Reduced Splenocyte Metabolism and Immune Function in Rats Implanted With the Morris Hepatoma 7777

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Although the immune system is important in antitumor defense, little is known about the immune response during progressive tumor growth. Sprague-Dawley rats (171 ± 3 g) of the Buffalo strain were implanted with the Morris Hepatoma 7777 ([MH 7777] a poorly differentiated, rapidly growing tumor) and killed either 2 (T2) or 3 (T3) weeks postimplantation when the tumor weighed 3.0 ± 0.4 and 14 ± 1 g, respectively. Splenocytes were isolated and their phenotypes, metabolism (metabolite production from glucose and glutamine), proliferative response ([^{3}H]thymidine incorporation in response to polyclonal mitogens), and natural killer (NK) cytotoxicity (lysis of YAC-1 cells) were determined. Five rats were killed with the T2 group to serve as non–tumor-bearing controls (T0). Food intake and nontumor body weight decreased ($P < .01$) 14 days after tumor implantation. There was a progressive decrease (T3 < T2 < T0) in splenic mitogen responses ($P < .05$) and plasma essential and nonessential amino acid concentrations ($P < .05$). Compared with T0, NK cytotoxic activity was significantly ($P < .05$) lower at T2 and higher at T3. The presence of the tumor at both T2 and T3 resulted in lower production of metabolites from glucose and glutamine by splenocytes. The proportion of CD8$^+$ cells was lower ($P < .05$) and the proportion of B cells and macrophages higher ($P < .05$) in spleens from tumor-bearing rats. In conclusion, the presence of even a small tumor burden (1.4% of body weight) significantly altered the host's immune function and metabolism. A larger tumor burden (6% of body weight) increased NK cytotoxic activity and further reduced cell-mediated immune function.

ALTHOUGH IT IS KNOWN that immunological defenses are important for the control of experimental tumors, many aspects of this basic phenomenon remain unknown. The Morris Hepatoma 7777 (MH 7777) is a poorly differentiated and rapidly growing transplantable tumor that results in host death approximately 4 weeks after implantation, when the tumor has reached approximately 10% of body weight.[1] Although this tumor is frequently used as a model of neoplasia, little is known about the host immune response to the MH 7777. Important contributors to immune surveillance for other tumors (induced, transplantable, and spontaneously occurring) include components of both the natural (nonspecific) and specific immune system.[2-4] The importance of these host defenses during the progressive growth of a tumor is not known.

There is a growing interest in lymphocyte energy metabolism and its possible role in immune function in health and disease.[5-8] The utilization and oxidation of glucose and glutamine, the most abundant amino acid in plasma, are essential for cells of the immune system.[6,7] Lymphocyte activation and proliferation are associated with a significant increase in metabolic activity.[7-9] Blocking the utilization of either glucose or glutamine in vivo[10] or in vitro[11] significantly reduces immune function. Recently, we demonstrated that the MH 7777 uses for protein synthesis and oxidizes a significant amount of the host's amino acid intake.[12] Tumor cells also use glucose and glutamine at high rates[13,14] and thus are in direct competition with the host for these important substrates. The effect of neoplasia on immune cell metabolism is not known. To aid in the design of interventions aimed at improving host antitumor defenses, a clear understanding of how the tumor affects the function and metabolism of the immune system is necessary. The following study was conducted to examine the progressive effect of the MH 7777 on host natural killer (NK) cell activity, immune response to mitogens, and immune cell metabolism.

MATERIALS AND METHODS

Chemicals

D-[U-^{14}C]glucose and L-[U-^{14}C]glutamine were obtained from New England Nuclear (Boston, MA). [^{14}C]glutamine was purified immediately before use on a Dowex AG1-X8 (200-400 mesh, acetate form; Bio-Rad Laboratories, Mississauga, Ontario, Canada) column (0.55 × 0.8 cm) by eluting it with 2 mL water. Sodium ^{51}Cr and [^{3}H]thymidine were purchased from Amersham (Oakville, Ontario, Canada). RPMI culture media was purchased from Fisher Scientific (Edmonton, Alberta, Canada), antibiotic-antimycotic from Gibco (Burlington, Ontario, Canada), and all other culture media ingredients and Ecolite scintillation fluid from ICN (Montreal, Quebec, Canada). Benzethonium hydroxide, bovine serum albumin (fraction V), amino acids, glucose, and trypan blue were purchased from Sigma Chemical (St Louis, MO). Lactate dehydrogenase, glutamate dehydrogenase, NAD, NADH, adenosine diphosphate, and α-ketoglutaric acid were purchased from Boehringer-Mannheim Canada (Laval, Quebec, Canada). Fluorescein isothiocyanate–conjugated goat antimouse IgG with no cross-reaction to rat IgG was obtained from Organon Teknika (Scarborough, Ontario, Canada). All diet ingredients were purchased from Teklad (Madison, WI).

