responses of C57BL/6J mice on different diets 11 days after i.p. inoculation with DBA/2 mastocytoma}

<table>
<thead>
<tr>
<th>Protein content as % total calories</th>
<th>Total calories/mouse/day</th>
<th>Target cell ( ^{14} )Cr release (cpm)</th>
<th>% specific lysis</th>
<th>Serum blocking % specific lysis (cells and serum)</th>
<th>Mean log cytotoxic antibody titer</th>
<th>Mean log hemagglutination titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>21</td>
<td>3085 ± 274</td>
<td>54</td>
<td>15</td>
<td>2.4</td>
<td>4.2</td>
</tr>
<tr>
<td>11</td>
<td>20</td>
<td>3002 ± 253</td>
<td>52</td>
<td>23</td>
<td>2.4</td>
<td>3.9</td>
</tr>
<tr>
<td>8</td>
<td>18</td>
<td>3249 ± 271</td>
<td>58</td>
<td>46e</td>
<td>1.6e</td>
<td>3.1e</td>
</tr>
<tr>
<td>8</td>
<td>9</td>
<td>3507 ± 286</td>
<td>64e</td>
<td>54e</td>
<td>1.5e</td>
<td>2.5e</td>
</tr>
<tr>
<td>5</td>
<td>18</td>
<td>3466 ± 293</td>
<td>63e</td>
<td>60e</td>
<td>1.2e</td>
<td>2.1e</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>3591 ± 301</td>
<td>66e</td>
<td>63e</td>
<td>0.9e</td>
<td>1.8e</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>1072 ± 123</td>
<td>10e</td>
<td>62e</td>
<td>0e</td>
<td>0e</td>
</tr>
<tr>
<td>Nonimmune</td>
<td>840 ± 63</td>
<td></td>
<td>0e</td>
<td>64e</td>
<td>0e</td>
<td>0e</td>
</tr>
</tbody>
</table>

\begin{itemize}
\item \textsuperscript{a} Mean cpm ± S.D. of radioactivity released in each test after adjustment of total releasable activity to 5000 cpm.
\item \textsuperscript{b} Percentage of specific lysis in each test with sensitized lymphocytes from mice on 28\% casein diets and inactivated serum from each dietary group at 1/10 final dilution.
\item \textsuperscript{c} Significantly different from 28\% casein group \((p < 0.01)\).
\end{itemize}
following repeated inoculation. Hence the deficits in humoral immune responses demonstrated in animals on restricted diets in the primary response could be partly overcome by repeated antigenic stimulation.

Numbers of Cytotoxic Cells in Nutrition-deficient Animals. Lower total numbers of lymphoid cells were found in spleens of immunized mice on low-protein or low-calorie diets than in normally fed controls. In previous studies, this deficit was corrected by adjusting all spleen cell suspensions to the same cell concentration before testing by in vitro assay. In this experiment, spleen cell suspensions were adjusted to a standard volume of suspending medium before testing and mixed with target cells in ratios equivalent to those existing in vivo. Results showed a similar degree of cytotoxicity by both lymphoid cell populations, in spite of a 2-fold reduction in the ratio of lymphocyte to target cells (Table 3). These findings may indicate that the specifically committed effector cells operate more efficiently, perhaps due to lack of serum inhibition, or that a greater proportion of spleen cells in the malnourished animals are specifically committed, although the total spleen cell number is reduced.

Tumor Growth and Immune Responses in Autochthonous Mice. Twelve-week-old C3Hf/Umc male mice were inoculated s.c. with 1 X 10^6 viable C3H/Bi/Umc mammary tumor cells and, after 1 week on normal diets, were divided randomly into different groups.

Radioactivity remaining in microcultures of target cells after incubation with spleen cells or with spleen cells and serum from animals in different groups.

### Table 2

<table>
<thead>
<tr>
<th>Diets</th>
<th>Total calories/mouse/day</th>
<th>Mean body wt (g)</th>
<th>cpm remaining in cultures</th>
<th>% inhibition of culture by spleen cells</th>
<th>cpm remaining in cultures</th>
<th>% total inhibition of cultures by spleen cells and serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>28% casein</td>
<td>20</td>
<td>18.6 ± 1.6</td>
<td>1435 ± 86^a</td>
<td>72</td>
<td>4885 ± 236^a</td>
<td>2</td>
</tr>
<tr>
<td>11% casein</td>
<td>19</td>
<td>16.4 ± 2.1</td>
<td>1594 ± 92</td>
<td>68</td>
<td>3221 ± 104</td>
<td>36^b</td>
</tr>
<tr>
<td>8% casein</td>
<td>18</td>
<td>12.8 ± 1.9</td>
<td>1659 ± 125</td>
<td>67</td>
<td>1914 ± 96</td>
<td>62^b</td>
</tr>
<tr>
<td>5% casein</td>
<td>17</td>
<td>11.2 ± 2.3</td>
<td>1322 ± 91</td>
<td>74</td>
<td>1593 ± 113</td>
<td>68^b</td>
</tr>
<tr>
<td>3% casein</td>
<td>13</td>
<td>9.8 ± 1.3</td>
<td>1417 ± 97</td>
<td>72</td>
<td>1615 ± 102</td>
<td>68^b</td>
</tr>
<tr>
<td>Nonimmune</td>
<td></td>
<td></td>
<td>4413 ± 151</td>
<td>12^b</td>
<td>1142 ± 88</td>
<td>78^b</td>
</tr>
</tbody>
</table>