Experimental Design

Experiments were reviewed by the University Animal Policy and Welfare Committee and conducted in accordance with the guidelines of the Canadian Council on Animal Care. Twenty-five female Sprague-Dawley rats (171 ± 3 g) of the Buffalo strain were housed individually in a temperature- and light (12-hour light/dark cycle)-controlled room with free access to water, and were fed ad libitum

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a nutritionally complete semipurified diet. The diet contained (wt/wt) 26% high-protein casein, 20% fat (providing a polyunsaturated to saturated fatty acid ratio of 0.9), 19% starch, 20% glucose, 4.8% nonnutritive cellulose, 4% glycine, 0.3% choline, 0.6% inositol, 0.4% L-methionine, 4.8% Bernhart-Tomarelli mineral mix, and 1% AOAC vitamin mix. After 1 week of feeding, 20 rats received two 20-μL subcutaneous injections, one in each flank, of finely chopped MH 7777. Body weight and food intake were recorded every 3 days throughout the first 2 weeks of the study. Rats were killed by CO_2 asphyxiation after 2 (T2, n = 10) or 3 (T3, n = 10) weeks following implantation. At necropsy, arterial blood was collected by cardiac puncture, the spleen was removed, and the tumors were excised from both flanks, pooled, and weighed. Five rats served as non–tumor-bearing controls (T0) and were fed the diet for the same time as the T2 group.

Preparation of Immune Cells

Splenocytes were isolated under sterile conditions[7] in Krebs-Ringer HEPES buffer supplemented with bovine serum albumin (5 g/L). For determination of NK cytotoxic activity, cells were prepared and cultured in RPMI containing fetal calf serum (50 g/L), 2-mercaptoethanol (2.5 μmol/L), glutamine (4 mmol/L), penicillin (1×10^5 U/L), streptomycin (100 mg/L), amphotericin B as Fungizone (0.25 g/L) (Gibco, Burlington, Ontario), and HEPES (25 mmol/L). Cell viability was assessed by trypan blue exclusion and was greater than 90% for all groups.

Splenocyte Energy Metabolism

Glucose conversion to lactate, pyruvate, and CO_2 by splenocytes was determined as previously described in detail.[8] Cells (4×10^9/L) were incubated in Krebs-Ringer HEPES buffer (described above) containing 4 mmol/L glucose (containing 7.4 MBq/L glucose) ± 1 mmol/L glutamine. Tubes were incubated while shaking for 2 hours at 37°C. Reactions were terminated by injection of 100 μL $HClO_4$ (1.5 mol/L), and shaking continued for 1 hour to trap evolved CO_2. $^{14}CO_2$ was trapped in benzethonium hydroxide, and its radioactivity was measured by liquid scintillation spectrophotometry in a Beckman 5000 beta counter (LS 5801; Beckman Instruments, Mississauga, Ontario, Canada). The incubation media (extracts plus cells) were stored at −20°C for later spectrophotometric determination of lactate[15] and, after neutralization, for pyruvate.[16] Glutamine conversion to glutamate, aspartate, and CO_2 was determined by incubating cells in 1 mmol/L glutamine (containing 18.5 MBq/L L-[U-^{14}C]glutamine) ± 4 mmol/L glucose under the incubation conditions already described. Upon terminating the reaction, trapped $^{14}CO_2$ was collected and counted as described for glucose. The incubation media (extracts plus cells) were neutralized, and ^{14}C-glutamate and ^{14}C-aspartate were separated on a Dowex AG1-X8 (200-400 mesh, acetate form) column as previously described.[7] Their radioactivities were determined in Ecolite scintillation cocktail and measured in the beta counter described earlier.

NK Cell Cytotoxicity

NK cell–sensitive YAC-1 cells (a gift from Dr A. Rabinovitch, Department of Medicine, University of Alberta, Edmonton, Alberta, Canada) were incubated with sodium chromate (^{51}Cr, 5.55 MBq) and seeded into 96-well V-bottom microtiter plates. Splenocytes were added in triplicate to the wells to achieve effector to target ratios of between 5:1 and 100:1. Following 4 hours of incubation at 37°C, an aliquot of the supernatant was counted in a gamma counter (Beckman gamma 8000) to determine ^{51}Cr release. Spontaneous release was determined from target cells incubated in the absence of effector cells. Maximum release was determined from detergent lysis of labeled target cells. Cytotoxic activity was calculated as follows: % specific lysis = 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Results were also calculated on a per-cell basis using the number of NK cells present, as determined by 3.2.3 monoclonal antibody binding. This was expressed in lytic units, with 1 lytic U being the number of effector cells ($\times 10^{-3}$) required to cause 15% lysis of target cells.

Splenocyte Mitogen Response

Splenocytes (2.5×10^9 cells/L) were cultured in 96-well microtiter plates with or without either Concanavalin A ([Con A] 5 μg/mL) or phorbol myristate acetate ([PMA] 30 μg/L) plus Ionomycin ([Iono] 0.75 μmol/L; Sigma) for 48 and 72 hours. Preliminary experiments determined that a 72-hour incubation produced the maximum [^{3}H]thymidine response to both mitogens by non–tumor-bearing animals. Experiments were conducted with splenocytes from both control and tumor-bearing rats to determine culture periods that corresponded to the maximum response for the mitogen concentrations used (data not shown). Cells were incubated in a humidified atmosphere at 37°C in the presence of 5% CO_2. Eighteen hours before harvesting the cells, each well was pulsed with 37 kBq [^{3}H]thymidine. Cells were harvested on glass-fiber filters using a multiwell harvester (Skatron, Lier, Norway) and counted using Ecolite in the beta counter described earlier. All assays were performed in quadruplicate, and stimulation indices were calculated for each condition as follows: [^{3}H]thymidine (kBq/min) incorporated by stimulated cells − [^{3}H]thymidine (kBq/min) incorporated by unstimulated cells)/[^{3}H]thymidine (kBq/min) incorporated by unstimulated cells.

Mononuclear Cell Phenotyping

Lymphocyte subsets from spleen were identified by immunofluorescence assay using supernatants from hybridomas secreting mouse monoclonal antibodies specific for different rat mononuclear cell subsets (kindly provided by Dr A. Rabinovitch, Edmonton, Alberta, Canada, and 3.2.3 by Dr P. Poussier, Toronto, Ontario, Canada). W3/25 recognizes a surface glycoprotein found on rat T-helper cells (CD4), OX8 recognizes T-cytotoxic/suppressor lymphocytes (CD8) and NK cells, OX42 reacts with a receptor found on most monocytes, granulocytes, and macrophages, OX12 recognizes a determinant on the rat κ-chain of Igs on B lymphocytes, and 3.2.3 reacts with rodent NK cells. Aliquots of 2 to 5×10^5 splenocytes were incubated for 30 minutes at 4°C with each antibody, washed three times in 200 μL phosphate-buffered saline containing fetal calf serum (40 g/L), and incubated for another 30 minutes at 4°C in 50 μL of a 1:300 dilution of fluorescein isothiocyanate–conjugated goat antimouse IgG. The cells were washed three times, fixed in paraformaldehyde (10 g/L in phosphate-buffered saline), and analyzed on a FACScan (Becton Dickinson, Sunnyvale, CA) according to relative fluorescence intensity. Resulting percentages were corrected for background fluorescence (5% to 10%), determined by incubating cells with fluorescein isothiocyanate–conjugated goat antimouse IgG only.

Plasma Amino Acids

Plasma was separated from arterial blood by centrifugation at 350 × *g* for 20 minutes and stored at −70°C for subsequent analysis. Amino acids were separated using a Varian 5000 (Palo Alto, CA) high-performance liquid chromatograph with a fluorochrome detector.[17] Samples were injected onto a Supelcosil 3-μm LC-18 reverse-phase column (4.6 × 150 mm; Supelco, Bellafonte, CA), and peak area integrations were calculated using a Shimadzu

Ezchrom Chromatography Data System (Shimadzu Scientific Instruments, Columbia, MA).

Statistical Analysis

The data were analyzed using SAS (Version 6; SAS Institute, Cary, NC) and are presented as the mean ± SEM. The effects of tumor were determined by one-way ANOVA. Significant ($P < .05$) differences between groups were identified by the Duncan multiple-range test.[18] NK cytotoxic activity, food intake, and body weight were compared among groups by a split-plot ANOVA. Paired *t* tests were used to compare food intake between days and rates of metabolite production with or without glucose or with or without glutamine.

RESULTS

Body Weight, Food Intake, and Tumor Weight

There was no significant effect of the tumor on rat body weight or food intake when analyzed over the first 2-week period (Fig 1). After 14 days, T3 rats continued to gain weight. However, most of the weight gain could be accounted for by the increase (11 g) in tumor weight between

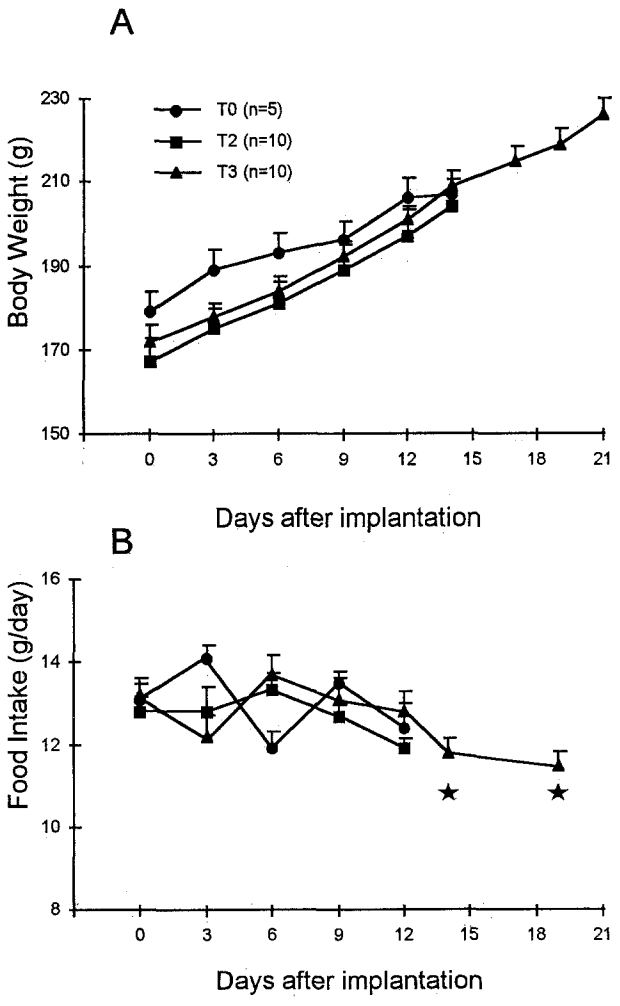

Fig 1. Effect of tumor progression on body weight and food intake. Points represent the mean ± SEM. *Significantly ($P < .01$) different *v* day 0, as determined by a paired *t* test.