^a Mean ± S.D. ^b Significantly different from 28% protein group (p < 0.05).

groups fed protein- and calorie-deficient diets. Five animals in each dietary group were sacrificed at weekly intervals and tumor size, cell-mediated immunity in vitro, and serum-blocking activity were measured. One week after inoculation, many animals had small palpable tumors and all had developed detectable cellular cytotoxic immunity and serum-blocking activity.

Two weeks after commencing the diets, mice fed high- or low-protein diets showed no change in either cellular immunity or blocking antibody, but animals on restricted-calorie-low-protein diets demonstrated a decrease in blocking activity (Table 4). Both low-protein and low-calorie animals showed absent blocking activity after 4 weeks on their respective diets, while cytotoxic cellular immunity in vitro remained vigorous. Tumor weight in animals on restricted diets was retarded to significantly greater extent (p < 0.01) than the reduction in tumor-free body weight of these diet-restricted animals, in comparison with animals on normal diets.

### DISCUSSION

The in vitro assays of cytotoxic cellular immunity used in this study are considered to measure specific thymus-dependent lymphocyte immunity for tumor target cells and may reflect a major component of the host defense against malignant cells (16, 17). Normal cellular cytotoxic responses to the immunizing tumor developed in animals fed at least 5% casein in their diets. Diminution in spleen cell numbers and changes in kinetics of the primary cellular response appeared in mice fed less than 10% casein and progressed to profound depression of cellular responses at a dietary level of 3% casein. Restriction of calories in animals on low-protein diets resulted in further weight loss and decreases in spleen cell number, but cytotoxic lymphocyte function was not impaired.

The major immunological difference detected between normal and protein-restricted animals in this study was the absence of serum-blocking activity in mice fed diets containing less than 10% casein. In the absence of this serum inhibition,
cancer immunity in dietary protein-restricted mice

Table 4

Relation of tumor growth, cellular immunity, and serum blocking in C3H mice commenced on diets 1 week after s.c. inoculation of 1 x 10⁶ mammary tumor cells

<table>
<thead>
<tr>
<th>Duration of diets (wk)</th>
<th>Mean tumor wt a</th>
<th>Tumor-free wt b</th>
<th>Cellular immunity c</th>
<th>Serum blocking d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28% casein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>98</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>94</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>26</td>
<td>89</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>34</td>
<td>81</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>41</td>
<td>78</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>5% casein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>92</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>13</td>
<td>83</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>81</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>79</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>79</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>5% casein, half-calorie</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>89</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>81</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>74</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>68</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>69</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

a Mean weight of excised tumor expressed as percentage of original host body weight (mean, 18 ± 3 g), 5 mice/group/week.
b Mean weight of tumor-bearing animal following excision of tumor, expressed as percentage of original body weight.
c Number of animals out of 5 showing 50% target cell lysis in vitro at 100/1 spleen cell to tumor ratio.
d Number of animals out of 5 the serum of which blocks cell-mediated lysis in vitro by 60% or more at 1/10 dilution.

The occurrence of tumor-specific IgG2a antibody at similar dietary protein levels, suggesting an effect on either component of a blocking antigen-antibody complex. Protein deficiency impairs immunoglobulin synthesis (5) and depresses numbers of plaque-forming cells and hemagglutinating antibodies to sheep red blood cells in rats and mice (8, 26). Powerful bacterial antigens have resulted in normal antibody responses at a similar level of protein deficiency (8).

The adverse effect of nutritional deprivation on the development, growth, and spread of malignant tumors in animals has been ascribed to limitation of nutrients essential for tumor growth (9), limitation of energy requirements for mitosis (4), or inhibition of the fundamental changes of carcinogenesis (25). The demonstration in these studies of profound depression of serum-blocking activity in animals with similar nutritional states suggests a possible direct antitumor effect through augmentation of cellular immune surveillance.

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REFERENCES


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