Table 1. Effect of Tumor Progression on Splenocyte Phenotypes

Antibody	Weeks After Implantation		
	0 (n = 5)	2 (n = 9)	3 (n = 9)
W3/25$^+$ (CD4$^+$)	35 ± 2^a	21 ± 1^b	34 ± 1^a
OX8$^+$ (CD8$^+$ + NK cells)	21 ± 1^a	18 ± 1^b	15 ± 1^b
OX12$^+$ (B cells)	17 ± 1^a	33 ± 3^b	31 ± 1^b
OX42$^+$ (macrophages)	6.7 ± 0.6^a	12 ± 1^b	9.8 ± 0.9^b
3.2.3$^+$ (NK cells)	6.7 ± 0.4	8.2 ± 0.7	7.8 ± 0.5
CD4/CD8 ratio	1.7 ± 0.1^a	1.2 ± 0.1^b	2.2 ± 0.1^c

NOTE. Values are the mean ± SEM percentage of total cells. Means within a row that do not share a common superscript are significantly ($P < .05$) different.

weeks 2 and 3. Compared with T0, food intake was lower ($P < .01$) on days 14 and 19 for T3 rats (Fig 1B). On days 14 and 21, the tumor weighed 3.0 ± 0.4 g (1.4% ± 0.1% of body weight) and 14 ± 1 g (6.1% ± 0.5% of body weight), respectively.

Splenocyte Numbers and Phenotypes

Spleen weight (0.46 ± 0.01 g, n = 25) did not differ among groups. However, compared with T0 ($155 \pm 11 \times 10^6$, n = 5), a significantly ($P < .05$) greater number of splenocytes were isolated from rats at T3 ($233 \pm 17 \times 10^6$, n = 10). The number of cells isolated from spleens at T2 ($187 \pm 10 \times 10^6$, n = 10) did not differ significantly compared with T0 or T3. Compared with non–tumor-bearing rats, there was a lower ($P < .05$) relative percent of CD8$^+$ (T-suppressor/cytotoxic) cells and a higher ($P < .05$) percent of macrophages (OX42-positive [OX42$^+$] cells) and B(OX12$^+$) cells at both T2 and T3 (Table 1). The relative percent of CD4$^+$ (W3/25$^+$) cells was lower at T2, but returned to a proportion not significantly different from T0 at T3. Compared with T0, the CD4/CD8 ratio was lower ($P < .05$) at T2 and higher ($P < .05$) at T3 (Table 1).

Plasma Amino Acid Concentrations

Amino acid concentrations in arterial plasma are listed in Table 2. Compared with T0, at T2 there was a lower concentration of ornithine (−43%), tryptophan (−19%), threonine (−17%), and valine (−15%). At T3, ornithine, tryptophan, and threonine levels remained lower than at T0, and there was a further decrease in the concentration of valine (32% < T0). In addition, at T3, concentrations of isoleucine (−31%), leucine (−32%), asparagine (−27%), tyrosine (−26%), glutamine (−18%), lysine (−18%), and alanine (−16%) were lower than those at T0 (Table 2).

NK Cytotoxic Activity

When analyzed across the five effector to target cell ratios measured, compared with T0, splenocyte NK cytotoxic activity was significantly reduced at T2 and increased at T3 (Fig 2). At each effector to target ratio, the percent specific lysis was greater ($P < .05$) for splenocytes from T3 rats as compared with T0 or T2 (Fig 2). In our laboratory, interassay variation is low, and the response of T0 rats was not different from that consistently observed in non–tumor-bearing rats. The relative percent of NK cells in spleen did not differ among groups (Table 1). Lytic units, the number

Table 2. Effect of Tumor Progression on Amino Acid Concentrations in Arterial Plasma

Amino Acid	Weeks After Implantation		
	0 (n = 5)	2 (n = 9)	3 (n = 10)
		μmol/L	
Alanine	427 ± 1[a]	408 ± 27[ab]	358 ± 16[b]
Arginine	78 ± 4	87 ± 6	74 ± 5
Asparagine	97 ± 3[a]	83 ± 5[ab]	71 ± 5[b]
Aspartate	16 ± 1	19 ± 2	15 ± 2
Glutamate	193 ± 4	157 ± 17	145 ± 14
Glutamine	1,109 ± 61[a]	977 ± 40[ab]	909 ± 61[b]
Glycine	530 ± 55	507 ± 32	534 ± 18
Histidine	56 ± 2	53 ± 4	60 ± 4
Isoleucine	157 ± 7[a]	135 ± 8[a]	109 ± 8[b]
Leucine	257 ± 12[a]	218 ± 13[a]	174 ± 14[b]
Lysine	854 ± 18[a]	815 ± 40[a]	702 ± 36[b]
Methionine	89 ± 1	88 ± 5	75 ± 5
Ornithine	79 ± 7[a]	45 ± 7[b]	40 ± 5[b]
Phenylalanine	70 ± 2	63 ± 2	64 ± 3
Serine	454 ± 19	404 ± 17	408 ± 16
Taurine	187 ± 15	219 ± 26	166 ± 11
Threonine	400 ± 19[a]	351 ± 14[b]	334 ± 14[b]
Tryptophan	235 ± 6[a]	190 ± 6[b]	175 ± 6[b]
Tyrosine	121 ± 9[a]	107 ± 8[ab]	90 ± 7[b]
Valine	343 ± 15[a]	292 ± 15[b]	232 ± 16[c]
Total	5,759 ± 108[a]	5,308 ± 207[a]	4,760 ± 140[b]

NOTE. Values are the mean ± SEM. Means within a row that do not share a common superscript are significantly ($P < .05$) different. Citrulline was less than the detection limit of the method.

of cells ($\times 10^{-3}$) required to induce 15% lysis of target cells, were significantly higher ($P < .05$) at T2 (7.8 ± 0.8, n = 10) than at T0 (5.4 ± 0.7, n = 5) or T3 (3.9 ± 0.2, n = 10).

Mitogen Responses

Compared with the unstimulated (control) condition, incubation with the mitogens Con A or the mixture of PMA + Iono significantly increased ($P < .001$) [^{3}H]thymidine incorporation by splenocytes from T0 and T2 rats at both 48 and 72 hours, but only after 72 hours for T3 (Fig 3, statistics not shown). A higher response to Con A occurred at 72 hours for all three groups (Table 3). A higher response to PMA + Iono occurred at 72 hours for T0 and T3, but was not different at 48 or 72 hours for T2 (Table 3). Compared with T0, the control (unstimulated) response increased ($P < .05$) at 48 hours for T2 and decreased ($P < .05$) at 72 hours for T3 (Fig 4). The [^{3}H]thymidine response to both mitogens at 72 hours by cells from T2 rats was lower than for T0 (Table 3 and Fig 3). The response by cells from T3 rats was lower ($P < .001$) than for T0 and T2 at both time points measured (Fig 4). However, because the unstimulated response at T3 was lower than at T2, the stimulation indices to both mitogens at 72 hours did not differ between T2 and T3 (Table 3).

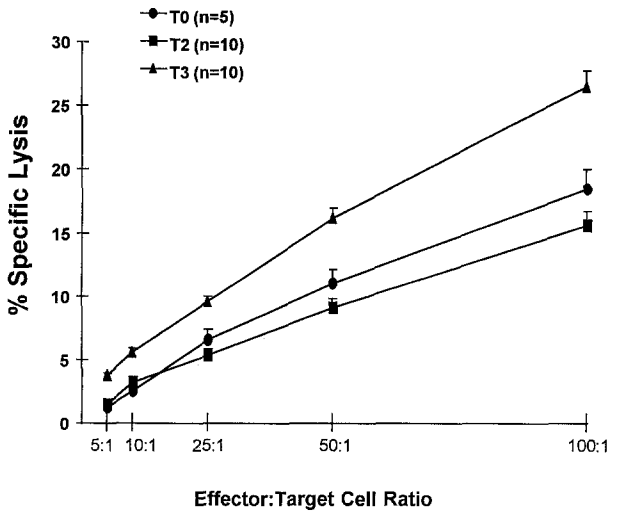

Fig 2. Effect of tumor progression on NK cytotoxic activity. Points represent the mean ± SEM. Lines for T0, T2, and T3 are all significantly ($P < .05$) different from each other, as analyzed by a split-plot ANOVA. At each effector:target cell ratio, the % specific lysis was greater ($P < .05$) for T3 *v* T0 cells.

Splenocyte Glucose Metabolism

When glucose was provided as the sole energy source, significantly lower rates of CO_2 and pyruvate production were observed at T2 and T3 compared with T0 (Table 4). Adding glutamine to the incubation media decreased the amount of glucose oxidized to CO_2 by all three groups (Table 4). When glutamine was present, the rates of metabolite production by cells from T2 were not different versus T0. With glutamine in the media, CO_2 production remained lower for T3 compared with T0 and T2 cells.

Splenocyte Glutamine Metabolism

When glutamine was provided as the sole energy source, lower rates of CO_2 and aspartate production were observed at T2 and T3 compared with T0 (Table 5). At T2 but not T3, the rate of NH_3 production was lower than at T0. Providing glucose in the incubation media reduced ($P < .05$) the rate of aspartate production by all three groups, CO_2 production by cells from T0 and T3, and glutamate by cells from T0 (Table 5). With the addition of glucose, the rates of CO_2 and NH_3 production were lower for T2 and T3 compared with T0 (Table 5).

DISCUSSION

Multiple effector mechanisms, immune and nonimmune, are involved in host resistance against tumor growth and metastasis. Specific immune responses against tumor-specific antigens are mediated by the cellular immune system,[2] and nonspecific immune responses by macrophages[19] and NK cells.[3] The tumor represents a considerable stressor/stimulant to the immune system. Mitogen-induced lymphocyte transformations provide a useful in vitro estimation of the cell-mediated immune response. The response to both the lectin, Con A (primarily a T-cell mitogen), and the combination of PMA + Iono (which stimulates most cell types) decreased progressively with tumor progression (Fig 3). At 3 weeks after implantation, cell-mediated immune function was suppressed to the point that cells did not respond to mitogens after 48 hours in culture. In other experimental tumor models, the very low response seen at this time is proposed to be due to

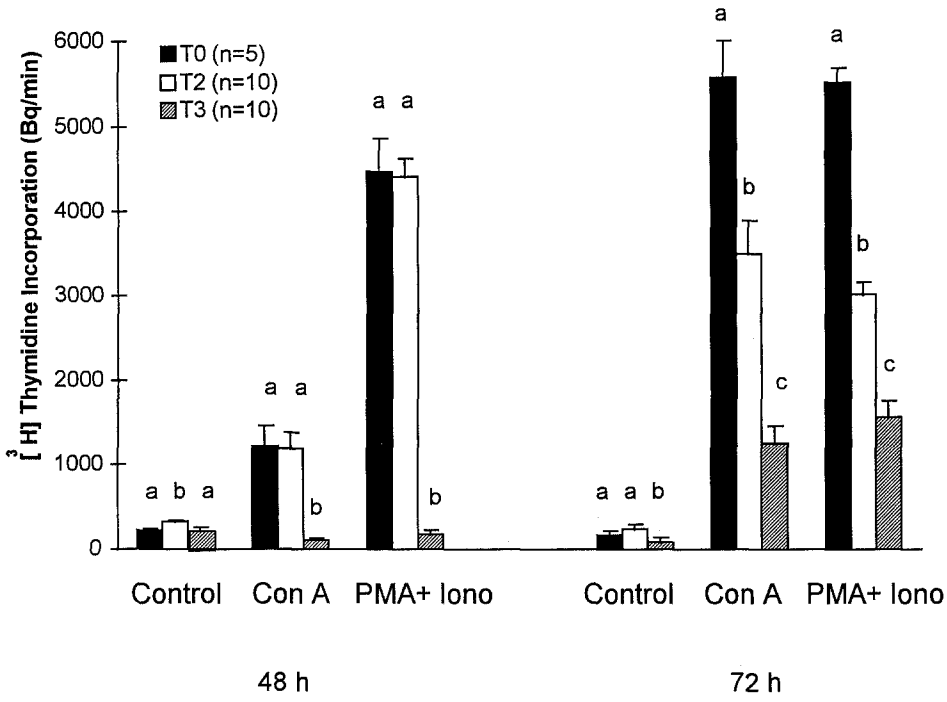

Fig 3. Effect of tumor progression on [^{3}H]thymidine uptake in response to incubation with mitogens. Bars represent the mean ± SEM. For each culture condition, bars that do not share a common superscript are significantly ($P < .05$) different as determined by ANOVA. Control, unstimulated cells.

enhanced suppression.[2] Once this suppressive activity is strongly established, removal of the tumor does not necessarily result in recovery of immune function.[2]

Underfeeding and energy restriction have been shown to alter tumor growth[20] and immune function.[21] At 2 weeks after implantation, rats had similar weight gain and food intake compared with control rats, and it is unlikely that the observed changes in immune function in these rats are due to energy malnutrition. At 14 days postimplantation, food intake by T3 rats was lower (9%) than before implantation of the tumor, and most of the weight gained after 2 weeks could be accounted for by the increase in tumor burden. Cancer-related anorexia and cachexia are important prognostic determinants of morbidity and mortality in cancer patients.[22] Therefore, energy/protein malnutrition likely contributed to the decreased mitogen responses of cells from T3 rats. In addition, casein is the basis for many of the current enteral formulas available to supplement cancer patients. Investigation with animals has suggested that exogenous nucleotides supplied by the diet increase immunity by contributing to the pool of nucleotides available to lymphocytes.[23,24] Restricting dietary nucleotides (by feeding purified casein diets) has been demonstrated to decrease NK cytotoxic activity[23] and cell-mediated immunity.[24] It is therefore possible that all our rats, including those not bearing tumors, demonstrated some immunosuppression due to the diet fed.

It has been suggested that the tumor may inhibit cell-mediated immune function by enhancing suppression in the host and/or depleting or inhibiting the function of accessory cells. However, in both tumor-bearing groups, compared with T0, there was a decrease in the relative percent

Table 3. Effect of Tumor Progression on Mitogenic Stimulation Index

	Stimulation Index		
	Weeks After Implantation		
Mitogen	0 (n = 5)	2 (n = 10)	3 (n = 10)
Con A			
48 h	4.8 ± 1.3^a	2.9 ± 0.9^a	0.01 ± 0.01^b
72 h	32 ± 3^a	13 ± 1^b	13 ± 2^b
PMA ± Iono			
48 h	20 ± 3^a	13 ± 1^b	0.2 ± 0.1^c
72 h	32 ± 1^a	12 ± 1^b	16 ± 2^b

NOTE. Values are the mean ± SEM. Means within a row that do not share a common superscript are significantly ($P < .05$) different. The stimulation index was determined as ([^{3}H]thymidine (kBq/min) incorporated by stimulated cells − [^{3}H]thymidine (kBq/min) incorporated by unstimulated cells)/[^{3}H]thymidine (kBq/min) incorporated by unstimulated cells.

Table 4. Effect of Tumor Progression on Glucose Metabolism by Splenocytes (nmol/2 h/10^6 cells)

	Weeks After Implantation		
Glucose Metabolite	0 (n = 5)	2 (n = 10)	3 (n = 10)
CO_2			
−Glutamine	7.0 ± 0.5^a	4.6 ± 0.2^b	4.0 ± 0.3^b
+Glutamine	4.1 ± 0.4^a *	3.7 ± 0.2^a *	2.9 ± 0.2^b *
Lactate			
−Glutamine	29 ± 3	25 ± 1	32 ± 3
+Glutamine	22 ± 1*	23 ± 2	27 ± 2
Pyruvate			
−Glutamine	7.8 ± 0.7^a	5.6 ± 0.6^b	5.8 ± 0.4^b
+Glutamine	7.7 ± 0.5	7.0 ± 0.5	7.6 ± 0.5*
Lactate/pyruvate ratio			
−Glutamine	3.9 ± 0.6^a	4.6 ± 0.4^{ab}	5.6 ± 0.3^b
+Glutamine	3.0 ± 0.3	3.4 ± 0.3*	3.5 ± 0.3*

NOTE. Values are the mean ± SEM. Means within a row that do not share a common superscript are significantly ($P < .05$) different as determined by ANOVA.

*Significantly different ($P < .05$) *v* −Glutamine as determined by paired *t* test.

Table 5. Effect of Tumor Progression on Glutamine Metabolism by Splenocytes (nmol/2 h/10^6 cells)

	Weeks After Implantation		
Glutamine Metabolite	0 (n = 5)	2 (n = 10)	3 (n = 10)
$^{14}CO_2$			
−Glucose	11 ± 1[a]	5.1 ± 0.7[b]	8.1 ± 0.7[c]
+Glucose	5.8 ± 0.3[a] *	4.3 ± 0.5[b]	4.1 ± 0.3[b] *
[^{14}C]glutamate			
−Glucose	14 ± 1	9.2 ± 0.6	13 ± 2
+Glucose	8.6 ± 0.5*	8.2 ± 1.3	9.8 ± 1.0
[^{14}C]aspartate			
−Glucose	8.1 ± 0.6[a]	4.1 ± 0.3[b]	5.1 ± 0.4[a]
+Glucose	1.8 ± 0.2[a] *	1.3 ± 0.2[b] *	1.7 ± 0.1[ab] *
Ammonia			
−Glucose	21 ± 2[a]	14 ± 1[b]	22 ± 2[a]
+Glucose	38 ± 4[a] *	16 ± 2[b]	26 ± 4[b]

NOTE. Values are the mean ± SEM. Means within a row that do not share a common superscript are significantly ($P < .05$) different as determined by ANOVA.

*Significantly different ($P < .05$) *v* −Glucose as determined by paired *t* test.

of CD8$^+$ (cytotoxic/suppressor T) cells in the spleen. T cells are important in mediating the immunological response to tumors.[2] Compared with T0, at T2 the relative percent of CD4$^+$ cells (T-helper/inducer subset) and the CD4/CD8 ratio were reduced. Decreases in the CD4/CD8 ratio have been associated with a reduced mitogen response and increased tumor growth.[2] The increase in the relative percent of CD4$^+$ cells and the increased CD4/CD8 ratio at T3 is interesting. The higher percent of CD4$^+$ cells at T3 could be related to increased NK activity; NK cells have been observed in vitro to activate CD4$^+$ cells.[25] Despite a relatively small proportion of macrophages in the spleen, the proportion of macrophages was elevated at both T2 and T3. This suggests that macrophages are involved in antitumor defenses or immune suppression of the MH 7777. Macrophages accumulate in considerable numbers at the tumor site in a variety of transplantable tumors, and in vitro can lyse or inhibit a variety of transformed cells.[3]

The high rate of protein synthesis in neoplastic tissue demands a continuous supply of amino acids and energy substrates.[26] One of the most important sources of molecular and energetic substrates necessary for tumor growth is the plasma pool. In this regard, the tumor is in direct competition with the host's immune system for energy substrates. It has been suggested that unsuccessful competition by the host's immune cells with the tumor for limited nutrients may suppress immune function.[13] Although tumor cells can use virtually any energy substrate to meet their high energy needs, having a remarkable ability to sustain a rapid rate of growth during starvation or semistarvation of the host,[12] glucose and glutamine appear to be the preferred energy substrate of most tumors, including the MH 7777.[14,27]

Consistent with previous reports, a decrease in plasma glutamine concentration was observed at T3. The MH 7777 has been reported to exhibit a high rate of glutamine utilization.[14] Glutamine concentration[28] and glutaminase activity[29] in several other rapidly growing tumors correlate with tumor growth, and this has provided the rational for chemotherapy interventions aimed at reducing glutamine availability.[30] In the present study, reductions in the concentration of other nonessential and essential amino acids were observed at both T2 and T3. Plasma amino acids represent the balance between intake, intestinal absorption, liver biosynthesis of nonessential amino acids, oxidative breakdown, muscle metabolism, and tumor demand.[12] Although all these factors were not measured in the present study, we have recently demonstrated that the MH 7777 is a significant "nitrogen trap."[12] Rats bearing the MH 7777 are reported to have lower intracellular concentrations of serine, glutamine, glutamate, threonine, valine, leucine, and isoleucine in muscle as compared with pair-fed non–tumor-bearing controls.[31] Asparagine, also decreased at T3, is preferentially used by malignant cells.[32] The high utilization of asparagine is the basis for the antitumor activity of asparaginase.[33] Ornithine concentrations were significantly reduced after 2 weeks of tumor implantation. Increased flux of ornithine from host tissues to the tumor, tumor polyamine biosynthesis, and ornithine decarboxylase activity have been correlated with increased growth of Erlich ascites tumors.[34] Malignant cells synthesize ornithine from glutamine, and as ornithine availability decreases, the demand for glutamine increases.[34]

An increase in energy metabolism is necessary to initiate and sustain a mitogenic response.[10,11] Although it is well established that glucose and glutamine are the major energy substrates for immune cells,[5-8] little is known about substrate utilization in the presence of a tumor. Consistent with the literature on non–tumor-bearing rats,[6-8] glucose was metabolized primarily to lactate and pyruvate, with approximately 79% and 17%, respectively, of the recovered glucose carbons found in these end products. Glutamine was metabolized mainly to ammonia, glutamate (66% of recovered glutamine carbons), and aspartate (25% of recovered glutamine carbons), a process termed "glutaminolysis,"[5] as distinct from the complete oxidation to CO_2. The inhibition of glutamine metabolism by glucose and inhibition of glucose metabolism by glutamine observed in T0 rats are consistent with previous reports.[7] It has been reported that glucose decreases glutamine metabolism via decreasing intracellular phosphate concentrations and a corresponding decrease in glutaminase activity, the rate-limiting step in glutamine metabolism.[35] In tumor-bearing rats, providing both glucose and glutamine did not alter the production of the major carbon end products (glutamate and lactate) of glucose and glutamine metabolism. This suggests that "normal" immune cell metabolism is altered in the tumor-bearing host.

Glutamine also serves as an important nitrogen precursor for the biosynthetic processes associated with proliferation.[6] It has been reported that rates of NH_3 production by immune cells closely approximates rates of glutamine utilization.[7] Lower rates of NH_3 production were observed in cells from tumor-bearing rats when glucose and glutamine were provided in concentrations similar to plasma levels (Table 5). Lower glutamine utilization rates at this time point are consistent with the reduced ability of

splenocytes to respond to immune challenges. It has been demonstrated in vitro[7] and suggested in vivo[5,6] that changes in glutamine availability inhibit immune responses. Thus, the effect of the tumor on immune metabolism could even be greater at T3, when plasma glutamine concentration was decreased. Providing glutamine was recently demonstrated to increase the tumoricidal effects of methotrexate.[36] Our diet did not contain free glutamine, but casein (the protein source of the diet) contains considerable glutamine; the diet provided approximately 2 to 3 g/100 kg protein-bound glutamine. This is more than in standard laboratory chow fed in other studies. Splenocytes represent a mixture of cell types, and the tumor altered the relative proportion of immune cell phenotypes in spleen. There are limited data available regarding the metabolic substrate utilization of different T-cell subsets. However, macrophages oxidize and use glucose at a very high rate.[37] Despite the greater proportion of macrophages in tumor-bearing rats, an increased glucose utilization was not observed.

NK cells are a discrete subpopulation of lymphoid cells with spontaneous cytotoxic reactivity against a variety of tumor cell lines and primary tumors.[3] In vivo, NK cells contribute to limiting the development of transplanted and primary tumors and metastasis of established tumors.[4] Cytotoxic assays in vitro led to the discovery of NK cells and remain an important means of assaying NK activity. There is considerable evidence in a variety of other transplantable tumors demonstrating a strong correlation between NK activity in vivo and that measured for splenocytes in vitro.[4] The cytotoxic activity of splenocytes against YAC-1 cells correlates well with the levels of NK cell activity in the host early in the establishment of other tumors.[38] After 2 weeks of implantation, there was a decrease in cellular NK activity (an increase in lytic units), not in the number of NK cells, suggesting a suppressive effect of the tumor on this host immune defense. It is suggested that the tumor may mediate suppression via production of prostaglandins or stimulation of NK-suppressor cells.[4]

After 3 weeks of tumor implantation, per-cell NK cytotoxicity against YAC-1 cells increased (Fig 2) over that at T0. Although the T0 rats were only fed the diet for 3 weeks, we have recently reported similar levels of NK activity in non–tumor-bearing rats fed the identical diet for 4 weeks.[39] It has been reported for other tumors that cytotoxic activity is highest when tumor growth peaks.[2] Death occurs with the MH 7777 within 4 weeks after implantation.[1] Despite the increase in activity, there is evidence that when the tumor represents a significant burden to the host, there is a diminished susceptibility of the tumor to NK activity.[4] This would not be evident using a NK-sensitive target cell line such as YAC-1 cells. Although the primary tumor may have developed NK resistance, for other tumors, splenocyte NK activity is negatively correlated with metastasis.[38] This may help to explain the low metastasis potential of the MH 7777.[1]

The present study demonstrated a progressive suppressive effect of the tumor on components of specific and nonspecific immune responses to tumors. Experimental tumors do not provide an exact reflection of the situation in spontaneously occurring human tumors; however, they provide valuable models to test basic principles of host immune function and metabolism in the tumor-bearing state. The information derived from these studies may be used to develop new approaches aimed at improving host defenses. Several variables of the experimental model used may be of importance for immune function, including tumor type, anatomical location (primary *v* metastasis, ectopic *v* orthoptic implantation), stage of tumor progression, tumor burden, and interaction with antitumor therapies. Further studies are needed to probe the relation between malnutrition in the tumor-bearing host and immune function, factors affecting differential availability of substrates to the host immune system, other host tissues, and tumor, and effects of supplemental nutrients applied enterally or parenterally on immune function in the tumor-bearing state.

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